Preclinical Assessment of Immunocal® as a Preventative Treatment for Traumatic Brain Injury (TBI) in a Mouse Model of Closed Head Injury

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PRECLINICAL ASSESSMENT OF IMMUNOCAL® AS A PREVENTATIVE TREATMENT FOR TRAUMATIC BRAIN INJURY (TBI) IN A MOUSE MODEL OF CLOSED HEAD INJURY

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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ABSTRACT

Over the past three decades, traumatic brain injury (TBI) has been considered a "silent epidemic" and recognized as an emergent public health problem by the Centers for Disease Control and Prevention (CDC). TBI is defined as a bump, blow, or jolt to the head that disrupts the normal function of the brain. Due to the debilitating effects and prevalence of TBI, novel preventative treatment regimens are highly desirable in at risk populations. According to the CDC groups disproportionately affected by TBI include athletes, people aged 75+, and service men and women, among others. Here, we investigated a unique whey protein supplement, Immunocal®, to determine its ability to boost resilience of neurons prior to injury. It has been previously described that Immunocal® functions specifically by increasing levels of the essential antioxidant glutathione (GSH). We hypothesize that boosting brain levels of GSH will help ameliorate cognitive, physical, and histopathological indices of brain injury post TBI in a mouse model of closed head impact injury. We found that twice daily oral supplementation with 3.30% Immunocal® for 28-35 days prior to impact significantly improved animal motor coordination and to a lesser extent, cognitive performance. Histopathological results also demonstrated a significant effect on corpus callosum width (axonal myelination) and a decreased presence of degenerating neurons in Immunocal®-treated animals compared to untreated TBI animals. However, no effect was seen in
measured clinical effects, or primary injury using MRI analysis. Finally, Immunocal®
treatment prevented an ~30% reduction in the brain GSH/GSSG ratio observed in
untreated TBI mice. These cumulative data suggest that Immunocal®, while unable to
attenuate damage done by the primary injury, was helpful in alleviating secondary injury
mechanisms in this TBI mouse model. In the future, this compound could hold promise
as a preventative agent for in TBI in certain high risk populations.
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CHAPTER ONE: INTRODUCTION

1.1. TBI: long term consequences and relationship to sports and military service

According to the Centers for Disease Control, there were at least 2.8 million incidences of TBI related emergency room visits, hospitalizations, and deaths in 2013 (Taylor et al., 2017). Most of these cases are categorized as forms of mild TBI including concussions. Repetitive incidences of mild TBI are linked to progressive neurological dysfunction and an increased risk of neurodegeneration. For many decades it has been recognized that repetitive mild TBI experienced by boxers resulted in a syndrome of progressive neurological deterioration originally known as dementia pugilistica. More recently, this term has been replaced with the more descriptive designation, chronic traumatic encephalopathy (Blennow et al., 2012). This complex neurological disorder is characterized by executive dysfunction, depression, memory impairment, and dementia, amongst other types of cognitive and affective dysfunction (Baugh et al., 2012). The pathology of chronic traumatic encephalopathy includes brain atrophy, aggregates of hyperphosphorylated tau protein (p-tau), and in some cases, amyloid plaque deposition and/or accumulation of TDP-43 inclusions. Chronic traumatic encephalopathy and other forms of dementia have been linked to repetitive mild TBI caused by sports related concussive and subconcussive head trauma in football, hockey, soccer, and wrestling (Guskiewicz et al., 2005; McKee et al., 2009; Broglio et al., 2011; Gavett et al., 2011). In a similar manner, blast-related TBI which is estimated to affect 10-20% of veterans
returning from the wars in Iraq and Afghanistan, is also associated with an increased risk of chronic traumatic encephalopathy and other types of dementia, as well as post-traumatic stress disorder (Elder and Christian, 2009; Goldstein et al., 2012; Barnes et al., 2014). Regardless of the cause or severity of TBI, even mild TBI is a significant risk factor for development of dementia including Alzheimer’s disease (Gavett et al., 2010; Johnson et al., 2010; Shively et al., 2012; Lee et al., 2013). Thus, identification of new strategies to enhance resilience (preventative) against TBI is of particular importance to people participating in “high risk” occupations, such as athletes or military personnel. In addition, novel therapeutic agents that improve recovery (restorative) from TBI would have broad applicability to the population as a whole.

1.2. Current therapeutic approaches for TBI

The pathophysiological processes underlying the short and long term injury sequelae associated with TBI are complex. The primary injury is mechanical, resulting from an external force, and leads to tissue deformation, tearing of blood vessels and neuronal axons, necrotic cell death, and initiation of secondary injury processes. Secondary injury mechanisms may include intracranial hemorrhage, excitotoxicity, ionic disturbances, decreased cerebral blood flow, edema, inflammation, mitochondrial dysfunction, oxidative stress, nitrosative stress, and (neuronal and glial) cell death by apoptosis. Although many patients might in theory, be able to significantly recover from the primary mechanical injury of TBI given appropriate acute surgical interventions and supportive care, the detrimental consequences of secondary injury often lead to long term physical, cognitive, and emotional impairments that markedly reduce quality of life. Given the multi-factorial nature of secondary injury, many different therapeutic
approaches have been investigated in an attempt to mitigate the post-acute neuronal damage caused by TBI including antioxidants, neurorestorative therapies, neuroprotective pharmacological agents, and drugs that modulate neuroinflammation (Hall et al., 2010; Xiong et al., 2010; Lulic et al., 2011; Kumar and Loane, 2012; McConeghy et al., 2012). Yet, despite some compelling results with specific agents in pre-clinical animal models of TBI and Phase I/II trials in patients, there are currently no FDA approved drugs for TBI which have shown significant therapeutic efficacy in large, randomized Phase III clinical trials. Therefore, novel therapeutic approaches for TBI that either enhance neuronal resilience (preventative) or improve recovery (restorative) are critically needed.

1.3. Nutritional interventions to enhance resilience and speed recovery from TBI

In this rapidly evolving research arena, the clinical use of nutritional supplements or nutraceuticals, particularly those with free radical scavenging or antioxidant activities (e.g., polyphenols like resveratrol), has generated significant attention as a possible preventative or restorative strategy for TBI. Recently, the Institute of Medicine convened a committee to review the existing evidence for the potential role of nutrition in providing resilience against or treating the effects of TBI. That committee included experts in neurology, clinical nutrition, psychiatry and behavioral science, biochemical and molecular neuroscience, epidemiology, and the pathobiology of TBI, among others. Their report concluded that nutrition (supplementation) be used “not only to augment overall defensive mechanisms against the effects of TBI but also as post-injury treatment to lessen the acute and subacute effects of TBI” (IOM, 2010). Furthermore, the committee recommended the continued study of the clinical application of novel nutritional approaches (see excerpt from Recommendation 3-1 below). In particular, the committee
concluded that nutraceuticals or nutritional supplements with intrinsic antioxidant activity should be evaluated for preventative or restorative effects in animal models of TBI (see Recommendation 14-1 below).

**RECOMMENDATION 3-1.** The committee recommends that DoD, in cooperation with others, refine the existing animal models to investigate the potential benefits of nutrition throughout the spectrum of TBI injuries, that is, mild/concussion, moderate, severe, and penetrating, as well as repetitive and blast injuries (p. 4; IOM, 2010).

