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THE EFFECTS OF DENDRO[60]FULLERENE ON NEUROBEHAVIORAL OUTCOME AND CEREBRAL VASCULAR FUNCTION AFTER TRAUMATIC BRAIN INJURY

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by

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Dedication

To my family and friends, especially my parents, for their constant encouragement to go one-step further for all these years.

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THE EFFECTS OF DENDRO[60]FULLERENE ON NEUROBEHAVIORAL OUTCOME AND CEREBRAL VASCULAR FUNCTION AFTER TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) causes an increase in reactive oxygen species (ROS) that leads to cerebral vascular dysfunction. The reduction of ROS can preserve vascular function and lead to improved outcomes. The antioxidant carboxyfullerene nanoparticle, dendro[60]fullerene (DF-1) was examined as a potential therapy for TBI because of its strong antioxidant capabilities. The effects of DF-1 on mean arterial pressure (MAP), cerebral perfusion and cerebral vascular resistance were evaluated. DF-1's ability to reduce neuronal cell injury after TBI was evaluated by Fluoro-Jade C staining in the hippocampus. Through these experiments a dose response curve was conducted to compare 10, 25 and 50-mg/kg doses of DF-1. 10-mg/kg was chosen as the most effective dose to be used in the remainder of experiments. Middle cerebral artery (MCA) experiments were performed to evaluate if DF-1 preserved vasodilatory responses after injury. DF-1's effect on cerebral perfusion was also tested in a TBI + hemorrhagic hypotension model. Additionally, behavioral experiments were performed to determine if DF-1 improved cognitive

performance. These experiments determined that DF-1 did not preserve vascular function, but it did result in improved performance in behavioral tests, as well as a preservation of neurons in the hippocampus after TBI. Due to the positive results in behavioral and neuropathological experiments, we believe that DF-1 deserves further examination as a therapeutic option for TBI.

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List of Abbreviations

AA	Arachidonic Acid
BB	Beam Balance
BBB	Blood Brain Barrier
BW	Beam Walk
CCI	Controlled Cortical Impact
CO_2	Carbon Dioxide
$CMRO_2$	Cerebral Metabolic Rate for Oxygen
CNS	Central Nervous System
CVR	Cerebral Vascular Resistance
DF-1	Dendro[60]fullerene
FJC	Fluoro-Jade C
FPI	Fluid Percussion Injury
HH	Hemorrhagic Hypotension
H_2O_2	Hydrogen Peroxide
IA	Impact Acceleration
MAP	Mean Arterial Pressure
MCA	Middle Cerebral Artery
MWM	Morris Water Maze
NO	Nitric Oxide
$PaCO_2$	Partial Pressure of Carbon Dioxide
PEG-HCCs	Poly(ethylene glycol)-functionalized Hydrophilic Carbon Nanoclusters

PID	Post-injury Days
PSS	Physiologic Salt Solution
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBI	Traumatic Brain Injury

Chapter 1: Introduction

Traumatic Brain Injury

TBI is an important Health Care Issue

Traumatic brain injury (TBI) is defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force (Menon 2010)." It is one of the most common, debilitating neurological conditions. TBI is a leading cause of morbidity and mortality worldwide (Coronado 2011, Natale 2003, Zasler 2007), affecting over 2.5 million people annually (Center for Disease Control 2014, McFadden 2011). Brain injury is a contributing factor to 30% of all injury related deaths in the U.S. (Center for Disease Control 2014, Yue 2013). In the United States, nearly 7,000 brain injuries occur each day (Center for Disease Control 2014, Yue 2013). Of these cases, approximately 275,000 individuals are hospitalized (Yue 2013) and over 50,000 deaths occur (Cecil 2010, Ling 2010, Yue 2013). Worldwide TBI is accounts for the greatest number of disability-adjusted life years lost (Ghajar 2000). Determine the amount of people worldwide is difficult because some countries lack the epidemiological monitoring necessary for accurately determining the number of affected patients. We do know that incidence ranges widely amongst countries. Many countries such as Sweden, Germany, South Africa and Brazil have much higher incidence rates than that of the United States. Brain injury is a substantial public health issue in worldwide in both the developed and developing world (Roozenbeek 2013).

TBI Results in Neurological, Neuropsychological and Cognitive Deficits

After brain injury, a cascade of biochemical processes are initiated that lead to neuronal cell death and cognitive dysfunction (Hovda 2014, Natale 2003). These processes

can result in long-term cognitive, psychological, emotional and motor impairments (Marklund 2006, Masel 2010, Wagner 2004). Half of the individuals, whose injuries required hospitalization, suffer from some impairment for the duration of their lifetime (Kurland 2012). There are a wide variety of serious disorders that can result after TBI, including psychiatric diseases, epilepsy, and sleep disorders (Fleminger 2008, Hauser 1991, Masel 2010, McLean 1984, Zasler 2007). TBI also increases the likelihood of suffering from other neurological disorders such as Alzheimer's and Parkinson's disease (Bower 2003, Masel 2010, Schofield 1997). Over 5.3 million Americans are permanently disabled due to past head injuries (Bramlett 2004, Zasler 200713). The total acute and long-term care medical costs related to TBI amount to over 60 billion dollars annually (Coronado 2011, Kurland 2012). Currently there are no commercially available pharmacological treatments for TBI (Agoston 2012, Golding 2002, Marklund 2006, Morales 2005), therefore there is a major need for the identification and/or development of effective therapeutic interventions.

TBI Results in Cerebral Vascular Dysfunction

Biochemical processes that are initiated after brain injury result in significant impairment of the cerebral vasculature (DeWitt 2003, Golding 2002). TBI can cause significant decreases in cerebral perfusion and reduce the cerebral metabolic rate for oxygen ($CMRO_2$) (Bouma 1991, DeWitt 2003, Gobiet 1975, Martin 1997). Cerebral perfusion after TBI has important implications to morbidity and mortality rates (DeWitt 2003). In some patients decreased cerebral perfusion is not problematic because $CMRO_2$ is also decreased and even reduced levels of cerebral perfusion are capable of meeting metabolic demands (Diringer 2002, Martin 1997, Obrist 1984). This is not always the case,

and in some patients cerebral perfusion can be too low to properly meet the brain's metabolic demands (Graham 1996). Autoregulation is also impaired following TBI (Cold 2012, Enevoldsen 1978, Golding 2002). TBI results in disruption of the blood brain barrier (BBB) (Golding 2002, Hicks 1997). Brain injury causes damage to components of the BBB, including the endothelium, pericytes and astrocytes. This results in the opening of the blood brain barrier. This allows for abnormal movement of water to the parenchyma, leading to edema. The breakdown of the blood barrier can also cause harmful proteins that are normally excluded to enter the brain. At the same time, the transport of nutrients that are vital to brain function can be impaired (Golding 2002). Impairments to cerebral perfusion, $CMRO_2$ and autoregulation are all contributing factors to increased morbidity and mortality (Chesnut 1993, DeWitt 2003, Luerssen 1988, Miller 1985).

Normal Cerebral Vascular Function

Reactivity of the Cerebral Circulation

To understand the pathophysiology of cerebral vascular dysfunction, we will first review normal vascular function. The cerebral circulation is highly reactive and arteries dilate and constrict in order to maintain proper cerebral perfusion and meet the brain's metabolic demands (Golding 2002, Kuschinsky 1978). Many factors act to control vascular reactivity including, arterial blood pressure, oxygen, carbon dioxide (CO_2) and metabolic activity (Kontos 1981, Kontos 1978, Kuschinsky 1978, Rumbaugh 1995). One way cerebral sympathetic nerves react to changes in arterial blood pressure is by constriction and dilation of blood vessels (Kontos 1978, Kontos 1981). The vasculature is highly reactive to CO_2 levels (Kontos 1981) and responds to changes in the partial pressure of carbon dioxide ($PaCO_2$) (Kuschinsky 1978, Rumbaugh 1995). When $PaCO_2$ is high, vasodilation of the arteries occurs to increase cerebral perfusion and decrease cerebral

vascular resistance (CVR). When $PaCO_2$ is low, vasoconstriction occurs and cerebral perfusion is decreased, while CVR is increased (Kontos 1981, Rumbaugh 1995). These changes in response to $PaCO_2$ levels are mediated by the vascular smooth muscle reacting to hydrogen ion concentrations (Kontos 1981). Adrenergic and cholinergic mechanisms in the brain stem are also thought to help mediate the cerebral vasculatures' response to changes in CO_2 levels (Kuschinsky 1978, Lance 1983). Changes in arterial oxygen tension also have an effect on vasodilation and constriction but not to the same degree that $PaCO_2$ does (Rumbaugh 1995). Cerebral vascular reactivity is also affected by metabolic demand, which is strongly coupled to cerebral perfusion (Kontos 1981). When metabolic rate rises in a region of the brain, blood flow to that region also increases (Ingvar 1976, Kontos 1981, Sokoloff 1977). This relationship is mediated by vasodilator metabolites such as adenosine, hydrogen ions and potassium ions (Kontos 1981).

Autoregulation

Autoregulation of the cerebral vasculature functions to maintain constant cerebral perfusion in response to changes in arterial blood pressure (Kontos 1981, Kuschinsky 1978, Strandgaard 1973). These compensatory mechanisms are able to maintain cerebral perfusion easily when blood pressure is between 60 mmHg and 160 mmHg. Outside of this range, the cerebral circulation is incapable of maintaining normal levels of cerebral perfusion (Golding 2002, Lassen 1959). Autoregulation is mediated by a wide variety of chemical factors; known as vasoconstrictors and vasodilators (Golding 2002), many of which are released by the vascular endothelium of arteries and arterioles (Golding 2002, Kuschinsky 1978, Strandgaard 1973, Vane 1990). Cerebral arteries/arterioles dilate in order to increase cerebral perfusion when blood pressure decreases. When blood pressure

increases, cerebral arteries/arterioles constrict to reduce blood flow (Kuschinsky 1978, Strandgaard 1973). The amount of dilation and constriction of the blood vessels is dependent on vessel size. Large arteries are more responsive than smaller arterioles and react to small changes in blood pressure. When blood pressure is high, approximately 110 mmHg to 160 mmHg, only the largest vessels reacted to maintain cerebral perfusion. The smallest vessels reacted only below 90 mmHg and above 160 mmHg; intermediate vessels began reacting after the largest vessels but before the smallest vessels. The larger arteries contribute more to CVR when blood pressure is high, than the smaller arterioles. However, as blood pressure changes, the portion of the vasculature that contributes to changes. When pressure is between 90 and 120 mmHg the larger vessels are dilated and it is the smaller arterioles that contribute the most to CVR. When pressure is very low, both arterioles and arteries are dilated and it is the passive vessels that contribute the most to vascular resistance (Kontos 1978).

Clinical Traumatic Cerebral Vascular Dysfunction

TBI Reduces Cerebral Perfusion in Patients

Brain injury impairs the body's normal control of the cerebral vasculature and this results in a variety of detrimental consequences (Bouma 1991, DeWitt 2003, Golding 2002). In some patients, TBI is associated with decreased cerebral perfusion (Bouma 1991, DeWitt 2003, Gobiet 1975, Martin 1997). Decreased cerebral perfusion is also referred to as hypoperfusion (Martin 1997). Hypoperfusion typically occurs within the first 12 hours of injury. It is sometimes accompanied by ischemia (Bouma 1991, Martin 1997). Decreased cerebral perfusion is associated with poor neurological outcomes and increases the brains susceptibility to secondary insults, such as ischemia (Giri 2000, Golding 2002, Hlatky 2003, Jenkins 1989, Robertson 1992). Early ischemia occurs in approximately one

third of patients (Bouma 1991). The cause of these changes to cerebral perfusion after TBI is not clear. However, the coupling between cerebral perfusion and metabolic demand that occurs in healthy individuals is no longer present after injury (Golding 2002), this can result in low $CMRO_2$ (DeWitt 2003, Martin 1997). Cerebral perfusion typically increases to baseline levels by 24 hour after injury (Bouma 1991).