**RECOMMENDATION 14-1.** Based on positive outcomes with curcumin and resveratrol in small animal models of TBI, DoD should consider conducting human trials. In addition, other flavonoids (e.g., isoflavones, flavanols, epicatechin, theanine) should be evaluated in animal models of TBI (p. 222; IOM, 2010).

Indeed, multiple studies in various animal models of TBI have shown significant neuroprotective or cognitive therapeutic benefits with a number of nutraceutical or nutritional therapies. For instance, post-TBI supplementation with the omega-3 fatty acid, docosahexaenoic acid (DHA), normalized levels of brain-derived neurotrophic factor (BDNF) and manganese superoxide dismutase which were reduced following fluid percussion injury in rats (Wu et al., 2011). It has also been demonstrated in a fluid percussion injury of rats that 7,8-dihydroxyflavone (7,8-DHF), a TrkB agonist, attenuated the effects of TBI on plasticity markers and memory function in Barnes maze testing (Agrawal et al., 2015). Multiple groups have also reported beneficial effects of dietary curcumin when given pre- and post-TBI to rats and mice, respectively, subjected to weight drop injury (Samini et al., 2013; Zhu et al., 2014). In a weight drop injury model, rats administered the natural antioxidant melatonin post-procedure showed reduced oxidative stress, decreased presence of degenerating neurons, and increased translocation.
of Nrf2 from the cytoplasm to the nucleus. (Ding et al., 2014). Post-TBI administration of resveratrol preserved hippocampal neuron survival and reduced microglial inflammation following controlled cortical impact (CCI) injury in rats (Sönmez et al., 2007; Gatson et al., 2013). Edaravone, a novel small-molecule synthetic free-radical scavenger, was found to promote CA3 neuronal survival in the hippocampus, as well as decreasing activation of astrocytes and glial cells and reducing the presence of inflammatory cytokines and blood brain barrier (BBB) permeability (Wang et al., 2011). Collectively, these findings demonstrate that nutraceuticals or nutritional supplements with intrinsic antioxidant or free radical scavenging activities demonstrate significant neuroprotective, anti-inflammatory, and beneficial cognitive effects in pre-clinical animal models of TBI.

1.4. Evidence supporting strategies to increase glutathione (GSH) as a therapeutic approach for TBI

Oxidative and nitrosative stress are key factors in the secondary injury processes following TBI (Bains and Hall, 2012). GSH is a key endogenous antioxidant that detoxifies these free radical species. Brain GSH levels are significantly reduced following TBI induced by CCI in rats (Tyurin et al., 2000). Genetic variations in the activity of glutathione-S-transferase-4, a GSH-dependent enzyme that reduces the toxic lipid peroxidation product 4-hydroxynonenal, is a determining factor in the extent of neurodegeneration after TBI in rats (Al Nimer et al, 2013). Moreover, mice homozygous for deletion of the GSH-dependent, free radical detoxifying enzyme, glutathione peroxidase-1, display enhanced susceptibility to brain mitochondrial dysfunction induced by TBI (Xiong et al., 2004). These findings suggest that a strategy aimed at sustaining or
enhancing brain GSH levels may be a viable approach to mitigate secondary injury and
the subsequent long term cognitive, physical, and emotional deficiencies induced by TBI.
Along these lines, several studies have shown that administration of the GSH precursor,
N-acetylcysteine, just prior or immediately after TBI significantly preserved brain tissue
and mitochondrial GSH levels, reduced measures of oxidative damage, and preserved
neuronal survival (Xiong et al., 1999; Hicsonmez et al., 2006). In a similar manner,
treatment with another GSH precursor, gamma-glutamylcysteine ethyl ester, reduced
indices of oxidative and nitrosative stress and preserved BBB function when given
immediately post-TBI (Reed et al., 2009; Lok et al., 2011). Finally, the GSH analog and
nitric oxide modulator, S-nitrosoglutathione, reduced BBB disruption, decreased
neuronal loss, reduced inflammation, protected axonal integrity, and increased the
expression of neurotrophic factors when administered post-TBI to rats subjected to CCI
(Khan et al., 2009; 2011). Each of these results indicates the potential of enhancing GSH
as a therapeutic approach for TBI. Unfortunately, none of these previous studies
evaluated the effects of GSH precursor supplementation on cognitive or motor deficits
induced by TBI and as a result, it is presently unclear what therapeutic benefit this
strategy might realistically hold for patients suffering from TBI.

The nutritional supplement, Immunocal®, is a non-denatured whey protein
supplement designed to augment the available intracellular GSH pool. Cellular GSH
concentrations are highly dependent on the availability of cysteine, which is the limiting
precursor in GSH synthesis (Tateishi et al., 1974; Meister, 1984). The cysteine precursor,
cystine, occurs in high levels in Immunocal® because the supplement is rich in serum
albumin, alpha-lactalbumin, and lactoferrin. These proteins have a significant number of
cystine residues in this non-denatured preparation. In addition, the direct GSH precursor, glutamylcysteine, is also present in the serum albumin fraction of this supplement. When cystine is provided in this peptide form, it is resistant to proteolysis by pepsin and trypsin but is readily cleaved and reduced to two cysteine molecules within the target cell. This is significant, as cysteine supplementation alone is cytotoxic (Bounous and Gold, 1991).

Immunocal® was initially developed as a nutritional supplement to increase immune system function after dietary amino acids were discovered to increase immune reactivity (Bounous and Kongshavn, 1978). It has been investigated in several human diseases and has been shown to significantly increase blood or lymphocyte GSH levels in HIV-seropositive or cystic fibrosis patients, respectively (Bounous et al., 1993; Grey et al., 2003). Immunocal® is one of only a handful of nutritional supplements that are included in the Physician’s Desk Reference and is comprised of natural food protein placing it in the FDA category of generally recognized as safe (GRAS) (PDR, 2016). We have recently found that Immunocal® supplementation preserves blood and spinal cord GSH levels and delays disease onset and progression in a transgenic mouse model of amyotrophic lateral sclerosis (Ross et al., 2014).

1.5. Justification for selection of the CCI model of TBI

We have chosen a model of TBI in which head motion of the animal is constrained and a closed head injury is induced using a stereotactically-guided electromagnetic force impactor. The advantages of this model are: 1) injury is moderate and highly reproducible, 2) injury is not focal in nature as with penetrating models, and 3) the oxidative damage, neuronal cell death, and cognitive and motor impairments
induced in this TBI model are well characterized (Shohami et al., 1997; Pineda et al., 2001; Leinenweber et al., 2006; Tavazzi et al., 2007; Lloyd et al., 2008; Hall et al., 2010).

1.6. Hypothesis and rationale

Given the observation that GSH is reduced in the brain following a TBI and recent research utilizing GSH precursors as both a preventative and restorative treatment in TBI, we pursued the use of Immunocal® as a novel therapeutic agent. Immunocal® is a preferred option as its safety is already well outlined, and there are no currently known side effects to using this whey protein (excluding allergenic). While this compound’s efficacy in treating neurodegenerative diseases has already been described, it has yet to be used in a model of TBI. This specific protein isolate is unique in its undenatured composition, leading to high levels of cystine which can then be converted to cysteine, the limiting precursor in GSH synthesis. The focus of the present study is to determine if supplementation with Immunocal® will decrease indices of oxidative and nitrosative stress, reduce neuronal cell death, diminish glial activation, and improve cognitive and motor function in a mouse model of closed head impact injury.