TBI Impairs Autoregulation

Autoregulation is frequently either partially or fully impaired after TBI and this can persists for several days after injury (DeWitt 2003, Engelborghs 2000, Golding 2002, Overgaard 1974). Arteries and arterioles become less responsive to changes in $PaCO_2$ in the first few days following TBI (DeWitt 2003, Overgaard 1974). Notably, the vasodilatory response is impaired more frequently than vasoconstriction after injury (Bouma 1990, DeWitt 2003). After TBI, levels of vasoconstrictors are increased, and vasodilators levels are diminished, leading to enhanced vasoconstriction (Armstead 2012, Enevoldsen 1978, Golding 2002, Overgaard 1974). In 50% of patients autoregulation is impaired and this is often accompanied by vasospasm (Martin 1997). Vasospasm is typically resolved within three or four weeks of injury (Martin 1995, Martin 1997, Zane 1992).

Clinical Consequences of Impaired Autoregulation

Trauma-induced impairment of autoregulation plays a direct role in the increased risk of posttraumatic hypotension or hypoxemia (Chesnut 2003, DeWitt 2003, Engelborghs 2000, Luerssen 1995). Hyperemia and impaired autoregulation may follow vasospasm in some patients (Rumbaugh 1995). Increased hypotension and hypoxemia, especially when coupled with impaired autoregulation, have been correlated with a rise in morbidity and mortality rates in TBI patients (Chesnut 2003, DeWitt 2003, Luerssen 1988, Luerssen

1995). TBI with associated hypotension more than doubles mortality and morbidity compared to normotensive TBI patients (Luerssen 1995). Because cerebral vascular function and autoregulation after TBI has important implications to patient outcome; it is an important area for potential therapeutic intervention (DeWitt 2003, Luerssen 1988, Luerssen 1995).

Cerebral Vascular Dysfunction In Experimental Animals

TBI Reduces Cerebral Perfusion and Impairs Autoregulation in Experimental Animals

There are several animal models designed to mimic the effects of TBI in human populations, including impairments to the cerebral vasculature. Many models reduce cerebral perfusion, which has been established as an important factor in clinical TBI (Dietrich 1994, Ishige 1987, Yamakami 1989). Fluid percussion injury (FPI) and controlled cortical impact (CCI) both significantly reduce cerebral perfusion to approximately 50% of baseline for period up to four hours post-injury (Cherian 2000, Yamakami 1989). FPI also impairs cerebral dilator responses to hypoxia or hypercapnia occurs (Ishige 1987, Lewelt 1982, Yamakami 1989). The impact acceleration (IA) model also results in decreased cerebral perfusion that lasted over five hours (Prat 1997). In addition to reductions in cerebral perfusion, TBI results in significant impairment of autoregulation in animal models, just as it does in the clinical population. FPI, CCI, IA and weight drop models all damage autoregulation (Forbes 1997, Nawashiro 1995, Prat 1997, Wei 1980b). FPI prevents the cerebral vasculature from properly responding to changes in $PaCO_2$ (Dixon 1987) and results in impaired vasoconstriction (DeWitt 1992, Lewelt 1980, Wei 1980b).

Cellular Mechanisms of Cerebral Vascular Dysfunction: Reactive Oxygen/Nitrogen Species

TBI Alters Levels of Cerebral Vasoconstrictors and Vasodilators

As previously stated, many chemical factors are involved in the control of arterial dilation and constriction (Golding 2002, Kuschinsky 1978). TBI causes alterations in cerebral vasoconstrictor and vasodilators. (DeWitt 1988). After brain injury, the imbalance of vasoconstrictors and vasodilators leads to heightened levels of vasoconstriction (Golding 2002). Nitric oxide (NO) is an important vasodilator, and its presence is necessary for maintaining a state of partial dilation (Brian 1996, Faraci 1994). After brain injury, NO levels are reduced and this leads to sustained vasoconstriction (Cherian 2000). Prostacyclin, a vasodilator, is decreased following trauma (Armstead 1998). Other vasodilators such as vasopressin and calcitonin gene related peptide are also reduced or impaired (Armstead 1996, Armstead 1999). Vasoconstrictors such as thromboxane, endothelin, leukotrienes and monohydroxy-eicosatetraenoic acids are elevated after TBI (Armstead 1996, DeWitt 1988, Golding 2002). Abnormally high levels are reactive oxygen species (ROS) and reactive nitrogen species (RNS) also contribute to impaired autoregulation after TBI (Bains 2012, Golding 2002, Kontos 1985, Kontos 1986b).

ROS & RNS Production After TBI

Brain injury results in an overproduction of ROS and RNS that are derived from multiple sources (Bains 2012, DeWitt 2003, Kontos 1985, Kontos 1989, Li 2015, Serizawa 2014). ROS (Fabian, et al., 1995; 1998) and RNS production begins to rise immediately following trauma and they are a major contributor to damage after TBI (Serizawa 2014). The influx of oxygen radicals after TBI is initiated by the creation of superoxide (Bains 2012). After injury, phospholipases C and A_2 are activated, releasing arachidonic acid

(AA). AA is catalyzed by cyclooxygenases into cyclic endoperoxides. Endoperoxide is metabolized to produce prostaglandins. It is during the production and metabolism of cyclic endoperoxides that superoxide is created and the rates of these reactions are accelerated after TBI (DeWitt 2003, Kontos 1985). Superoxide is catalyzed by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2) and oxygen. The hydroxyl radical is created when ferrous iron is oxidized in the presence of H_2O_2 (Bains 2012, Kontos 1989). The hydroxyl radical is more reactive than superoxide, but superoxide plays in important role in the formation of peroxynitrite by reacting with NO. Peroxynitrite then reacts to form RNS such as nitrogen dioxide (Bains 2012, Wei 1985).

The respiratory chain that takes place within mitochondria is the source responsible for much of the ROS and RNS after brain injury. An influx of calcium into the mitochondria that occurs after TBI results in disruption of the electron transport chain and an overproduction of ROS (Carriedo 2000, Merenda 2006). Superoxide is the primary ROS that is generated by the mitochondria (Keller 1998). The mitochondria are also damaged by ROS/RNS release after TBI. This damage disrupts mitochondrial energy metabolism and lead to ROS-induced apoptosis (Lifshitz 2004, Merenda 2006). ROS also damage mitochondrial cell membranes through lipid peroxidation and lead to oxidative damage and cleavage of the mitochondrial genome (Fiskum 2004, Nakahara 2004, Merenda 2006). Lipid peroxidation is induced in mitochondria when superoxide interacts with NO and peroxynitrite is created (Keller 1998). Damage of the mitochondrial membrane through lipid peroxidation causes mitochondrial dysfunction and disrupt energy metabolism (Vagnozzi 1999).

Antioxidant Systems

The body has natural antioxidant systems that are able to maintain proper homeostasis of ROS and RNS in healthy individuals (DeWitt 2003, Golding 2002, Kontos 1986b). Antioxidants such as SOD, glutathione peroxidase and peroxiredoxin, act to control levels of ROS and RNS. However, TBI overwhelms the bodies' natural antioxidant system (DeWitt 2003, Golding 2002, Kontos 1986b, Serizawa 2014). The central nervous system is more susceptible to damage from ROS and RNS because it has lower antioxidants levels and higher levels of iron and polyunsaturated lipids compared to other regions of the body (Fabian 1998). This is important because ROS and RNS are thought to be major contributors to cerebral vascular dysfunction after TBI (DeWitt 1997, Kontos 1989). Levels of oxidative stress have been found to parallel injury severity (Ansari 2008b, Cernak 2000, Lin 2002). In addition to vascular injury, ROS and RNS also contribute to neuronal damage and death after TBI (Cornelius 2013, Kontos 1989, Tyurin 2000). Increased ROS and RNS release after TBI causes direct damage to the vasculature by protein oxidation, DNA damage, mitochondrial damage, lipid peroxidation, as well as inducing apoptosis (Bains 2012, Kontos 1989, McIntosh 1998, Serizawa 2014, Werner 2007). Reducing ROS and RNS levels after traumatic cerebral vascular injury (Bains 2012, DeWitt 1997; DeWitt 2003, Wei 1981) has the potential to improve trauma-induced cerebral vascular dysfunction (Bains 2012, Hall 1993b, Yoshida 2006).

Effects of ROS/RNS on Cerebral Perfusion & Autoregulation

Pathological increases in ROS and RNS levels after TBI result in abnormal arteriole dilation, impaired cerebral vascular reactivity, reduce oxygen metabolism, create endothelial lesions and damage the BBB (Abdul-Muneer 2014, DeWitt, 2003, Kontos

1986a, Kontos 1989, Smith 1994). ROS affect autoregulation by reducing vasoconstrictors and impairing vasodilation (Kontos 1980). The increase in hydroxyl radicals that occurs after injury affect vasodilation, damage the endothelium and cause peroxidation of polyunsaturated fatty acids that damages the BBB (Golding 2002, Smith 1994). The overproduction of ROS that overwhelm the brain's natural antioxidant system, leads to the formation of peroxynitrite (Golding 2002, Wei 1985). Peroxynitrite plays a role in ischemia, causes cerebral vascular dysfunction and impairs autoregulation after TBI (Beckman 1994, DeWitt 2003). Multiple studies have demonstrated that reduction of ROS and RNS after TBI by antioxidants improved cerebral vascular reactivity (Abdul-Muneer 2014, Hall 1993b) preserved the BBB (Smith 1994, Zhao 2007) and improved cognitive outcome after TBI (Beni 2004, Kelso 2011).

Antioxidant Mechanisms

There are multiple mechanisms in which antioxidants protect against oxidative stress. One way is through the inhibition of ROS and RNS generation (Ansari 2008a, Ozturk 2005). Ginkgo biloba is one example of an antioxidant that acts by preventing ROS and RNS formation. A study by Bastianetto, et al. demonstrated that the extract protected hippocampal cells from β-amyloid induced toxicity in an *in vitro* Alzheimer's disease model by inhibiting free radical production (Bastianetto 2000, Lin 2002). Tirilazad mesylate is another antioxidant that works by inhibiting ROS formation, specifically hydroxyl radicals (Hall 1992, Hall 1993a, Smith 1994). Antioxidants can also act by binding the metals that are needed to form ROS (Ansari 2008a, Ozturk 2005). Additionally, antioxidants can reduce oxidative stress by neutralizing ROS and RNS (Ansari 2008a, Ozturk 2005). Previous laboratory studies have shown that free radical

scavengers such as dimethyl sulfoxide, and human serum albumin are capable of improving motor outcomes after TBI. Grip test, and neuroscore tests were performed to measure these three outcomes (Wheaton 2011). Glutathione peroxidase functions by neutralizing peroxides and glucose-6-phosphate also neutralized ROS and RNS by donating electrons (Ansari 2008a, Dringen 2005). Superoxide dismutase converts superoxide to H_2O_2 and oxygen, then catalase and/or glutathione peroxidase converts H_2O_2 to oxygen and water (Lewen 1998).

Antioxidants Improve Cerebral Perfusion/Autoregulation

It is well established that antioxidant reduction of the ROS and RNS that damage the cerebral vasculature is a significant point for therapeutic intervention after brain injury (Bains 2012, Kontos 1980, Muir 1995, Wei 1980a). Previous antioxidant studies have demonstrated that antioxidants are capable of improving trauma-induced impairments to autoregulation and cerebral perfusion in animal experiments (DeWitt 1997, Kontos 1986a, Kontos 1986b, Kontos 1992, Lin 2002, Wheaton 2011, Zasler 2007). The protective mechanism of these antioxidants to preserve cerebral vascular function can vary (Ansari 2008a, DeWitt 2003). Cyclooxygenase inhibitors and oxygen radical scavengers can be used to scavenge ROS and preserve autoregulation and reduce endothelial lesions (Kontos 1986a). Treatment with SOD has been shown to increase cerebral perfusion after TBI (DeWitt 1997, Muir 1995). Tirilazad mesylate is capable of preserving the BBB after TBI through inhibiting lipid peroxidation (Hall 1992, Hall 1993a, Smith 1994). Previous clinical trials that attempted to use antioxidants to treat TBI have been unsuccessful thus far at improving patient outcomes (Marshall 1998, Young 1996). This is thought to be due to the difference in time administration in animal studies (immediately following injury)

versus clinical treatment (six hours after injury) (Dewitt 2003, Muir 1995, Wei 1981). However, investigation of antioxidants to reduce cerebral vascular damage are worthwhile because studies have indicated a longer therapeutic window in cerebral vascular injury lasts as long as 12 hours after injury (Ji 2012).