1.7. Summary of findings

Using a mouse model of closed head injury, we show here that Immunocal® improved several measures of secondary injury mechanisms in TBI, but was largely unable to combat the primary mechanical injury caused by impact. Initial clinical data related to apnea time and righting reflex time pointed to an obvious difference between injured animals and sham controls, with Immunocal® not accounting for any difference between treated and untreated animals. Magnetic resonance imaging (MRI) showed both a stark difference in injury between sham and TBI animals, as well as TBI animals that
had received Immunocal® prior to injury. It also showed that this compound was unable to prevent BBB permeabilization caused by injury. We also discovered that levels of p-Tau, a primary injury marker, increased with TBI, but were unchanged by the administration of the compound at 72 hours post procedure. Interestingly, at this same time point, Iba-1 and S100β levels – markers for reactive microglia and astrocytes respectively – were unchanged in both TBI and Immunocal®-treated animals compared to TBI (data not shown). We also observed a marked decrease in reduced GSH to oxidized GSSG ratio in TBI animals alone, with this effect being ameliorated by prior treatment with Immunocal®. Motor coordination tasks though, and to a lesser extent behavioral tasks, showed significant improvement with the administration of Immunocal® prior to impact. Significant improvement was seen between TBI and Immunocal®-treated animals both in rotarod and challenging beam walk tests. Improvement in spatial memory was also seen in Barnes maze data, when on day 6 of the acquisition period mice pre-treated with Immunocal® demonstrated a reduced latency time compared to TBI mice. Working memory as tested by a modified Y-maze, also suggests a trend toward improvement in mice treated with Immunocal® as compared to TBI animals. Various histopathological indices displayed an improvement in secondary injury as well, through looking at both degenerating neurons present, and the width of the corpus callosum 18 days post injury.
CHAPTER TWO: MATERIALS AND METHODS

2.1. Animal care and treatment

All animal work was conducted under a protocol approved by the University of Denver IACUC. Male CD1 Elite (CD1-E) mice were purchased from Charles River Laboratories (Hollister, CA) aged 35 days. Mice received a numbered ear tag upon arrival for identification purposes, and then were allowed one week to acclimate to the animal facility at the University of Denver before beginning the study. Mice were then randomly assigned and evenly distributed among one of three groups: Sham, TBI, or TBI+Immunocal®. Mice in the TBI+Immunocal® group were dosed twice daily by oral gavage with 0.25 mL of a 3.30% solution of Immunocal® in sterile drinking water. Dosing was performed 5 days a week over a period of 28-35 days. After this point, mice were subjected to TBI as described below. The day on which TBI was induced was considered Day 0. Following TBI, mice were monitored closely each day for signs of infection, bleeding, and general distress until the study concluded at Day 18. The MRI portion of the study concluded at Day 3.

2.2. Traumatic Brain Injury

Following the 28-35 day dosing regimen, traumatic brain injury was induced by controlled cortical impact (CCI) using The Leica Impact One system (Leica Biosystems, Buffalo Grove, IL). Briefly, mice were anesthetized via isoflurane inhalation, and monitored throughout the procedure for the depth of anesthesia by toe pinch reflex.
anesthetized, temperature was maintained at approximately 37 ± 1°C by placement on a thermal pad. A midline incision approximately 1 cm in length was made along the head, and the skin was pulled aside using small bulldog clamps. With the skull exposed, a dental scraper was used to partially remove the fascia in order to better visualize anatomical markers. Bregma was located, and a concave 22 gauge steel disk 5mm in diameter was affixed to the skull using tissue adhesive just caudal to this point. Animals were then placed in a stereotaxic frame, and the head was secured to prevent movement during impact. The arm of the impactor was then positioned such that the impactor probe was directly centered over the metal disk. The probe was then set to the desired impact depth of 2.75mm and a velocity of 5.75-6 m/s to induce moderate injury as described by Lloyde et al. (2008). Mice in the TBI and TBI+Immunocal® groups were subjected to injury at this time, after which animals were monitored for signs of TBI-induced apnea. Once apnea was overcome, animals were removed from the stereotaxic frame. Sham animals were simply removed from the stereotaxic frame following identification. Mice were returned to the thermal pad, and the metal disk was removed from the skull.

Bupivacaine (0.25% solution in sterile water) was applied generously to the open incision, which was then closed using tissue adhesive. The mice were allowed to recover on the thermal pad during which time their righting reflex time was measured. Righting reflex was defined as the point at which the animal was able to return to and maintain a sternal position after being placed on its side during recovery from anesthesia. Mice were returned to their home cage once they became fully ambulatory.
2.3. Assays of cognitive and motor function

All behavioral assays were recorded using a video camera, and quantified as described below.

![Figure 1. Summary of the dosing and behavioral testing schedule before and after TBI induction.](image)

2.3.1. Challenging beam walk task

The challenging beam walk test for motor function and coordination was performed as previously described by Fleming et al. (2013). The apparatus for the challenging beam walk was composed of four segments supplied by Starks Plastics (Forsest Park, OH), each of which was 25cm in length. The first segment had a width of 3.5cm, with each subsequent section decreasing by one centimeter in width to a final measurement of 0.5cm. These segments were secured together and suspended at a height of approximately 14.5cm above a level surface (Fig. 1A, C).

Mice were allowed a two day training period prior to TBI on Days -4 and -3. The training set up for the beam apparatus is shown in Figure 1A. On the first training day, mice were placed at the wide end of the beam. The investigator then held an empty cage containing clean bedding on its side a few centimeters in front of the mouse as incentive for the animal to navigate the beam (Fig. 1B). As the mouse moved toward the cage, the investigator pulled the cage away from the mouse such that the animal was forced to traverse the beam, and the mouse was only allowed to enter the cage once it had successfully reached the end of the beam. This procedure was repeated until the mouse
could traverse the beam without the need for prompting or correction from the investigator. On the following day, a cage with clean bedding was placed on its side at the narrow end of the beam in a fixed position (Fig. 1A). Mice were then placed on the wide end of the beam and allowed to traverse the full length of the beam to reach the empty cage. This phase of training was repeated until the mouse could consistently traverse the entire beam without prompting or correction from the investigator.

Following TBI induction on Day 0, mice entered the testing phase of the challenging beam walk task on Day 1. For this phase, wire grids with openings measuring 1cm² were placed securely over each beam segment creating a space between the top of the grid and the surface of the beam (Fig. 1C). This was done to increase the difficulty of the task and to enhance visual scoring of foot faults. As before, an empty cage was placed at the narrow end of the beam and served as the goal for successful completion of the beam walk. Mice were placed at the wide end of the beam on top of the grid and allowed to traverse the entire length of the beam a total of three times. The number of foot faults for the right hind foot was quantified for each animal on each segment of the beam and averaged across the three attempts. Foot faults were defined as any point at which the mouse stepped through the metal grid or gripped the plastic beam instead of the wire grid. The time it took the mouse to traverse the full length of the beam was also recorded for each of the three attempts on the beam.
Figure 2. The challenging beam walk task. A. Set up of the beam walk apparatus on days -4 and -3 for training. B. Mouse training for the challenging beam walk on day -4. A cage was held just in front of the mouse by the investigator in order to entice the mouse to traverse the beam. C. Set up of the beam walk apparatus on day 1 for testing of motor coordination. Following training, a wire grid was placed over the beam to increase the difficulty of the task and allow for better visualization of foot faults.