Advantages & Disadvantages of Nanoparticles

Nanoparticle antioxidants have advantages over other classes of antioxidants, as they are able to fully quench ROS without the aid of a second molecule (Bitner 2012). Carboxyfullerenes differ in their antioxidant potential and relative toxicity. These properties are dependent on addends, the attachments used to make them biologically functional (Beuerle 2008). Each addend renders fullerenes particles less stable and potentially more toxic and they can also reduce their antioxidant strength. Due to this property of addends, DF-1, a monoadduct, should have a greater affinity for ROS while being less toxic than other carboxyfullerenes with multiple addends (Beuerle 2008). While DF-1 and other carboxyfullerenes are both "highly soluble in aqueous buffers," DF-1 (Beuerle 2008) should be safer and more effective than other multiple addend carboxyfullerenes (Nielsen 2008).

Antioxidant Nanoparticles

Carboxyfullerene Nanoparticles

Carboxyfullerenes nanoparticles have been described as "radical sponges" and experiments in biological systems have demonstrated their strong antioxidant properties (Beuerle 2008, Brown 2010). It was first prepared in 1985 (Nielsen 2008, Partha 2009), but later was discovered to occur in nature (Buseck 1992). These truncated icosahedrons (Partha 2009) are comprised entirely of carbon atoms (Taylor 1993) arranged in a hollow spherical shape with a diameter of 0.7 nm (Nielsen 2008). C-60 fullerene has over 30

double bonds. The curved structure and series of π-double bonds gives carboxyfullerenes their strong antioxidant properties (Beuerle 2008). It has not been determined if fullerenes' donate or accept electrons to neutralize ROS. Suggested mechanisms include the attachment of stoichiometric ROS to the fullerenes surface followed by later elimination and the donation of the outer electrons of the carboxyfullerenes for radical reduction (Beuerle 2008). Furthermore, the modified fullerenes, dendro[60]fullerene (DF-1) and C3, can both scavenge NO. They also prevent NO formation by inhibiting NO synthase (Nielsen 2008).

Because unmodified carboxyfullerenes have a long biological half-life, there is some potential for bioaccumulation and toxicity. While there are few studies on the toxicity of carboxyfullerenes, currently, it is thought that their toxicity is low (Nielsen 2008). Unmodified carboxyfullerenes are almost completely insoluble in water (solubility = 0.1 ng/l (Nielsen 2008)), resulting in aggregations that alter their properties (Partha 2009). Fullerenes are naturally hydrophobic but the addition of functional groups creates water-soluble molecules for biological applications (Dugan 1997, Quick 2008, Theriot 2010). Functional attachments, or addends, that confer solubility have implications on other aspects of carboxyfullerene functions, such as absorption, distribution and excretion (Beuerle 2008).

Carboxyfullerene Nanoparticles Use in TBI & CNS Injury

While carboxyfullerenes have yet to be investigated in relation to TBI, they have been demonstrated to be effective in reducing ROS and improving outcome in other central nervous system (CNS) disorders (Baati 2012, Dugan 1997, Partha 2009). Modified carboxyfullerenes reduced apoptosis and excitotoxic necrosis (Partha 2009) and decrease

neuronal cell death induced by NMDA or AMPA in cultured cortical neurons (Dugan 1997). C3 nanoparticles were able reduce cognitive deficits in Morris Water Maze (MWM) testing and extended the life span when orally administered to mice (Quick 2008). The well-studied trisadduct carboxyfullerene C3 has been tested in many models including for Alzheimer's disease, ALS and aging (Dugan 1997, Quick 2008). Other carboxyfullerenes have also been shown to reduce neuronal cell death in rats caused by ischemic injury (Lin 2002). While carboxyfullerenes have not been investigated in TBI, an antioxidant nanoparticle with a similar mechanism of action has been used (Bitner 2012). Poly(ethylene glycol)-functionalized hydrophilic carbon nanoclusters (PEG-HCCs) improved cerebral perfusion when administered during resuscitation in a rat TBI + hemorrhagic hypotension (HH) model (Bitner 2012).

Introduction of Experiments

Hypothesis & Aims

For all experiments we choose to work with the carboxyfullerene nanoparticle, DF-1. DF-1 has only one addend and therefore should have a greater affinity for ROS. It also has the advantage of being less toxic than other carboxyfullerenes with multiple addends (Beuerle 2008). The central hypothesis of this dissertation work was that the administration of DF-1 would improve cerebral vascular function, neuropathologic and behavioral outcomes after TBI with and without posttraumatic hemorrhagic hypotension. Aim one examined how DF-1 affected cerebral perfusion, cerebral vascular reactivity, vasodilatory responses of the middle cerebral artery (MCA) to reduced intravascular pressure and neuropathological outcome in the hippocampus. Aim two was to test if DF-1 would improve the cerebral perfusion after TBI + HH. In the final aim, we determined if DF-1

would improve motor function in beam walk (BW) and beam balance (BB) testing and working memory function MWM testing.

Fluid Percussion Injury

The fluid percussion device (figure 1) used in all of our experiments consists of a fluid filled cylinder reservoir. The reservoir is 60 cm long and 4.5 cm in diameter. At one end of the reservoir is a piston mounted on O-rings and at the other end of the reservoir is a transducer attached to a tube that is 2 mm in diameter and has a male Luer Lock adapter attached to the other end (Dixon 1987). Prior to injury a 3 mm craniotomy was trephined 2.5 mm from the midline, midway between the lambda and bregma. When the animal is fully prepared for injury, the 2 mm tube is connected to the craniotomy site at the opening of the skull (Dixon 1987, Hawkins 2013). Fluid is then rapidly injected into the epidural space by a pendulum, that is approximately 5 kg, striking the fluid reservoir (McIntosh 1989). This produces an injury to the brain as a result of tissue strain (Dixon 1987, Thibault 1992). The predetermined height of the pendulum corresponds to injury level. The transducer measures the pressure pulse and verifies that injuries between animals are uniform (McIntosh 1989). This model produces physiological, histopathological and neurological changes that mimic clinical head injuries in humans. We choose moderate parasagittal FPI in Sprague-Dawley rats as the injury method for a number of other reasons. Parasagittal FPI is one of the most common types of injury methods used in TBI research and therefore offers the advantage of being well described and understood (Meaney 1995, Morales 2005). It is also well established for producing results that are measureable in behavioral tests (Morales 2005). Additionally, compared to other animal's rats are low cost and easy to handle (Morales 2005).

Fluid Percussion Injury Clinical Relevance

It has been well established that the FPI model is representative of clinical head injuries in human patients (McIntosh 1989). Physiologically, FPI produces a short hypertensive period immediately after TBI, followed by a period of hypotension. Hypotension is common in clinical TBI patients and is a major secondary complication with important implications to outcome (McIntosh 1989). Hypertension occurs after FPI for a brief period and in cases of severe injury is followed by a period of hypotension (McIntosh 1989). FPI results in initial, transient cerebral perfusion increases followed by prolonged reductions (Morales 2005). Cerebral autoregulation is also impaired following FPI (DeWitt 2003). The fact that FPI is known to cause neuronal damage in the hippocampus, along with changes in cerebral perfusion, blood pressure and autoregulation is important because these are all aspects of TBI that we studied in this project. This rodent injury model is also well established for behavioral tests, another component that we plan to test (Morales 2005).

Biomechanics of FPI

Histological studies of FPI show that damage is seen primarily in the cortex, as well as in the brainstem (McIntosh 1989). These are areas that are frequently damaged in clinical TBI (McIntosh 1989). FPI results in the diffuse axonal damage that is characteristic of human head injury (Marmarou 1994). The histological damage that occurs to neurons due to TBI has been found to correlate with injury severity (McIntosh 1989). It has been well established through the use of Nissl, silver and Fluoro-jade staining, that hippocampal neuronal death occurs bilaterally in this model. The diffuse white matter damage is similar to clinical observations (Morales 2005). It has been well established that when neuronal

damage and death occur in the hippocampus, the damage is bilateral and the diffuse white matter damage is similar to that observed in humans (Morales 2005). The model can be modified to create both diffuse and focal injury (Morales 2005).

FPI causes an increase in intracranial pressure that results in displacement and strain of brain tissue in the foramen magnum. In severe FPI the strain that occurs is not uniform (Dixon 1987, Thibault 1992). The lower brain stem receives higher levels of strain than the rest of the brain tissue (Dixon 1987, Thibault 1992). Strain caused by FPI results in tissue herniation and results in enough force to stretch axons (Thibault 1992). The majority of axonal damage seen after lateral FPI is seen at the site of injury and extends into the ipsilateral internal capsule and fimbria (Meaney 1994). Central FPI causes axonal damage at the site of injury and in the brain stem. Some injury occurs in the brain stem after lateral FPI though it is less extensive than that which occurs after central FPI (Meaney 1994). The strain that occurs in the brain stem is great enough to cause functional impairments of axons (Thibault 1992).

Description of Experiments

Our hypothesis was that DF-1, a powerful antioxidant nanoparticle, would improve vascular function after injury, reduce hippocampal cell death and behavioral outcomes after TBI with and without posttraumatic hemorrhagic hypotension. In all experiments, male Sprague-Dawley rats were subjected to moderate FPI. In aim one we examined the effects of DF-1 administration on cerebral perfusion (laser Doppler flowmetry), CVR and MAP after injury. Twenty-four hour after injury, brains were stained with Fluoro-Jade C (FJC) and the numbers of FJC positive neurons were counted in hippocampal cell layers CA1, CA2 & CA3. Additionally, in another set of animals, MCA were harvested two hours after

injury and their responses to incremental decreases in pressure from 100 to 20 mmHg were tested. In aim two, rats were subjected to moderate fluid percussion TBI or sham TBI with and without HH. Five minutes after injury, DF-1 was administered and MAP was lowered to 40 mmHg by removal of blood via the jugular vein for 50 min. This was followed by resuscitation with saline and then shed blood. Relative cerebral perfusion (laser Doppler flowmetry) and MAP were measured during TBI, hemorrhage and resuscitation. In aim three, Sprague-Dawley rats subjected to moderate FPI or sham FPI were given DF-1 one hour after injury. Animals were tested using BB and BW tasks on post-injury days (PID) 0-4 and the MWM on PID 11 to 15.

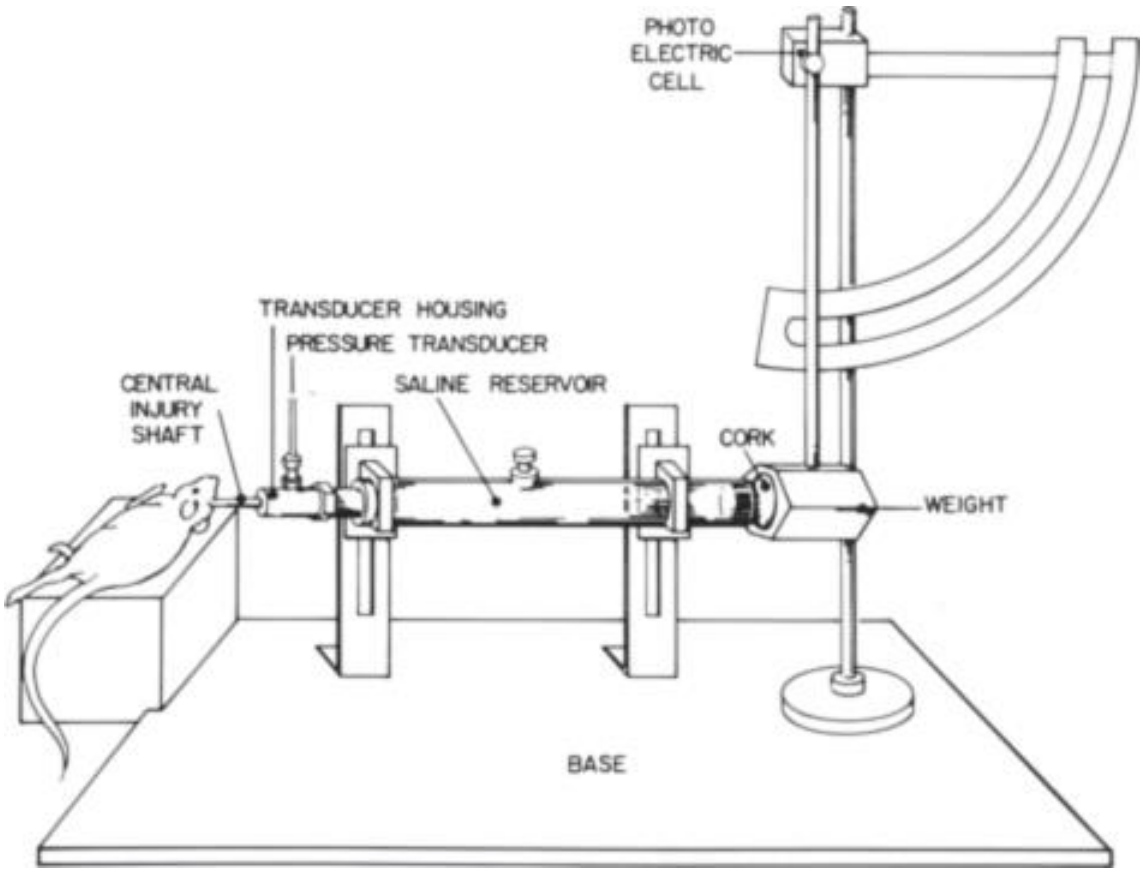

Figure 1 - Shows a diagram of the fluid percussion device that was used in these experiments (Dixon 1987).

Chapter 2: Materials & Methods

Animals

All animal experiments adhered to the National Institutes of Health's guidelines for ethical animal care and use and were approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee. Adult (3-6 month, 350-425 g) male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed two per cage at constant temperatures (21°–23°C) and humidity levels (45–50%). They were housed in a 12-hour light and dark cycle with food and water provided *ad libitum*.

Animal Surgical Preparation

Rats were anesthetized with 4% isoflurane in an anesthetic chamber, intubated, and mechanically ventilated (1.5-2.0% isoflurane in O_2: room air (30:70) using a volume ventilator (EDCO Scientific, Chapel Hill, NC)). All survival surgeries were performed under aseptic conditions (clean surgical gowns, sterile gloves and head covers). Instruments were autoclaved prior to use and drapes were used during the procedures. Incision sites were shaved, scrubbed, then rinsed with alcohol and painted with iodine prior to procedures. Throughout the procedure, rectal and temporalis temperatures were monitored and maintained at 37.0±0.5 °C with an overhead lamp and water blanket (Gaymar, Orchard Park, NY). Rats were prepared for parasagittal fluid percussion TBI as described by Dixon, et al (Dixon 1987). Anesthetized rats were placed in a stereotaxic head holder and the skin over the dorsal surface of the skull was incised at the midline and reflected along with the temporalis muscles bilaterally. A 3 mm craniotomy was trephined 2.5 mm from the midline between the lambda and bregma and the dura was left intact. A

modified plastic Luer-lok needle hub was cemented over the craniotomy using cyanoacrylic and dental cement (Hawkins 2013a).

Cannulation of Jugular Vein and Tail Artery

For some experiments, the right jugular vein and/or the tail artery of the animal were cannulated. The tail artery catheter was used so that mean arterial pressure could be monitored, while the jugular vein was used for removing blood to produce the hemorrhage injury. This was done in order to deliver DF-1 nanoparticles. For the jugular vein silastic tubing was used whereas polyethylene tubing was used for the carotid artery and tail vein.

Parasagittal Fluid Percussion Injury

Following the surgical preparation, rats were connected to the fluid percussion device at the craniotomy site and subjected to fluid percussion TBI. Immediately after injury, rats were disconnected from the FPI device. Post-injury, the Luer Lock trauma adapter was removed and the scalp was sutured. In the case of survival experiments, rats were placed in a cage and monitored hourly for at least four hours. For non-survival experiments, animals were euthanized at the conclusion of the experiment by decapitation while deeply anesthetized (4.0% isoflurane).

Hemorrhagic Hypotension

For these experiments a jugular catheter and tail vein cannulation were inserted during surgical preparations prior to FPI. HH was initiated five minutes following FPI. In the first phase of the hemorrhage model blood was withdrawn into heparinized tubes through the jugular catheter until MAP reached 40 mmHg. Prior to beginning HH an approximately volume to achieve an MAP of 40 mmHg was calculated. The predicted volume was calculated as 2.0 mL/100g. When HH was initiated the first 50% of the blood

volume calculated was removed in the first five minutes, 25% in the second five-minute interval and the remaining volume in the next five minutes. If 40 mmHg was not achieved 1.0 mL per minute could be removed until the proper level was achieved. This level was sustained for fifty minutes following the start of HH injury. In the second phase, one mL boluses of saline were given every minute until MAP reached 50 mmHg. This was sustained for 30 min. The final phase, reperfusion, lasted 30 minutes. During this time blood was reinfused at a rate of two mL per minute and the animal was ventilated on 100% oxygen (Bitner 2012, Navarro 2012).

Laser Doppler Flowmetry

To measure relative cerebral perfusion using laser Doppler flowmetry (Haberl 1989, Stern 1977), the skull to the left of the sagittal suture was thinned using an air-cooled mini drill and a black rubber tube was glued in place. A laser Doppler probe was placed over the thinned skull and positioned to avoid large blood vessels using a stereotaxic electrode holder.

Middle Cerebral Arteries Experiments

Animals subjected to moderate FPI were treated with a 10-mg/kg dose of DF-1 one hour after injury. One hour after treatment (two hours after injury), the MCA was isolated from the rats. A 2 mm section of the MCA was mounted on an Arteriograph (Living Systems, St. Albans, Vermont). A physiologic salt solution (PSS) was prepared of 130-mmol/L NaCl, 4.7 mmol/L KCl, and 1.17 mmol/L $MgSO_4 7H_2O$, 5-mmol/L glucose, 1.50 mmol/L $CaCl_2$, and15 mmol/L $NaHCO_3$. The MCA segment was bathed in this PSS at room temperature for 60 minutes with the intravascular pressure set at 50 mmHg. During this time, the vessel was monitored for leaks indicated by a decrease in pressure. If there

was a leak that could not be corrected, the vessel was excluded from the study (DeWitt 2001).

Prior to experiments the vessel was exercised by alternating the pressure between 20 mmHg and 40 mmHg three times in five minutes intervals. After this period, pressure was increased to 100 mmHg. Using an inverted microscope, the vessels were monitored and the arterial inner diameter was measured by a video scalar and a calibrated and optical micrometer. In order to measure vasodilatory response, we decreased intravascular pressure from 100 mmHg to 20 mmHg in 20 mmHg increments. After each decrease, the vessel was allowed 10 minutes to equilibrate and then a measurement of diameter was taken (DeWitt 2001).

Histochemical Staining

In preparation for staining, frozen 10 μm coronal sections were cut on a cryostat (Lecia Microsystems, Inc., Bannockburn, IL) and mounted onto pre-cleaned Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Ten sections were taken in the region containing the hippocampus with 15 μm between each section (Hellmich 2007).

After sectioning, slides were fixed for thirty minutes in ice-cold formalin and then washed two times in Milli-Q water (EMD Millipore, Darmstadt, Germany). Slides were allowed to air dry for one hour. They were then immersed in a basic alcohol solution for five minutes. Next, the sections were immersed in 70% ethanol for two minutes, followed by a two-minute rinse in Milli-Q water and immersion in potassium permanganate for ten minutes (Gu 2012, Wang 2011). This was followed by a two minute rinse in Milli-Q water before they were stained with 0.0001% FJC (Histochem, Inc., Jefferson, AR) for ten

minute. Finally they were rinsed 3 times in Milli-Q water for one minute per rinse (Gu 2012).

Neuronal Counting

FJC positive neurons were counted blindly in the CA1/CA2 and C3 regions of the ipsilateral hippocampus. For each rat FJC positive neurons were counted in 10 slides. The numbers from each hippocampal subfield on each slide were added to give a total number of FJC positive neurons per rat.

Beam Balance

Vestibulomotor function was assessed using beam balance and beam walk tasks. Animals were trained on the beam balance on the day prior to injury. For training animals were placed on a narrow wooden beam that is 60 cm in length, 1.75 cm in width, 4.0 cm in height and elevated 1 m from the floor. The time the animal remained on the beam out of the 60-second trial was recorded (Dixon 1987). If the animal reached the sixty seconds, the trial was ended. If it was unable to balance it was allowed to fall into a safety box placed below the beam. Three trials were performed per day with 15 seconds of rest given between each trial (Kline 2000). A pre-assessment was done on the day of injury and testing was conducted on PIDs 1 through 4. Each trial was also scored with a rating from 1 to 6. The scoring was: 1 = balances with steady posture (grooms, climbs barrier); 2 = grasps sides of beam and/or has shaky movements; 3 = hugs beam or slips or spins on beam; 4 = attempts to balance, but falls off after 10 s; 5 = drapes over beam or hangs from beam and falls off in less than 10 s; or 6 = falls off, makes no attempt to balance or hang from beam (Sell 2008).

Beam Walk

The beam was 100 cm in length by 2.5 cm in width by 4.0 cm in height and elevated 1 m above the floor. The beam also had four equally spaced pegs 2 cm in height and 25 cm apart. The goal box used was 28 cm in length, 18 cm in height and 18 cm in width (Dixon 1987). To begin animals were first placed in the goal box with no light or noise stimulus for two minutes. Next the animal was placed at one end of the narrow beam, opposite from the goal box (Kline 2000). At the start of the trial a bright light and white noise source near the starting position of the task was turned on. Time to reach the escape chamber was recorded. Once the animal reached the goal box the light and white noise were discontinued (Dixon 1987). Three trials were performed each day with thirty seconds between each trial, during which time the animal remained in the darkened goal box (Dixon 1987, Kline 2000, Sell 2008). Animals were trained on the beam walk test the day prior to injury. A pre-assessment was done on the day of injury and testing was conduct on PIDs 1 through 4 (Sell 2008).

Morris Water Maze

A variation of the MWM was used that examines spatial working memory after TBI (Hamm 1996). This water maze was a tank approximately 180 cm in diameter and 75 cm deep was filled with water to a depth of 28 cm. A Plexiglas goal platform (10 cm diameter) was submerged 2.5 cm below the surface of the water. Distal clues were present on the 4 walls of the room surrounding the water maze. A platform (10cm diameter) was placed in water 2.5 cm below the surface. Testing began 11 days post-surgery and continued until day 15. Each day consisted of four two-minute trials. Both the location of

the platform and start location were randomized to one of four quadrants for each trial (Kline 2000, Clark 2007). Time to reach the platform was measured (Hamm 1996)

Statistical Analysis

Physiologic Data

Data were expressed as mean ± SEM. Mean arterial pressure, cerebral perfusion and cerebral vascular resistance were analyzed using a two-way ANOVA in Prism® (Graphpad Software, Inc., La Jolla, CA). *Post hoc* comparisons of means were performed using Tukey's multiple comparison tests with an α level of significance of 0.05.

Neuronal Counts

Data for neuronal counts were expressed as mean ± SEM and were analyzed using one-way ANOVA analyses in Prism®. For *post hoc* comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05.

Behavioral Data

For the beam balance experiments, data were expressed as mean ± SEM. A Friedman's test was performed and Dunn's multiple comparison tests were performed with an α level of significance of 0.05.

For beam walk experiments, data were expressed as mean ± SEM and were analyzed using a two-way ANOVA. For *post hoc* comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05.

MWM latencies were expressed as mean ± SEM and was analyzed using two-way ANOVA in Prism®. Each set of measurements from the same animal was considered a correlated cluster of observations. For *post hoc* comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05 (Sell 2008).

Chapter 3: The Effects of Dendro[C60]Fullerene DF-1 on Cerebral Vascular Reactivity After Fluid Percussion Injury

Abstract

Since reactive oxygen species contribute to cerebral vascular dysfunction after traumatic brain injury (TBI), antioxidant therapy may preserve vascular function and reduce posttraumatic neuronal injury. We tested the hypothesis that dendro[60]fullerene (DF-1), a powerful antioxidant nanoparticle, would improve vascular function after injury and reduce hippocampal cell death. Male Sprague-Dawley rats were subjected to moderate FPI and treated with 10-mg/kg, 25-mg/kg, or 50-mg/kg of DF-1 one hour post-injury. We examined the effects of DF-1 administration on cerebral perfusion (laser Doppler flowmetry), and arterial blood pressure after injury. Additionally, MCAs were harvested two hours after injury and their responses to incremental decreases in pressure from 100 to 20 mmHg were tested. In a second set of animals, brains were harvest twenty-four hour after injury, stained with FJC and the numbers of FJC positive neurons were counted in hippocampal cell layers CA1/CA2 & CA3. Although the numbers of FJC-positive hippocampal neurons were reduced significantly ($p<0.05$, TBI vs. TBI+DF-1), cerebral perfusion and MCA responses to reduced intravascular pressure were not improved by DF-1 administration. These results indicated that DF-1 reduced hippocampal injury without improving cerebral vascular function suggest that DF-1 act through extravascular mechanisms. Since carboxyl-functionalized fullerenes (e.g. DF-1) are thought to readily cross the blood brain barrier, the neuroprotective actions of DF-1 might be due to direct antioxidant effects on neurons and/or glia.