2.3.2. Modified Y maze

Following the challenging beam walk, mice were tested on Day 1 in a modified version of the Y-maze (Stoelting, Wood Dale, IL) designed to test spatial recognition and working memory as described by Adamczyk et al. (2014). In the first phase of testing, one arm of the maze was blocked as shown in Fig. 2a. The mouse was placed into the entry arm, and allowed to explore the open areas of the maze for a period of 5 minutes...
without interference. After this period, the mouse was removed from the maze and returned to its home cage for a period of 10-15 minutes. The blocked arm of the maze was then opened, as shown in Fig. 2b. The mouse was again placed into the entry arm and allowed an additional 5 minutes to explore the entirety of the maze. The time it took the animal to enter the newly opened arm was recorded as lag time, and the time spent in the unblocked arm versus the two previously opened arms was determined. Additionally, the number of entries into each arm were quantified to ensure that all mice were equally active throughout the testing period.

Figure 3. Modified Y-maze for working memory. A. During the first phase of testing, the upper arm of the maze is blocked to prevent entry by the mouse. The mouse is then placed into the entry arm and allowed to explore both this arm, and the open arm. B. During the second phase of testing, the upper arm of the maze is unblocked, and the mouse is free to explore all areas of the maze.

2.3.3. Rotarod

Rotarod testing for motor coordination and function was performed on days 9 and 16 following TBI. Mice were placed on a rod, 30 mm in diameter, rotating at 4 rpm. Animals were placed in individual lanes as shown in Fig. 4 to prevent interference between mice while the test was being conducted. When the mice had acclimated to the slow speed, the rod was accelerated from 4-40 rpm over the course of 5 minutes. Mice
were given three attempts on the apparatus before testing ended. The duration of time that the mouse spent on the rod was recorded by depression of a lever triggered upon the mouse falling, and averaged across the three attempts.

![Rotarod testing for motor coordination. The mouse was placed into an individual lane on a rotating rod and a lever was raised. The rod was then accelerated. When the mouse falls, the lever is depressed and the time that the mouse spent on the rod is recorded.](image)

2.3.4. **Barnes maze**

Barnes Maze (ANY-maze, Wood Dale, IL) testing was performed on days 10-16 as described by Mouzon *et al.* (2012). The first 6 days were training days, followed by a probe/test day. The circular table was divided into quadrants (Fig. 5), with an arrow on the wall indicating the escape pod. On training days, mice were placed in each quadrant, beginning in 1 and ending in 4, and allowed 90 seconds to find the escape pod. If the mice were unable to find the pod after the allotted time, they were directed to it and remained in the pod for 30 seconds. If they found the pod and entered on their own, the pod was then covered and they remained there for 30 seconds. Videos were reviewed, and latency times were recorded. On probe day, the pod was blocked so that mice could not enter. Mice were placed in the middle of the table and allowed to search the maze for
60 seconds, then removed. Videos were reviewed and latency times to the zone were recorded, as well as the percent of the time spent in the zone (data not shown). The zone was defined as the hole on either side of the escape pod. In some cases mice performed multiple probes.

Figure 5. Barnes maze testing for spatial memory. The mouse is placed in one of the four quadrants as indicated, and allowed 90 seconds to explore the maze and find the escape pod, as indicated by the pink and red arrows.

2.3.5. Elevated plus maze

Elevated Plus Maze (ANY-maze, Wood Dale, IL) testing was performed on Day 17 as described by Petraglia et al. (2014). Mice were placed in the middle of the maze between the open and enclosed arms (Fig. 6), and allowed to explore the maze for 5 minutes. Percent of time in the open arms and closed arms was quantified. Mouse had to have all four paws on the arm for it to be quantified as in the open or closed arms.
Figure 6. Elevated plus maze testing for anxiety. Mouse was placed facing the wall in the middle of the maze indicated by the red arrow. The mouse was allowed to explore the maze for five minutes.

2.4. Reagents

Primary antibodies to beta actin, S100β and Iba-1 were purchased from Abcam (Cambridge, MA). The primary antibody to phosphorylated Tau S369, and the protein standard used for western blotting (Novex® sharp pre-stained protein standard) were purchased from Invitrogen (Carlsbad, CA). Purified oxidized (GSSG) and reduced (GSH) glutathione was purchased from Sigma Aldrich Co. LLC (St. Louis, MO). Cy3-conjugated secondary antibody was purchased from Jackson Immunoresearch (Westgrove, PA). Fluoro-Jade C staining kit was purchased from Biosensis (Temecula, CA). Luxol fast blue staining kit was purchased from American Mastertech (Lodi, CA). All imaging was performed using a Zeiss Axiovert-200M epi-fluorescence microscope in a blinded fashion.
2.5. Fluoro-Jade C staining

2.3.1. Tissue processing

Frozen brains were cryosectioned either by the Histology Core at the University of Colorado Anschutz medical campus or AML Laboratories Inc. (St. Augustine, FL). Briefly, 12µm coronal sections were created starting at bregma and proceeding towards the posterior of the brain. Tissue sections were mounted on adhesive microscope slides discarding three to four tissue sections between each mounting. Following mounting, tissue was fixed in 4% paraformaldehyde for one hour.

2.3.2. Slide staining

Fluoro-Jade C staining was performed as specified by the manufacturer. Briefly, coronal brain sections were immersed in a 1:9 solution of 1% sodium hydroxide and 70% ethanol for five minutes followed by a two-minute wash in 70% ethanol. Next, tissue sections were immersed in a 1:9 solution of 0.06% potassium permanganate and distilled water for ten minutes then washed with distilled water for two minutes. Tissue was then incubated in a solution 1:2:8 solution of DAPI, 0.0004% Fluoro-Jade C and distilled water for ten minutes, taking precaution to protect the solution from light. Sections were then washed three times in distilled water and dried at 50-60°C for ten minutes. Sections were imaged under 40x magnification for all fluorescent foci. The total number of Fluoro-Jade C positive foci were then quantified for at least two tissue sections per animal.
2.6. Luxol fast blue staining

Tissue processing was done as described above for Fluoro-Jade C staining. Brain sections were incubated in Luxol fast blue stain solution at 60 °C overnight, followed by washing with distilled water. Sections of gray and white matter were differentiated by dipping brain tissue into 0.05% lithium carbonate and 70% ethanol. Slides were then immersed in cresyl violet stain for ten minutes followed by further differentiation in 70% ethanol. Following the staining process, tissue sections were imaged at 20x magnification to visualize the corpus callosum. Images of the midbody of the corpus callosum were captured for at least two tissue sections per animal. The health of the corpus callosum was assessed by measuring the width of the corpus callosum.

2.7. Magnetic resonance imaging (MRI)

All MRI studies were performed in the Colorado Animal Imaging Shared Resources (AISR). All animals underwent MRI imaging 72 hours after injury, using pre- and post-gadolinium-enhanced (0.2 mmol/kg Omniscan® IV) T1-weighted and T2-weighted sequences. The mice were anesthetized with 2.5% isofluorane. Scans were performed on a 4.7 Tesla Bruker PharmaScan. A quadrature birdcage coil (inner diameter 38 mm) tuned to the 1H frequency of 200.27 MHz, was used for RF transmission and reception. T2-weighted MRI (to confirm and quantify injury) was acquired using a rapid acquisition with relaxation enhancement (RARE, Bruker manufacturer label for a fast spin echo sequence) protocol with following parameters: Field of view (FOV) = 36 mm; echo time/ repetition time (TE/TR) = 100/ 4,000 msec; slice thickness = 1 mm; no interslice gaps; number of slices = 16; number of averages = 8; matrix size = 128x256;
total acquisition time = 8 min 31 sec. T1-weighted MR images (for BBBD assessment) were acquired using a multi-slice multi-echo (MSME, Bruker manufacturer label for a spin echo sequence, in this case with one echo) sequence, before and 5 minutes after administration of 0.2 mmol/kg Omniscan® via tail vein. The following acquisition parameters were used: FOV = 36 mm TE/TR = 11/900 msec; slice thickness = 1 mm with no gaps applied; number of slices = 16; number of averages = 2; matrix size = 128x256; total acquisition time = 3 min 50 sec. All images were acquired in the axial orientation.

2.8. Western blotting

2.7.1. Tissue processing

Whole half brains were thawed from liquid nitrogen (-196°C). 1 mL of Wahl lysis buffer was added, with 1µL of leupeptin (5mg/mL) and 1 µL of aprotenin (5mg/mL) per half brain. The brains were then homogenized using a Dounce homogenizer; 20 strokes with the loose pestle and 20 strokes with the tight pestle. Samples were centrifuged for 5 minutes at 10,000 rpm, and the supernatant was isolated. The samples were diluted 1:100 for a BCA protein assay (Thermo Scientific, PA).

2.7.2. Blotting

Western immunoblotting was done to immunochemically detect proteins on a polyvinylidene difluoride (PVDF) membrane. Protein samples (80 µg/mL) and ladder were loaded into 3.9% stacking polyacrylamide gel on top of a 7.5% polyacrylamide gel and allowed to run for one hour at 35 mA per gel. The current was then turned down to 7mA and the gels were allowed to run overnight (17-20 hours). Proteins were then transferred from the gel to the PVDF membrane using an Amersham TE62 transfer tank
transfer unit at 1V for 1.5 hours. The membrane was then removed and allowed to sit in 1X blocking buffer for 1 hour while rocking. The blocking buffer was drained, and the membrane was allowed to sit in primary antibody (1:1000). The membrane was washed 3x for 15 minutes in PBS-T, then allowed to incubate in the secondary antibody (1:5000) for 1.5 hours while rocking. The secondary was then removed, and the membrane was washed again in PBS-T, 3x for 15 minutes. An ECL chemiluminescent kit (GE Healthcare; Pittsburgh, PA) was used to stain the gels. 6 mL of reagents 1 and 2 were added to the membrane and hand shaken for 1 minute. The membrane was then wrapped in saran wrap and taped to a film cassette. The membrane was exposed to a piece of film for an appropriate amount of time (depending on the blot), and then developed using a CP 1000 developer (AGFA; Mortsel, Belgium).

2.9. High performance liquid chromatography with electrochemical detection (HPLC-ECD)

2.8.1. Tissue processing

Full half brains were obtained from mice 72 hours post injury, following isofluorane overdose, and were immediately frozen in liquid nitrogen (-196°C). For HPLC-ECD analysis, 2.5 M perchloric acid was added to the half brains and the brains were roughly chopped using pointed surgical scissors. Samples were then sonicated 3 times for 15s intervals. Samples were then centrifuged for 5 minutes at 13,000 rpm, and the supernatant was removed. 20 uL of solution was used for a BCA protein assay (Thermo Scientific, PA). The remainder of each solution was neutralized with 500 uL of
4 M KOH and vortexed thoroughly. Samples were then centrifuged for 15 minutes at 13,000 rpm, and stored at -80°C until the HPLC machine was ready to run the samples.

2.8.2. HPLC-ECD

GSH and GSSG in samples and known standards were separated by reversed-phase HPLC on a C18 bonded silica column at 35°C (5 μm, 4.6 x 250 mm) from Dionex, Inc. (Sunnyvale, CA). Analytes were detected using a CoulArray® detector (model 5600, ESA) on three coulometric array cells in series; electrochemical detectors were set between 0 and 900 mV at increments of 75 mV. Concentrations were determined with a standard curve of each identified analyte. Mobile phase consisted of 50 mM Lithium acetate and 1% acetonitrile in water, pH 3.8. The flow rate was set to 0.4 mL for all samples. CoulArray® software was used for baseline correction and peak analysis.
CHAPTER THREE: RESULTS

3.1. Immunocal® is unable to ameliorate primary damage caused by a closed head injury

Figure 7. Immunocal® does not protect brain from primary injury. A. MRI imaging, without contrast, of gross injury. The red circle indicates the area of injury in a mouse that received an impact (left) compared to a sham mouse (right). B. Quantification of volume of injury in mm$^3$. Results are shown as mean ±SEM, n=6. Abbreviations used: ICAL, Immunocal®.
Animals were treated with 0.25 mL of 3.30% Immunocal® in water twice a day, 5 days a week for 28-35 days prior to impact. Imaging occurred 72 hours post injury. Gross injury caused by primary injury mechanisms is apparent in an MRI lacking contrast and indicated by a dark disruption in the normal gray colored brain (Fig. 7A). Injury was quantified by measuring the area of damage across images to find the volume, showing no significant difference between TBI animals and TBI+Immunocal® animals (Fig. 7B).

Figure 8. Immunocal® does not protect brain from BBB permeabilization. A. MRI imaging of BBB integrity using Gadolinium contrast. The red circle indicates the area of disruption in an injured mouse (left), compared to a sham animal (right). B. Quantification of volume of BBB disruption in mm$^3$. Results are shown as mean ±SEM, n=6. Abbreviations used: ICAL, Immunocal®.
BBB disruption has been commonly described as a mechanical injury process in TBI, and is seen on an MRI with Gadolinium contrast (Fig. 8A). This permeabilization was present in both TBI and TBI+Immunocal® treated animals (Fig. 8B).

Figure 9. Clinical measures from surgery. A. Mean (±SEM) righting reflex times assessed post-surgery. Both untreated and ICAL pre-treated mice subjected to TBI displayed markedly increased righting reflex times when compared to sham animals (n = 43 sham, and 39 TBI and TBI+ICAL). B. Mean (±SEM) apnea times. All of the mice subjected to a TBI, including those treated with ICAL, displayed apnea following impact, with no differences between groups (n = 26 sham, 20 TBI, and 22 TBI+ICAL). C. Mean (±SEM) body weights were assessed at Day 0 prior to TBI or sham surgery. Mice pre-treated for 28 days with ICAL displayed a statistically significant (p<0.01) reduction in body weight compared to sham animals. However, no significant differences in body weight were observed between mice assigned to TBI versus TBI+ICAL groups (n = 43 sham, and 39 TBI and TBI+ICAL). Abbreviations used: ICAL, Immunocal®.
Animals subjected to a TBI, both Immunocal®-treated and not, experienced lengthened righting reflex time compared to sham controls (Fig. 9A). Righting reflex was defined as the point at which the animal was able to return to and maintain a sternal position after being placed on its side during recovery from anesthesia. Similar results were seen in the apnea time data, with only animals that were exposed to an impact were apneic during the procedure (Fig. 9B). There was a statistically significant difference in weight between sham and Immunocal® treated animals prior to surgery, likely due both to supplementation with Immunocal® as it is a whey protein and will increase lean muscle mass, as well as stress to the animal through chronic oral dosing (Fig. 9C).