Introduction

Worldwide, TBI is a leading cause of morbidity and mortality (Coronado 2011, Natale 2003). There are over 2.5 million new cases of TBI annually (Center for Disease Control 2014). Medical costs associated with TBI amount to over 60 billion dollars each year (Coronado 2011, Kurland 2012). Brain injury results in a cascade of biochemical processes that lead to neuronal cell death and cognitive dysfunction (Hovda 2014, Natale 2003). Among these processes are increased generation and release of ROS (Cornelius 2013, Ozturk 2005). ROS (e.g. superoxide anion and hydroxyl radicals) are thought to be major contributors to cerebral vascular dysfunction after TBI (DeWitt 1997, Kontos 1989). In addition to vascular injury, ROS cause neuronal damage and death (Cornelius 2013, Kontos 1989). Reducing ROS levels after traumatic cerebral vascular injury (Wei 1981; DeWitt 1997; DeWitt 2003) has potential to reduce cerebral vascular dysfunction caused by brain injury (Bains 2012, Hall 1993, Yoshida 2006).

Elevated levels of ROS are derived from multiple sources after TBI (Bains2012, Kontos 1989, Li 2015). Brain injury activates phospholipases C and A_2, releasing AA from cell membrane phospholipids (Wei 1982, Wei 1986b). AA is metabolized by cyclooxygenases into cyclic endoperoxides, which are further converted to prostaglandins (DeWitt 1988, Ellis 1981). When cyclic endoperoxides are metabolized the generate superoxide. These reactions are accelerated after TBI (Wei 1982, Wei 1980b). Superoxide is then catalyzed by SOD into H_2O_2 and oxygen. When H_2O_2 reacts to oxidize ferrous iron hydroxyl radicals are formed (Bains 2012, Kontos 1989). Elevation of ROS after TBI has a profound effect on the cerebral vasculature. Abnormal arteriole dilation, abnormal vascular reactivity, reduced oxygen metabolism and damage the BBB

can all occur (Abdul-Muneer 2014, DeWitt, 2003, Kontos 1986a, Kontos 1989, Smith 1994). It has been established through multiple studies that the reduction of ROS after TBI can improve cerebral vascular reactivity (Abdul-Muneer 2014, Hall 1993), preserve the BBB (Smith 1994, Zhao 2007) and lead to improved cognitive outcomes (Beni 2004, Kelso 2011).

Carboxyfullerenes nanoparticles are "radical sponges" and have been shown to have strong antioxidant properties (Beuerle 2008, Brown 2010). They are naturally hydrophobic but by adding functional groups, fullerenes become water-soluble molecules and applicable for biological applications (Bitner 2012, Dugan 1997, Theriot 2010, Quick 2008). Carboxyfullerenes are effective in reducing ROS and improving outcome in CNS disorders (Baati 2012, Dugan 1997, Partha 2009). Carboxyfullerenes have been tested to reduce apoptosis and excitotoxic necrosis (Partha 2009) and found effective in preventing neuronal cell death induced by NMDA or AMPA in cultured cortical neurons (Dugan 1997). They have postponed the onset of ALS symptoms and significantly delayed cell death in a mouse model, as well as (Dugan 2001). Nanoparticles reduced cognitive deficits in MWM testing and extended life span when orally administered to mice (Quick 2008).

There have been few studies of the effects on antioxidant nanoparticle administration after TBI (Bitner 2012, Hicks 2013). PEG-HCCs improved cerebral perfusion when administered during resuscitation in a rat TBI + hemorrhagic hypotension model (Bitner 2012). To our knowledge, no studies of carboxyfullerene antioxidant nanoparticles in TBI models have been conducted. Of the modified carboxyfullerene nanoparticles, we chose to work with the monoadduct carboxyfullerene DF-1 (Da Ros 2008). When multiple functional attachments are added to a fullerene it can sometimes

makes them less stable, increase toxicity and reduces their antioxidant properties (Beuerle 2008). Therefore this monoadduct fullerene, DF-1, with only one attachment, should have a greater affinity for ROS while being less toxic than other carboxyfullernes with multiple attachments (Beuerle 2008).

To determine the effect of DF-1 on cerebral vascular function after TBI, cerebral perfusion and arterial blood pressure were measured in rats subjected to moderate fluid percussion TBI *in vivo* and vasodilatory responses to reduced intravascular pressure were assessed in MCA segments harvested from rats after TBI. Hippocampal neuronal cell death was measured using FJC in rats treated with DF-1 or a vehicle one hour after TBI.

Methods

Animals

All animal experiments adhered to the National Institutes of Health's guidelines for ethical animal care and use and were approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee. Adult (3-6 month, 350-425 g) male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed two per cage at constant temperatures (21°–23°C) and humidity levels (45–50%). They were housed in a 12-hour light and dark cycle with food and water provided *ad libitum*.

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Rats were anesthetized with 4% isoflurane in an anesthetic chamber, intubated, and mechanically ventilated (1.5-2.0% isoflurane in O_2: room air (30:70) using a volume ventilator (EDCO Scientific, Chapel Hill, NC)). All survival surgeries were performed under aseptic conditions (clean surgical gowns, sterile gloves and head covers). Instruments were autoclaved prior to use and drapes were used during the procedures. Incision sites were shaved, scrubbed, then rinsed with alcohol and painted with iodine prior

to procedures. Throughout the procedure, rectal and temporalis temperatures were monitored and maintained at 37.0±0.5 °C with an overhead lamp and water blanket (Gaymar, Orchard Park, NY). Rats were prepared for parasagittal fluid percussion TBI as described by Dixon, et al (Dixon 1987). Anesthetized rats were placed in a stereotaxic head holder and the skin over the dorsal surface of the skull was incised at the midline and reflected along with the temporalis muscles bilaterally. A 3 mm craniotomy was trephined 2.5 mm from the midline between the lambda and bregma and the dura was left intact. A modified plastic Luer-lok needle hub was cemented over the craniotomy using cyanoacrylic and dental cement (Hawkins 2013a).

Parasagittal Fluid Percussion Injury

Following the surgical preparation, rats were connected to the fluid percussion device at the craniotomy site and subjected to fluid percussion TBI. Immediately after injury, rats were disconnected from the FPI device. Post-injury, the Luer-lok trauma adapter was removed and the scalp was sutured. In the case of survival experiments, rats were placed in a cage and monitored hourly for at least four hours. For non-survival experiments, animals were euthanized at the conclusion of the experiment by decapitation while deeply anesthetized (4.0% isoflurane).

Laser Doppler Flowmetry

To measure relative cerebral perfusion using laser Doppler flowmetry (Haberl 1989, Stern 1977), the skull to the left of the sagittal suture was thinned using an air-cooled mini drill and a black rubber tube was glued in place. A laser Doppler probe was placed over the thinned skull and positioned to avoid large blood vessels using a stereotaxic electrode holder.

Middle Cerebral Artery Experiments

Animals subjected to moderate FPI were treated with a 10-mg/kg dose of DF-1 one hour after injury. One hour after treatment (two hours after injury) the MCA were harvested and a 2 mm section of the MCA was mounted on an Arteriograph (Living Systems, St. Albans, Vermont) in a physiologic salt solution (PSS) (130-mmol/L NaCl, 4.7 mmol/L KCl, 1.17 mmol/L $MgSO_4 7H_2O$, 5-mmol/L glucose, 1.50 mmol/L $CaCl_2$, 15 mmol/L $NaHCO_3$). The MCA segments monitored for leaks at room temperature for 60 minutes with the intravascular pressure set at 50 mmHg (DeWitt 2001).

Prior to experiments, the vessel was exercised by alternating the pressure between 20 mmHg and 40 mmHg three times in five minutes intervals and then pressure was increased to 100 mmHg. Using an inverted microscope, the vessels were monitored and the arterial inner diameter was measured by a video scalar and a calibrated and optical micrometer. In order to measure vasodilatory response, we decreased intravascular pressure from 100 mmHg to 20 mmHg in 20 mmHg increments. After each decrease, the vessel was allowed 10 minutes to equilibrate and then a measurement of diameter was taken (DeWitt 2001).

Histochemical Staining

In preparation for staining, frozen 10 μm coronal sections were cut on a cryostat (Lecia Microsystems, Inc., Bannockburn, IL) and mounted onto pre-cleaned Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Ten sections were taken in the region containing the hippocampus with 15 μm between each section (Hellmich 2007).

After sectioning, slides were fixed for thirty minutes in ice-cold formalin and then washed two times in Milli-Q water (EMD Millipore, Darmstadt, Germany). Slides were allowed to air dry for one hour. They were then immersed in a basic alcohol solution for

five minutes. Next, the sections were immersed in 70% ethanol for two minutes, followed by a two-minute rinse in Milli-Q water and immersion in potassium permanganate for ten minutes (Gu 2012, Wang 2011). This was followed by a two-minute rinse in Milli-Q water before they were stained with 0.0001% Fluoro-Jade C (FJC), (Histochem, Inc., Jefferson, AR) for ten minute and then rinsed 3 times in Milli-Q water for one minute per rinse (Gu 2012).

Neuronal Counting

FJC positive neurons were counted blindly in the CA1/CA2 and C3 regions of the ipsilateral hippocampus. For each rat, FJC positive neurons were counted in 10 slides. The numbers from each hippocampal subfield on each slide were added to give a total number of FJC positive neurons per rat.

Statistical Analysis

For physiologic data, results were expressed as mean ± SEM. Mean arterial pressure, cerebral perfusion and cerebral vascular resistance were analyzed using a two-way ANOVA in Prism® (Graphpad Software, Inc., La Jolla, CA). Post hoc comparisons of means were performed using Tukey's multiple comparison tests with an α level of significance of 0.05. Data for neuronal counts were expressed as mean ± SEM and were analyzed using a one-way ANOVA in Prism®. For *post hoc* comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05.

Results

Cerebral Vascular Resistance

CVR was calculated as CVR= MAP x LDF^{-1}. CVR was significantly increased by TBI ($p<0.05$ TBI vs. Sham) and remained elevated throughout the duration of the

experiment (Figure 1). For both the TBI + 25-mg/kg DF-1 and TBI + 50-mg/kg DF-1 groups, CVR was significantly increased ($p<0.05$, TBI vs. TBI + 25 mg/kg DF-1 or TBI + 50 mg/kg DF-1). There was no significant difference between the three treatment groups.

Mean Arterial Blood Pressure

Mean arterial pressure was not affected by TBI but was significantly ($p<0.05$) increased by all doses of DF-1 compared to the sham and TBI groups (Figure 2). DF-1 was administered at 60 minutes after injury. Fifteen minutes after injury, MAP had significantly increased in the TBI + 10-mg/kg DF-1, TBI + 25-mg/kg DF-1 and the TBI + 50-mg/kg DF-1 groups ($p<0.05$ vs. TBI). MAP remained elevated in these groups for the remainder of the experiments. The three doses did not differ significantly from each other.

Relative Cerebral Perfusion

Relative cerebral perfusion was significantly reduced by TBI compared to the sham group ($p<0.05$) (Figure 3). After thirty minutes, cerebral perfusion began increasing towards baseline in all injured groups. There was a trend towards reduced relative cerebral perfusion in all TBI groups, but only statistically significant ($p<0.05$) reductions were observed only in the TBI + 25-mg/kg DF-1 group compared to the TBI group. This reduction occurred prior to DF-1 administration and thus this has no implication on treatment effect.

Middle Cerebral Artery Studies

In the sham group, MCA segments dilated as intravascular pressure was reduced. TBI significantly reduced vasodilation in response to reduced intravascular pressure, ($p<0.05$, TBI vs. Sham) (Figure 4). Middle cerebral arterial dilation during reductions in intravascular pressure did not differ significantly between the TBI alone and TBI + DF-1

groups indicating that DF-1 treatment did not improve vasodilation in response to reduced intravascular pressure in MCA segments.

Fluoro-Jade C Cell Counts

TBI resulted in significantly higher numbers of FJC-positive neurons in hippocampal cell layers CA1/2 and CA3 ($p<0.05$, TBI vs. Sham) (Figure 5). All doses of DF-1 reduced cell death in both CA1/2 and CA3 regions of the hippocampus ($p <0.05$, TBI vs. TBI + 10-mg/kg or 25-mg/kg or 50-mg/kg DF-1). There were no significant differences in the numbers of FJC-positive neurons among the different doses of DF-1 in the CA1/2 region. In the CA3 region of the hippocampus, the 10-mg/kg dose of DF-1 seemed to be more effective than the higher doses. The TBI + 10-mg/kg group had significantly fewer FJC-positive neurons that the TBI + 50-mg/kg group ($p<0.05$). There was a trend towards TBI + 10-mg/kg group reducing FJC-positive neurons compared to the TBI + 25-mg/kg group ($p<0.0692$).

Discussion & Conclusion

While some work has been done with nanoparticles for drug delivery, diagnostics and other applications in TBI research (Bitner 2012, Hicks 2013), the use of antioxidant nanoparticle is relatively unexplored (Cho 2012, Hockey 2013, Weinstein 2010). Bitner, et al., (2012) examined the use of the antioxidant nanoparticles, PEG-HCCs, to reduce ROS in a model of cortical contusion injury and hemorrhagic hypotension. PEG-HCCs reduced oxidative stress and increased endothelial cell survival *in vitro* and reduced oxidative stress and increased brain tissue NO levels and cerebral perfusion after TBI + hemorrhagic hypotension and resuscitation *in vivo* (Bitner 2012). Hicks, et al., (2013) reported that treatment with cerium oxide nanoparticles improved beam balance and MWM behavioral

outcome after FPI (Hicks 2013). Our studies are the first to use the carboxyfullerene nanoparticle DF-1, as a therapeutic agent as opposed to other nanoparticles. Also, to our knowledge, this is the first use of DF-1 in any model of central nervous system injury.

Compared to other carboxyfullernes, DF-1 has advantages in stability and ROS scavenging capabilities. DF-1 is a monoadduct carboxyfullerene; therefore it has only one functional attachment (Beuerle 2008). Since the addition of functional attachments render fullerenes less stable, more toxic and reduces their antioxidant properties, the monoadduct fullerene DF-1, with only one attachment, should have a greater affinity for ROS while being less toxic than other carboxyfullernes with multiple attachments (Beuerle 2008). Prior to our work, most of the existing research with DF-1 was done in studies looking at its therapeutic effects for radiation-induced damage. (Partha 2009, Theriot 2010) Although others have examined other carboxyfullerene nanoparticles in aging and neurodegenerative disorders (Baati 2012, Dugan 1997, Partha 2009), to our knowledge this is the study of DF-1 as a potential therapeutic agent for any central nervous system disorder.

DF-1 treatment did not improve CVR, cerebral perfusion, MAP or cerebral dilatory response compared to untreated TBI animals, as we had initially hypothesized. CVR, MAP and cerebral perfusion were all either unaffected or worsened, depending on the dose of DF-1. Trauma-induced reductions in vasodilation in response to reduced intravascular pressure in isolated, pressurized MCA segments were not reversed by DF-1 administration. The absence of a vasoprotective effect of DF-1 may have been due to administration one-hour post-TBI. Superoxide anion radical levels increase within minutes of TBI and continue to increase for at least 45 min post-injury (Fabian 1998, Kontos 1986b). Hydroxyl radicals have an initial peak in release at five minutes after injury (Smith 1994). This is

accompanied by an increase in lipid peroxidation (Abdul-Muneer 2014, Smith 1994). Because ROS peak early after TBI, it is possible that ROS-mediated cerebral vascular injury was far advanced by the time DF-1 was administered. The one-hour time point of DF-1 administration may have been too late for DF-1 to mitigate cerebral vascular injury.

PEG-HCCs were effective at improving cerebral perfusion after TBI and hemorrhagic hypotension (Bitner 2012). Unlike our study that used only one dose of DF-1, these studies used multiple doses of PEG-HCCs at different time points. Animals were also given PEG-HCCs at a different time point that coincided with blood reinfusion. Blood reinfusion is known to cause additional spikes in ROS levels (Bitner 2012, Fabian 1995). Administering DF-1 at the same time as reinfusion might have been an important factor in why PEG-HCCs were able to have an effect on cerebral perfusion in this model (Bitner 2012).

One explanation for the increased CVR and reduced cerebral perfusion that we observed following DF-1 administration might be that DF-1 is scavenging of nitric oxide (NO). Nielson, et al., (Nielsen 2008) reported that DF-1 scavenged NO and reduced NO formation by inhibiting NO synthase. Nitric oxide contributed to the maintenance of resting vasodilation in cerebral arteries and the inhibition of NO synthesis reduced cerebral perfusion (Cherian 2000, DeWitt 2003). Scavenging of NO would result in vasoconstriction of blood vessels and reduced cerebral perfusion (Cherian 2000, Förstermann, 2012). This is supported by the observation that PEG-HCCs increased brain tissue levels of NO (Bitner 2012).

While DF-1 administration did not improve the cerebral vascular resistance, cerebral perfusion or cerebral dilatory responses to reduced intravascular pressure, we

observed a reduction in the numbers of FJC-positive neurons in the hippocampus. This indicates that DF-1 treatment is preserving neurons after FPI and suggests that may have a potential therapeutic benefit after TBI. While the time of administration in this study may not have been early enough to protect the cerebral vasculature, it may have reduced subsequent oxidative stress in the brain. ROS are elevated in the parenchyma for several hours up to a few days after injury (Hall 2010, Tyurin 2000), which would permit DF-1 administration one-hour post-TBI to exert a neuroprotective effect.

Although ROS scavengers may exert neuroprotective effects without traversing the blood brain barrier (BBB) (Hall 2010), DF-1 would be more likely to have neuroprotective activity if it were capable of traversing the BBB. There is abundant evidence that the BBB is permeable to water soluble carboxyfullerenes such as DF-1 (Baati 2012, Beuerle 2007, Cagle 1999, Ji 2006, Qingnuan 2002, Quick 2008, Riviere 2009, Yamago 1995). DF-1's ability to cross the BBB would aid the nanoparticle's ability to reduce ROS levels and preserve neurons. Published research also indicates that carboxyl-functionalized fullerenes cross the blood brain barrier (Quick 2008, Riviere 2009). Two studies reported that all water-soluble fullerenes such as DF-1 are able to cross the BBB (Baati 2012, Yamago 1995). Other reports indicated that functionalized fullerenes (e.g. DF-1) readily cross the blood brain barrier (Beuerle 2007, Cagle 1999, Ji 2006, Qingnuan 2002). We believe that these reports support our research indicating that DF-1 does not improve cerebral vascular function after TBI, but crosses the BBB to provide neuroprotection through local anti-oxidant activity.

These experiments were also performed to help us determine the range for the most effective dose of DF-1 for TBI treatment. In order to determine the effects of different

doses, we measured cerebral perfusion, arterial blood pressure and MCA dilator responses to progressive reductions in intravascular pressure after moderate FPI followed by the administration of 10, 25 or 50 mg/kg DF-1. Our preliminary experiments had demonstrated that DF-1 improved cognitive outcome after TBI at a 10-mg/kg dose (data not shown). We utilized a dose response curve to determine whether the same was true for the use of DF-1 in respect to TBI. Our physiological experiments did not show a benefit for DF-1 doses > 10 mg/kg. Our FJC experiments indicated that the 10-mg/kg dose was most effective at reducing the amount of FJC positive neurons.

In summary, these results demonstrate that DF-1 treatment did not improve cerebral perfusion, MAP or CVR after a TBI. The 25-mg/kg dose of DF-1 reduced cerebral perfusion, and all doses increased MAP. While DF-1 did not have a significant effect on cerebral vascular reactivity it did preserve neurons in the hippocampus. DF-1 may be an effective neuroprotective therapy after TBI and further studies of the behavioral effects of DF-1, as well as its mechanisms of action, are worth investigating.

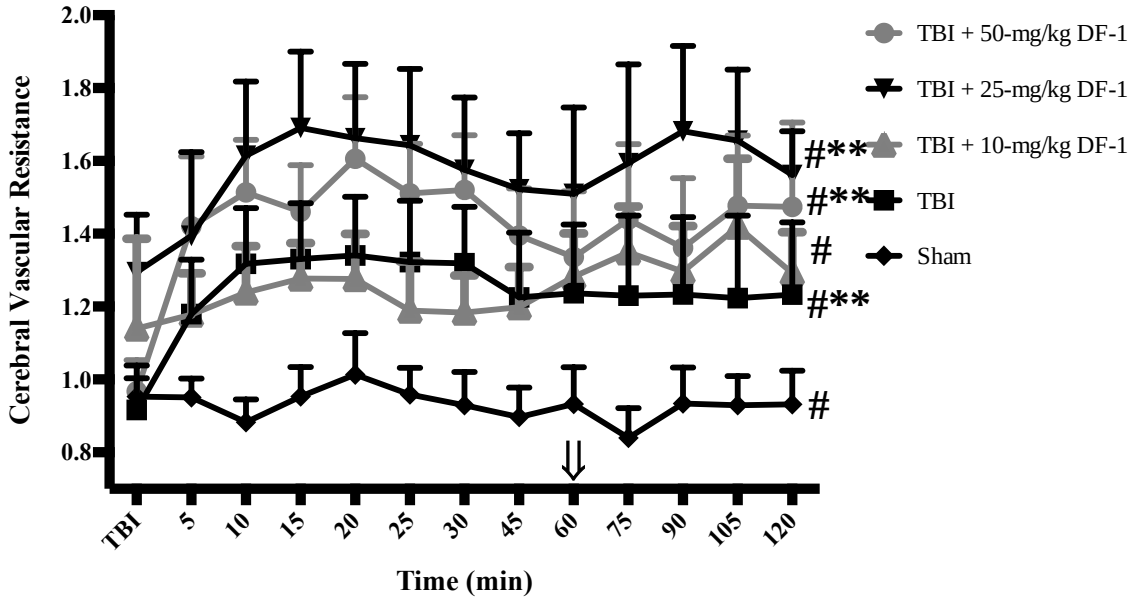

Figure 2 - Cerebral vascular resistance was significantly increased after traumatic brain injury (TBI) in TBI + 25-mg/kg DF-1 and TBI + 50-mg/kg DF-1 groups but there was not a difference between the three treatment groups. Arrow indicates when DF-1 was administered. (The # indicates significant difference from sham ($p<0.05$) and ****** indicates significant difference from TBI ($p<0.05$))

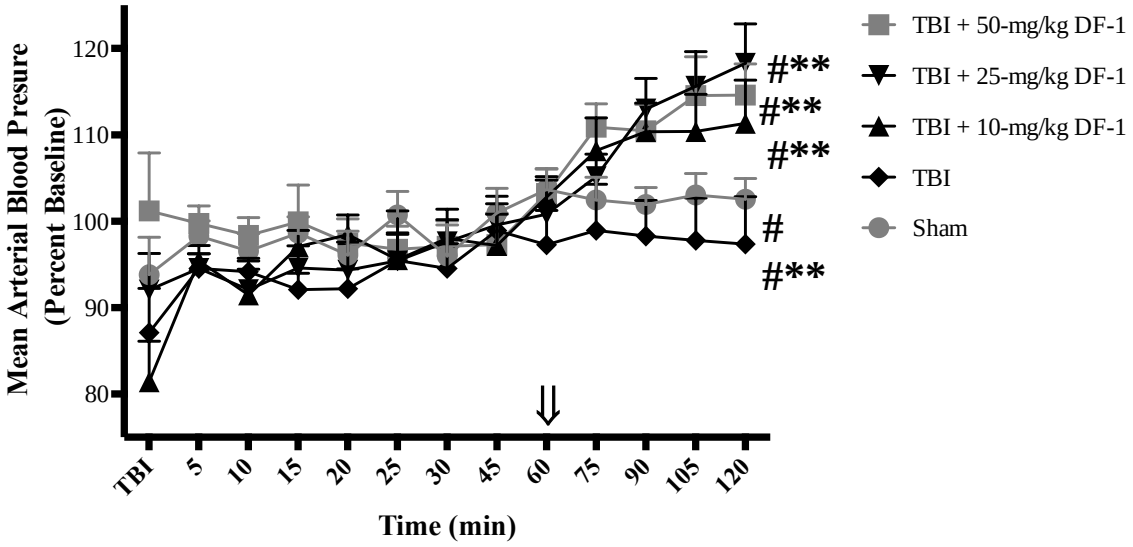

Figure 3 – Mean Arterial Blood Pressure (MAP) was significantly increased by all doses of DF-1 compared to the TBI group. Arrow indicates when DF-1 was administered. (The # indicates significant difference from sham ($p<0.05$) and ** **i**ndicates significant difference from TBI ($p<0.05$))