3.2. p-Tau levels post injury are unaffected by increased levels of GSH

The increase in brain GSH as shown in our HPLC results, was not sufficient to counter a marked increase in p-Tau levels induced by a closed head impact.

**phospho-Tau (Ser396)**

![Immunoblotting indicates increased levels of p-Tau post TBI in untreated and pretreated animals. Sham animals had normal levels of p-Tau following surgery. Injury increased the amount of p-Tau both in TBI and ICAL-treated animals. Abbreviations used: ICAL, I, Immunocal®; S, Sham; T, TBI.](image)
3.3. Immunocal® ameliorates GSH depletion after TBI

We have previously shown in vitro that Immunocal® demonstrates its neuroprotective abilities through enhancing GSH synthesis by providing the key precursors cystine and cysteine. We have also established this mechanism in vivo in lumbar spinal cord and whole blood taken from treated animals in a mouse model of amyotrophic lateral sclerosis (Ross et al., 2014). To investigate whether Immunocal® was able to ameliorate any decreases in brain GSH caused by TBI, we collected whole brains from mice 72 hours after impact and analyzed them for reduced GSH and oxidized GSSG using HPLC-ECD. The ratio of GSH to GSSG significantly decreased in TBI mice compared to shams, and exhibited an increase in mice that were pretreated with Immunocal® compared to TBI (Fig. 11).

![Brain GSH/GSSG Ratio](image)

Figure 11. HPLC-ECD detection of GSH/GSSG. GSH/GSSG ratio in brain tissue decreased after TBI compared to sham controls (*p<0.05). A significant increase was also seen in ICAL treated animals post injury (##p<0.001). Statistical significance determined by a paired Student’s t test, n=5. Abbreviations used: ICAL, Immunocal®.
3.4. Assays indicate improved motor coordination secondary to Immunocal® treatment

3.4.1. Rotarod testing

Motor coordination as measured by rotarod, showed a significantly shorter performance time in the untreated TBI animals compared to sham, with an improvement seen with Immunocal® pretreatment, when assessed on day 9 (Fig. 12A). By day 16, no deficits in rotarod performance were observed for either of the TBI groups in comparison to sham animals, and mice in all groups showed improved performance (i.e., longer latency times) compared to testing on day 9 (Fig. 12B).

![Figure 12. Rotarod performance post-injury improved with Immunocal® supplementation. A. Latency time spent on the accelerating rotarod apparatus significantly decreased in untreated TBI animals compared to sham (*p<0.05). Time spent on the apparatus significantly increased in ICAL treated animals compared to TBI (#p<0.05). Statistical significance determined by one way ANOVA with a post hoc Tukey’s test, n=7. B. All animals demonstrated longer times on the apparatus when tested on day 16, with no significant differences between groups, n=6. Abbreviations used: ICAL, Immunocal®. Data collected by Aimee Winter and Evan Manning.](image-url)
3.4.2. *Challenging beam walk*

In the beam walking task, another motor coordination measure, sham animals showed a progressive increase in right hind foot faults as the width of the beam narrowed from segment 1 (widest) to 4 (narrowest). For example, only approximately 3% foot faults were observed in sham animals on the widest segment 1 and this error rate increased to approximately 40% on the narrowest segment 4 (Fig. 13 A-D). Untreated mice subjected to TBI did not display any apparent increase in right hind foot faults when compared to Sham controls. However, mice pre-treated with Immunocal® prior to TBI trended towards a decrease in foot faults when compared to Sham or untreated TBI mice. This was particularly evident in the data for the narrowest segment 4 for which the percent right hind foot faults for Immunocal® pre-treated TBI mice was statistically significantly different than for untreated TBI mice.
Figure 13. Percent of right hind foot faults in narrowing beam segments. A-C demonstrate an increasing percent of foot faults between groups with the narrowing beam (A&B n=8; C n=6). D. In the narrowest section of the beam (section 4) the percent right hind foot faults of ICAL treated animals was statistically significant compared to TBI animals (*p<0.05; n=8; unpaired Student’s t-test).Abbreviations used: ICAL, Immunocal®. Data collected by Aimee Winter.

Total time to traverse the beam was also measured. This result trended toward Immunocal® treated animals traversing the beam faster than both sham and TBI animals, but did not reach significance (Fig. 14).
Figure 14. Time taken to traverse the entire beam and percent change in time. A. The mean time taken during the final trial (3) for the mice to cross the beam (n=8). B. The average time between all trials for mice to cross the beam (n=8). C. The percent change in time for mice to cross the beam between the first (1) and final (3) trials. All data is mean (±SEM), no statistical significance seen between groups (n=6). Abbreviations used: ICAL, Immunocal®. Data collected by Aimee Winter.

3.5. Cognitive assays indicate improved performance secondary to Immunocal® treatment

3.5.1. Barnes maze testing spatial memory

Mice were allowed 6 training days to learn the maze and use the visual cue to locate the escape pod (Fig. 5). By day 5, the TBI animals have a clear delay in finding the pod compared to sham, with a trend toward improvement seen in Immunocal®-treated
animals (Fig. 15A). By day 6, the TBI animals are still experiencing this effect compared to sham animals, while the Immunocal®-treated animals show a statistically significant improvement in pod latency time compared to TBI animals (Fig. 15B). The probe test as well indicates a similar result as seen in acquisition days 5 and 6 with impairment seen in the TBI animals and improvement with Immunocal®-treated animals (Fig. 15D).

Figure 15. Improvement in Barnes maze task in Immunocal®-treated animals. A. TBI animals show a significantly (*p<0.05) increased latency time to pod compared to sham animals on day 5 of the acquisition period. There is no significant effect seen in ICAL treated animals (n= 10 sham, 4 TBI, 6 TBI+ICAL). B. There is a significant (***p<0.01) increase in latency in TBI mice compared to sham. There is also a significant (###p<0.01) decrease in latency time of ICAL-treated animals compared to TBI. Statistical significance in A&B determined by one way ANOVA with a post hoc Tukey’s test (n= 10 sham, 6 TBI, 7 TBI+ICAL). C. Mean latency time to the pod throughout the acquisition period (days 1-6) (n=6). D. ICAL-treated animals show significantly (***p<0.01) decreased latency to zone times compared to TBI animals in an unpaired Student’s t-test (n= 6 sham, 5 TBI, and 7 TBI+ICAL). Abbreviations used: ICAL, Immunocal®.
3.5.2. Modified Y-maze testing working memory