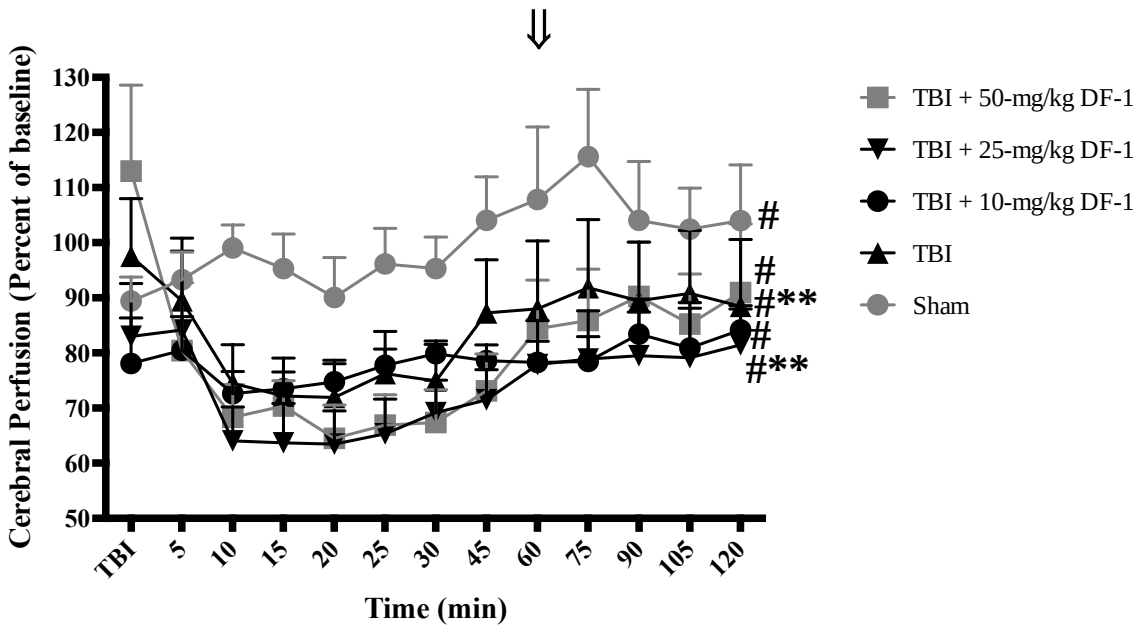

Figure 4 – Although there were trends towards reduced relative cerebral perfusion in all TBI groups, statistically significant reductions were observed only in the TBI + 25-mg/kg DF-1 group compared to the TBI group. Arrow indicates when DF-1 was administered. (The # indicates significant difference from sham ($p<0.05$) and ** indicates significant difference from TBI ($p<0.05$))

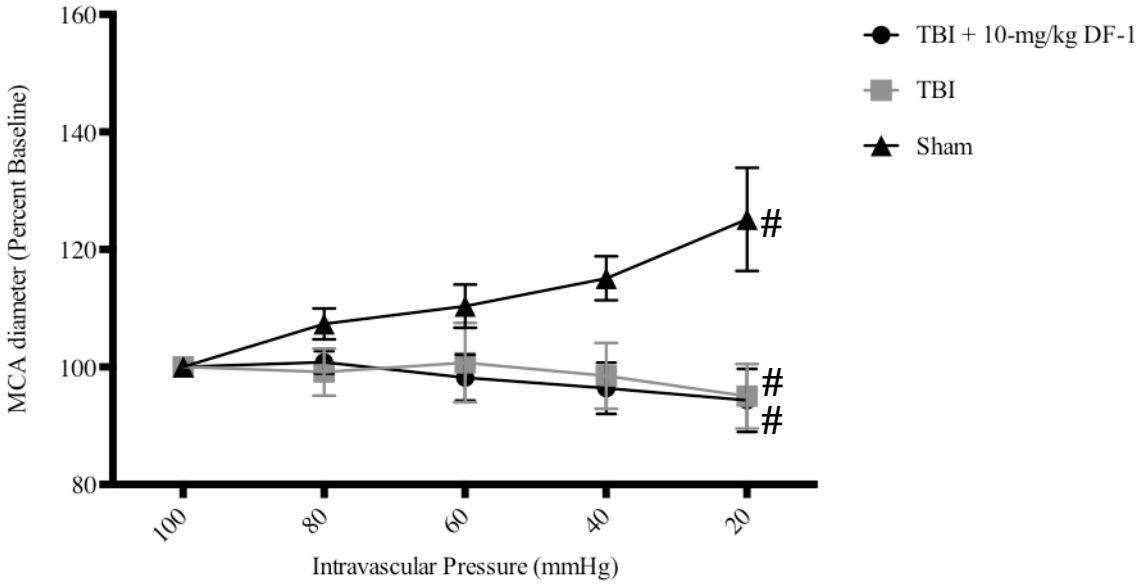

Figure 5 – Middle cerebral arterial (MCA) diameters during progressive reductions in intravascular pressure (percent of baseline diameters). In the sham group, MCA diameters increased as intravascular pressure was reduced. Sham differed significantly from both injured groups. Vasodilation as a response to decreased pressure is the normal response. In TBI animals this response is impaired. DF-1 did not improve the vessels ability to dilate after injury. (# Indicates significant difference from sham ($p<0.05$))

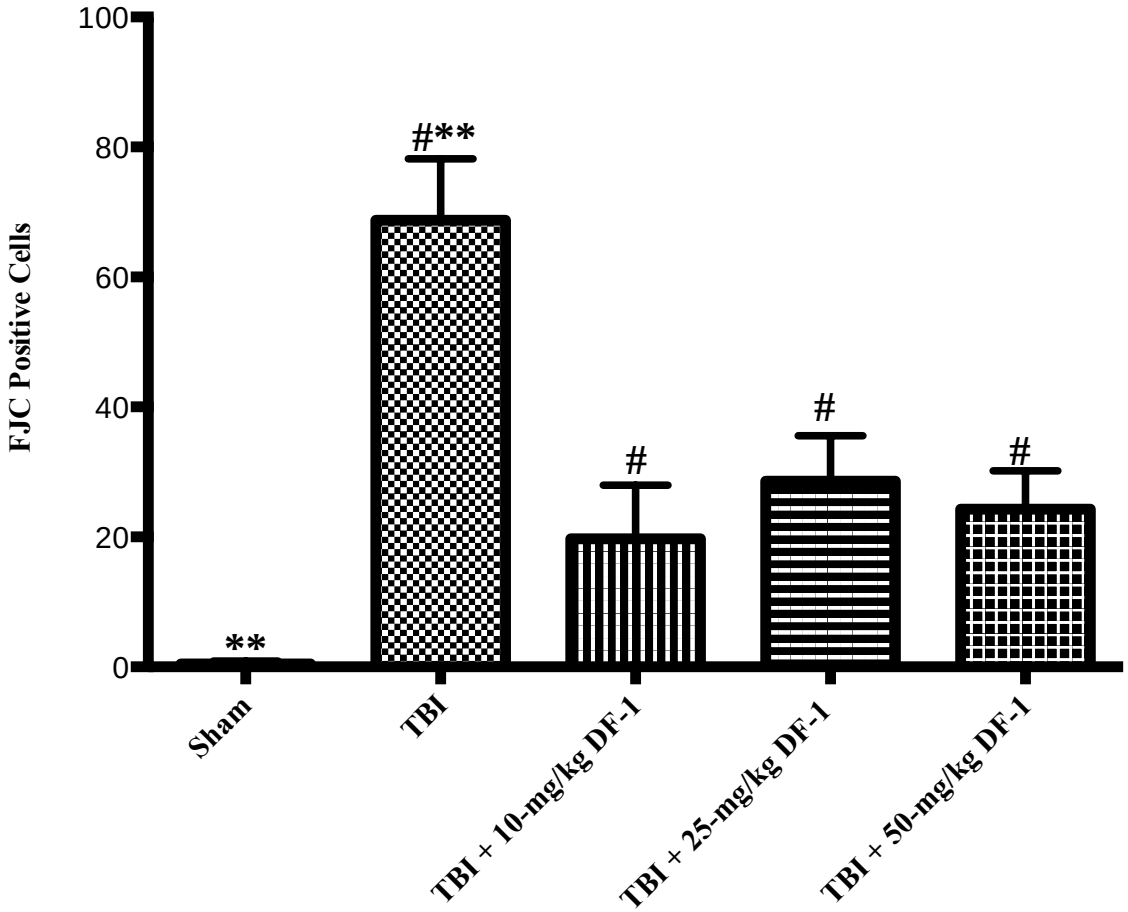

Figure 6 – The number of Fluoro-Jade C (FJC) positive cells in the CA1/2 region of the hippocampus were reduced by all doses of DF-1. Doses did not differ significantly from each other (**= $p < 0.05$ vs. Sham; # = $p<0.05$ vs. TBI)

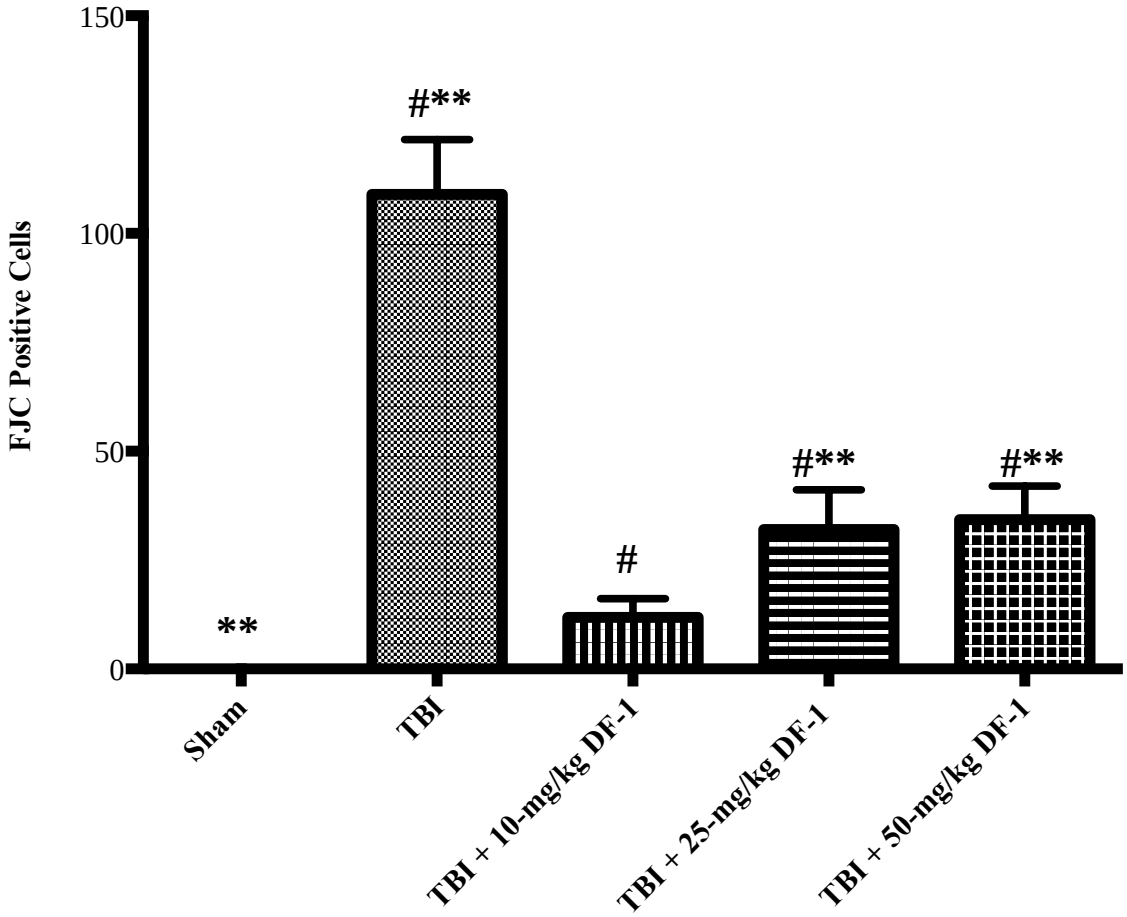

Figure 7 – The number of Fluoro-Jade C (FJC) positive cells in the CA3 region of the hippocampus were reduced by all doses of DF-1. Doses did not differ significantly from each other. The 10-mg/kg dose did not differ significantly from sham (The asterisk indicates significant difference from sham ($p<0.05$) and circle indicates significant difference from TBI ($p<0.05$))