We observed a clear difference in the lag time (the time it took the animal to enter the previously blocked arm of the maze) between sham and untreated TBI mice (Fig. 14B). In general, sham mice very quickly sought to explore the newly opened arm of the maze. Whereas TBI animals showed a wide distribution of lag times with some animals taking more than 1 minute to explore the newly opened arm of the maze. Mice pre-treated with Immunocal® prior to TBI showed a profile of lag times that were somewhat intermediate but which more closely mimicked the untreated TBI mice than sham controls. We next determined the percentage of animals that chose the previously blocked arm on the first attempt. If each mouse showed no preference for choosing one arm of the maze over another, then this value should approach 50% or random chance. Sham mice showed a preference for choosing the previously blocked arm with 75% of these animals choosing to enter this arm of the maze on the first attempt (Fig. 16A). Untreated TBI mice chose the previously blocked arm of the maze at a rate equivalent to random chance. Immunocal® pre-treated mice subjected to TBI chose the previously blocked arm of the maze at an intermediate rate relative to sham and untreated TBI mice.
Figure 16. Performance in Y-maze shows no effect of Immunocal® on working memory.
A. The percent of animals that chose the newly unblocked arm on the first attempt decreased in injured animals, but exhibited a noticeable increase in ICAL-treated animals. B. The lag time to the previously unexplored arm was determined for each individual mouse, with the TBI animals demonstrating an increased average time, and the ICAL-treated animals again showing an intermediate lag time compared to sham and TBI (n=8). Abbreviations used: ICAL, Immunocal®. Data collected by Aimee Winter and Evan Manning.

3.6. Elevated plus maze testing indicates no change in anxiety post-TBI

There was no statistical significance or trend seen in anxiety behavior between the three groups as measured by the elevated plus maze. The percent of time spent in the open arms of the maze did not significantly differ between groups (Fig. 17).
Figure 17. Performance in elevated plus maze shows no effect on anxiety. There is no statistical significance between any groups in the elevated plus maze. Abbreviations used: ICAL, Immunocal®.

3.7. Histopathological indices indicate that Immunocal® has an effect on secondary injury mechanisms

3.7.1. Luxol fast blue imaging demonstrates a marked increase in corpus callosum width of Immunocal® treated animals compared to TBI animals

Luxol fast blue was used to stain the corpus callosum of mice 18 days post-surgery as measure of axonal integrity. Maximum width of the corpus callosum, as measured by arbitrary units using photoshop, experienced substantial thinning in TBI mice compared to sham controls (Fig. 18A-B). There was an increased width in Immunocal®-treated animals compared to TBI.
Figure 18. Immunocal® treatment improves axonal integrity in the corpus callosum. Top panels show Luxol Fast Blue stained imaging at 20x magnification. A. Representative image of sham animal corpus callosum on the left, followed by a TBI animal in the middle. White arrows indicate areas of demyelination secondary to injury. ICAL-treated animal image is seen on the left, with a slightly lower maximum width, but no obvious demyelination. B. Quantification of corpus callosum measurements. TBI images show a significantly (**p<0.001) thinner corpus callosum compared to sham controls. ICAL-treated images display a significant (#p<0.05) increase in width. Statistical significance determined by one way ANOVA with a post hoc Tukey’s test (n= 7 sham, 5 TBI, 6 TBI+ICAL). Abbreviations used: ICAL, Immunocal®. Imaging credit to Tyler Wallace.

3.7.2. Fluoro-Jade C staining indicates a neuroprotective effect of Immunocal®

We observed that brain sections from sham mice displayed little-to-no Fluoro-Jade C staining at 18 days post-injury, indicating little to no degeneration was occurring. In contrast, multiple foci of Fluoro-Jade C-positive neurons were detected throughout the
brain in untreated TBI mice (Fig. 19A & B). Interestingly, mice pre-treated with Immunocal® prior to TBI showed fewer and typically smaller foci of Fluoro-Jade C-positive neurons compared to untreated TBI mice (Fig. 19C & D).

Figure 17. Immunocal® treatment decreases neuronal degeneration. Top panels show staining with Fluoro-Jade C (green) imaged at 40x magnification. A. Demonstrates an example of two Fluoro-Jade C positive foci in a TBI animal, indicated by the white arrows. Foci are shown imposed with a brightfield image of the tissue. B. Demonstrates an example of a diffuse Fluoro-Jade C positive foci in a TBI animal. C. Quantification of number of fields with Fluoro-Jade-positive foci measured in fold change compared to control. TBI images show a significant (***p<0.001) increase compared to control. ICAL-treated images show a significant (##p<0.001) decrease in presence of degenerating neurons compared to TBI. D. TBI images contain more fields with multiple Fluoro-Jade-positive foci compared to sham controls measured by fold change compared to control. ICAL-treated animal trend towards a decreasing number of these fields compared to TBI. Statistical significance determined by one way ANOVA with a post hoc Tukey’s test,
n=4. Abbreviations used: ICAL, Immunocal®. Quantification and imaging credit to Tyler Wallace.
CHAPTER FOUR: DISCUSSION

As the prevalence of TBI continually increases in society, it is imperative that new therapies be explored to address this injury and its underlying pathologies. Antioxidants and GSH targeted treatments specifically, have been suggested as a promising research area in TBI (Shohami et al., 1997; Reed et al., 2009; Rodriguez-Rodriguez et al., 2014). Studies that point to readily available and non-prescription relief for TBI are also desirable compared to studies yielding a pharmaceutical product. The Food and Drug Administration (FDA) pharmaceutical approval process can take anywhere from 7.5 to 19 years. With the already devastatingly large presence of TBI and its ever-growing financial and debilitating effects on society, a nutritional supplement as an option for treatment and prevention is attractive. Therefore, the unique ability of Immunocal® to boost levels of the endogenous antioxidant GSH in the brain made it an appealing compound to study in TBI (Fig. 19; Song et al., 2017).

Here, we show that pretreatment of mice with Immunocal® prior to CCI, is unable to protect the brain tissue from primary injury mechanisms. This was seen in both our clinical and MRI data; as righting reflex and apnea times were unchanged with the pretreatment of Immunocal®, and the primary injury volume as measured by MRI was also unchanged between TBI and pre-treated animals. Interestingly, blotting revealed an increase in p-Tau levels induced by CCI at only 72 hours post procedure, with no effect seen with Immunocal® pre-treatment. McKee and Daneshvar suggest that axon shearing,
caused by the primary injury, could be responsible for this rapid tauopathy (2015). This further indicates that Immunocal® is unable to attenuate damage done by the mechanical injury. However, our motor coordination, behavioral, and histopathological data indicates that Immunocal® is able to alleviate some of the secondary injury mechanisms. We saw improved motor coordination in Immunocal®-treated mice in both the challenging beam walk and rotarod tasks. Animals pre-treated with Immunocal® showed a significantly longer time spent on an accelerating, rotating rod compared to TBI animals 9 days after CCI. Furthermore, treated animals showed a decreased number of right hind foot faults on the thinnest, most difficult section of the challenging beam walk, compared to TBI animals. Immunocal® treatment also improved spatial memory in both the Barnes maze and modified Y maze. Latency time on day 6 of the acquisition period in Barnes maze, showed a statistically significant increase in TBI animals compared to sham controls, indicating TBI animals had an impaired ability to remember the visual cue and subsequently find the escape pod. There was a subsequent decrease (improvement) in latency time seen between Immunocal®-treated animals, and TBI animals. There was also a decrease in delay time to the pod “zone” seen during the probe trial in Immunocal® animals compared to TBI. While there was no significant effect seen in either TBI or treated animals in the modified Y maze, there did appear to be an intermediate recovery in the percentage of animals investigating the unblocked arm on the first attempt, caused by Immunocal® treatment.

Our histopathological indices also indicate a protective effect, as indicated by decreased foci in Fluoro-Jade C staining. The decreased number of positive foci in sham
and Immunocal®-treated animals compared to TBI, indicate a lessened presence of degenerating neurons. Additionally, Luxol fast blue staining indicated a restorative effect in Immunocal® treated animals as compared to TBI. The TBI animals showed significantly thinner corpus callosum pathologies, suggested a demyelinating effect caused by CCI. Subsequently, we observed wider corpus callosum pathologies in pre-treated animals. Finally, in agreement with recently published data, an increase in GSH was observed in Immunocal® treated mice, despite a drop seen in mice with TBI alone (Song et al., 2017). Collectively, these data suggest that pre-treatment with Immunocal® does not help protect against the primary injury induced by CCI, but does ameliorate secondary injury mechanisms while also increasing levels of GSH in the brain post-injury. These results encourage the continued investigation into nutraceuticals and GSH modulators as therapeutic agents in TBI.

Unfortunately, much of the recent clinical research on TBI with antioxidant compounds demonstrates a modest to negligible effect (Maas et al., 2010; Rodriguez-Rodriguez et al., 2014). Currently, researchers are having difficulties mapping pre-clinical data into clinical practice for a multitude of reasons. Primarily, patients presenting with TBI often also present with a variety of comorbid injuries. Current research suggests that having other injuries along with a TBI can cause a systemic inflammatory response thereby changing the prognosis of the head injury (Xiong et al., 2013). Specifically, Maegele et al examined the effect of a fluid percussion induced TBI in rats, with or without a comorbid tibia fracture on cytokine-mediated inflammatory responses by monitoring biomarker levels in the blood (2007). Interestingly, the levels of
inflammatory markers increased with the two injuries compared to either one alone. Similarly, hypoxia has been shown to exacerbate axonal damage, and ultimately increase astrogliosis in rats following a weight drop model of injury (Hellewell et al., 2010). More research needs to be done to further elucidate the effects of comorbid injuries on TBI.

Aside from complications seen with comorbid injuries, it is also unlikely that any single compound is capable of attenuating all the primary and secondary injury profiles seen in TBI. Because of this, many researchers are starting to look into the concept of combination therapies (Margulies and Hicks, 2009; Xiong et al., 2013). For example, atorvastatin in combination with marrow stromal cells – both known effective monotherapies prior to combination therapy attempts – exhibited improved modified neurological severity scores and Morris water maze performance in female rats, compared to either one alone (Mahood et al., 2007).

Furthermore, several limitations still exist in animal models of TBI, which also impacts the ability of researchers to discover therapies that will maintain efficacy in humans. One such constraint is the current lack of a widely accepted scoring system to determine injury severity in animals (Xiong et al., 2013). This constrains researchers to determine impact in only histopathological indices. While this approach is more quantitative, it does not necessarily directly coincide with motor and behavioral abnormalities. There also exist a plethora of models of TBI in animals that are all currently in use. Different models present diverse injury pathologies, as well as varying physiological relevance in humans. While some of these differences in models are justifiable (i.e. a blast model as compared to a CCI), many of the models still in use today
are either poorly controlled, or generally lack physiological relevance. For instance, weight drop models commonly present with rebound injuries, creating potential issues with replicability between animals (Cernak, 2005). Similarly, fluid percussion procedures require a craniotomy, and careful attention must be paid to create the same injury in every case (Thompson et al., 2005, Xiong et al., 2013). This procedure also lacks physiological relevance as humans do not report to the emergency department presenting with a prior craniotomy and resulting cerebral contusion. Due to these differences, it seems as though an increase in homogeneity of models used to some extent is needed; or compounds of interest need to be tested across multiple models of TBI before being introduced clinically.

Admittedly, there are also inherent limitations to studying preventative treatment in TBI, and such trials are not necessarily meant to serve the entire population at risk for an injury. That being said, the main groups that will best be served from studies such as this would be servicemen and women, and athletes; namely because these groups of people are, or can be, generally on a fixed diet. The easiest way to administer Immunocal® would be through the diet, so any population that has an already controlled or monitored diet (and is at risk for a TBI), would be the most advantageous to work with.

Nonetheless, Immunocal® is still an attractive therapy for a potential clinical study. As far as safety and efficacy, this compound would likely exhibit minimal side effects such as modest weight gain and potentially allergenic-related reactions. The most noteworthy side effect likely being the interaction of GSH and anticancer drugs (Meister,
1991). Using Immunocal® to boost levels of GSH could potentially interfere with chemotherapy in patients that are receiving cancer treatment; though this specific population would likely be minimal in size in the already limited groups that any preventative clinical study would be investigating.

Immunocal® as a compound has the distinct ability to increase GSH levels in the brain. The research suggesting GSH as an appropriate target for TBI therapies, thereby suggests that Immunocal® could potentially be a beneficial preventative treatment of TBI.
CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

We suggest that 28-35 day pretreatment, twice daily, of mice with 3.30% Immunocal® ameliorates the secondary injury mechanisms induced by CCI (summarized in Table 1). There are a number of possible future directions with which to take this project.

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Table 1. Summary of Findings. The table summarizes the findings of the study and ranks the effects seen from various tests on a scale of – to +++: - indicates no ICAL effect, and +++ indicates $p<0.001$. 0 was assigned to elevated plus maze, as no effect was seen with TBI compared to sham, or ICAL+TBI. Abbreviations used: ICAL, Immunocal®.
First, investigating Immunocal® as a restorative treatment would be more relevant to the population at large. Recently, studies have been done using other antioxidants administered post-injury that have been successful. For instance, continual dosing with Epigallocatechin-3-gallate post CCI produced a decrease in free radical generation caused by TBI in rats (Itoh et al., 2013).

Additionally, the TBI research field as a whole is lacking in longitudinal studies. With increasing prevalence and awareness of TBI in society, the research is still largely catching up. Longitudinal studies on the impact of some of these potentially neuroprotective compounds on secondary outcomes to TBI such as chronic traumatic encephalopathy, are lacking. Going forward, the effect of treatment on future prognosis also needs to be investigated to inform potential treatments. The current approaches in this arena either assume that immediate and effective treatment of TBI symptoms will help these long term disabilities, or disregard the issue all together. It would be interesting to determine if pre- or post- injury treatment with Immunocal® improved long term outcomes commonly seen in TBI.

Finally, another potential direction would be to explore Immunocal® as a compound in a combination therapy. Since we describe the ability of Immunocal® to attenuate secondary injury mechanisms as caused by CCI, pairing it with a compound shown to protect against the primary mechanical would be appropriate. For instance, edaravone might be an appropriate compound to combine Immunocal® with for treatment, as this agent has been shown to lessen BBB disruption as a result of TBI.
(Wang et al., 2011). Future studies with Immunocal® in TBI are promising, and there are a variety of different directions in which this research could go.
REFERENCES


Wang, Guo-Hua, Zheng-Lin Jiang, Yong-Cai Li, Xia Li, Hong Shi, Yan-Qin Gao, Peter S Vosler, and Jun Chen. "Free-Radical Scavenger Edaravone Treatment Confers