Chapter 4: Dendro[60]Fullerene Does Not Improve Cerebral Perfusion After TBI and Hemorrhagic Hypotenstion

Abstract

Traumatic brain injury (TBI) causes an increase in reactive oxygen species (ROS) that can result in cerebral vascular dysfunction. Damage to the cerebral vascularature is further compromised when it occurs in conjunction with hemorrhagic hypotension (HH). The use of antioxidants to reduce ROS levels can serve to preserve vascular function and lead to better outcomes after injury. Dendro[60]fullerene (DF-1) is a powerful antioxidant nanoparticle. We examined if DF-1 might improve cerebral vascular function and/or cerebral perfusion after TBI & HH in rats. Isoflurane-anesthetized, Sprague-Dawley rats were subjected to moderate parasagittal FPI or sham TBI. Five minutes after injury, DF-1 was administered and mean arterial pressure (MAP) was lowered to 40 mmHg by removal of blood via the jugular vein for 50 min followed by resuscitation with saline then shed blood. Relative cerebral perfusion (laser Doppler flowmetry) and MAP were measured during TBI, hemorrhage and resuscitation. DF-1 did not improve relative cerebral perfusion after moderate TBI + HH. DF-1 is capable of acting as both a pro-oxidant and an antioxidant. It is possible that DF-1 is acting as a pro-oxidant in this model and producing superoxide that would inhibit vasodilation.

Introduction

TBI is a devastating injury that produces a cascade of dysfunction that affects the cerebral vasculature (Madamanchi 2005, Wei 1980a). The severity of secondary injuries that occur due to the primary insult is closely linked to a patient's outcome (Timofeev 2011). Maintenance of vascular function, especially cerebral perfusion, plays an important role in patient outcome (DeWitt 2003, Luerssen 1995). When TBI occurs in conjunction with HH, the rates of morbidity and mortality double (Coker 1989, Luerssen 1995, Schmoker 1992). Both TBI and HH can result in reduced cerebral perfusion and impaired autoregulation (Chen 1984, Hawkins 2013b, Navarro 2012). In the case of patients with the comorbidities of TBI and HH, due to injury, the brain's innate ability to adjust to hypotension is impaired and thus the effects of injury are exacerbated (Marmarou 1991, Navarro 2012).

Both TBI and HH result in elevated levels of ROS (Fink 2002, Wei 1980a). ROS are a significant contributing factor to vascular damage and they cause neuronal death and other complications after injury (Kontos 1989, Madamanchi 2005, Smith 1994). Elevated ROS play a role in impairing vasodilatory reactivity, reducing oxygen metabolism and damaging the BBB (Kontos 1986, Kontos 1989, Smith 1994). Blood reinfusions to treat hypotension also result in additional increases in ROS levels (Bitner 2012, Fabian 1995, Halliwell 1999). Antioxidants have been reported to be effective in reducing ROS and improving cerebral vascular reactivity post TBI (Abdul-Muneer 2014, Hall 1993). Treatment with antioxidants has also been shown to result in improved cognitive outcome (Beni 2004, Kelso 2011). The spikes in ROS from reinfusion at later time points presents a good therapeutic time point for intervention with antioxidants (Bitner 2012, Fabian 1995).

Because ROS are a major contributor to cerebral vascular damage and subsequent dysfunction in TBI and HH, antioxidants may reduce oxidative stress and improve outcome after TBI and HH (Bitner 2012, Hall 1993, Wei 1980a). DF-1 is a powerful antioxidant nanoparticle (Brettreich 1998). Nanoparticle antioxidants have advantages over other classes of antioxidants, as they are able to fully quench ROS without the aid of a second molecule (Bitner 2012). Another nanoparticle, with a similar structure to DF-1, PEG-HCCs effectively maintained normal cerebral perfusion for several hour after TBI + HH (Bitner 2012). Therefore, we hypothesized that cerebral vascular dysfunction would be reduced by treatment with DF-1, which would result in increased cerebral perfusion after TBI + HH.

The objective was to determine DF-1's effect on cerebral perfusion. In the TBI + HH model, mean arterial blood pressure is held constant, which allows for an examination of a substance's effect on cerebral perfusion (Navarro 2012). We wanted to look at DF-1's effect on the trauma hemorrhage model because reinfusion after hemorrhage causes increased levels of ROS at later time points, this injury model provides additional, clinically relevant time points for antioxidant intervention that are not available using a TBI model (Bitner 2012).

Methods

Animals

All animal experiments adhered to the National Institutes of Health's guidelines for ethical animal care and use and were approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee. Adult (3-6 month, 350-425 g) male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed two per cage at constant temperatures (21°–23°C) and humidity levels (45–50%) in a 12-hour light and dark cycle with food and water provided *ad libitum*.

Animal Surgical Preparation

Rats were anesthetized with 4% isoflurane in an anesthetic chamber, intubated, and mechanically ventilated (1.5-2.0% isoflurane in O_2: room air (30:70) using a volume ventilator (EDCO Scientific, Chapel Hill, NC)). All survival surgeries were performed under aseptic conditions (clean surgical gowns, sterile gloves and head covers). Instruments were autoclaved prior to use and drapes were used during the procedures. Incision sites were shaved, scrubbed, then rinsed with alcohol and painted with iodine prior to procedures. Throughout the procedure, rectal and temporalis temperatures were monitored and maintained at 37.0±0.5 °C with an overhead lamp and water blanket (Gaymar, Orchard Park, NY). Rats were prepared for parasagittal fluid percussion TBI as described by Dixon, et al. (Dixon 1987). Briefly, anesthetized rats were placed in a stereotaxic head holder and the skin over the dorsal surface of the skull was incised at the midline and reflected along with the temporalis muscles bilaterally. A 3 mm craniotomy was trephined 2.5 mm from the midline between the lambda and bregma and the dura was left intact. A modified plastic Luer-lok needle hub was cemented over the craniotomy using cyanoacrylic and dental cement (Hawkins 2013a).

Cannulation of Jugular Vein and Tail Artery

Prior to fluid percussion injury, the right jugular vein, and the tail artery of the animal were cannulated. The tail artery catheter was used so that mean arterial pressure could be monitored. The jugular vein catheter was inserted in order to induce the HH injury. Polyethylene tubing was used for the jugular vein and silastic tubing was used for the tail vein.

Parasagittal Fluid Percussion Injury

Following the surgical preparation, rats were connected to the fluid percussion device at the craniotomy site and subjected to fluid percussion TBI. Immediately after injury, rats were disconnected from the FPI device. Post-injury, the Luer Lock trauma adapter was removed and the scalp was sutured. In the case of survival experiments, rats were placed in a cage and monitored hourly for at least four hours. For non-survival experiments, animals were euthanized at the conclusion of the experiment by decapitation while deeply anesthetized (4.0% isoflurane).

Laser Doppler Flowmetry

To measure relative cerebral perfusion using laser Doppler flowmetry (Haberl 1989, Stern 1977), the skull to the left of the sagittal suture was thinned using an air-cooled mini drill and a black rubber tube was glued in place. A laser Doppler probe was placed over the thinned skull and positioned to avoid large blood vessels using a stereotaxic electrode holder.

Hemorrhagic Hypotenstion

For these experiments a jugular catheter and tail vein cannulation were inserted during surgical preparations prior to FPI. HH was initiated five minutes following FPI. In the first phase of the hemorrhage model blood was withdrawn into heparinized tubes through the jugular catheter until MAP reached 40 mmHg. Prior to beginning HH an approximately volume to achieve an MAP of 40 mmHg was calculated. The predicted volume was calculated as 2.0 mL/100g. When HH was initiated the first 50% of the blood volume calculated was removed in the first five minutes, 25% in the second five-minute interval and the remaining volume in the next five minutes. If 40 mmHg was achieved 1.0

mL per minute could be removed until the proper level was achieved. This level was sustained for fifty minutes following the start of HH injury. In the second phase, one mL boluses of saline were given every minute until MAP reached 50 mmHg. This MAP was sustained for 30 min. The final phase, reperfusion, lasted 30 minutes. During this time blood was reinfused at a rate of two mL per minute and the animal was ventilated on 100% oxygen (Bitner 2012, Navarro 2012).

Statistical Analysis

Data were expressed as mean ± SEM. Mean arterial pressure, cerebral perfusion and cerebral vascular resistance were analyzed using a two-way ANOVA in Prism® (Graphpad Software, Inc., La Jolla, CA). *Post hoc* comparisons of means were performed using Tukey's multiple comparison tests with an α level of significance of 0.05.

Results

Figure 1 shows the MAP levels of all groups during both the injury and resuscitation periods. MAP levels returned to baseline after the heparinized shed blood was reinfused in the third phase of the experiment for both groups. MAP was controlled throughout the experiment during the hypotension period. Figure 7 demonstrates that there was not a significant difference in MAP reduction between HH, HH + DF-1, TBI + HH and TBI + HH + DF-1 groups. Therefore hypotension was effectively achieved and uniform as desired.

Cerebral perfusion was significantly reduced in all injured groups compared to sham animals ($p<0.05$). The TBI + HH + DF-1 groups showed significantly lower cerebral perfusion when compared to the untreated TBI + HH group ($p<0.05$) (Figure 8). Sham animals treated with DF-1 did not differ from the untreated sham group (Figure 9), nor did

the hemorrhage animals treated with DF-1 differ from the untreated hemorrhage animals (Figure 8).

Discussion & Conclusion

Our studies showed that cerebral perfusion was decreased after TBI + HH, which is consistent with previous literature (DeWitt 1992, Matsushita 2001, Navarro 2012, Schmoker 1992). Because autoregulation is impaired after TBI, the cerebral vasculature is unable to maintain normal cerebral perfusion when systemic arterial blood pressure is reduced. Cerebral perfusion may be decreased further after head injury due to arterial hypotension or elevated intracranial pressure (Lewelt 1980, Oddo 2011). HH further impacts cerebral vascular reactivity (Hawkins 2013b, Kong 1991). Although we had hypothesized that DF-1 would improve cerebral perfusion after TBI + HH, we found that cerebral perfusion was lower in the TBI+HH+DF-1 group than in the untreated TBI+HH group during the resuscitation period. Since autoregulation is needed to maintain proper cerebral perfusion (Chan 1992, DeWitt 2003), our results suggest that autoregulation is impaired after TBI and these impairments are worsened by DF-1 treatment.

It is notable that for the groups that received HH alone, cerebral perfusion increased to baseline and was not significantly different from sham in the final resuscitation phase. In the TBI + HH and TBI + HH + DF-1 groups, cerebral perfusion remained significantly reduced during the resuscitation phase. Cerebral perfusion in the TBI + HH + DF-1 group was significantly reduced compared to the TBI + HH. Reduced cerebral perfusion in the resuscitation phase is correlated with poor restoration of MAP (Hawkins 2013), and reductions in MAP are associated with higher rates of cerebral ischemia (Dandapani 1995, Zhu 1995).

These results may be explained by the fact that DF-1 is capable of acting as both a pro-oxidant (Foley 2002). Due to this property, it has been demonstrated that fullerenes can inhibit vasodilation that is NO dependent by producing superoxide radicals. If DF-1 is acting as pro-oxidant in our model of TBI + HH, then it may be acting in the vasculature to prevent vasodilation (Satoh 1997). It is also possible that DF-1 is acting as an antioxidant and that the impaired vasodilatory response may be caused by DF-1 scavenging NO. Fullerene nanoparticles have been demonstrated to be capable of scavenging NO. It is also able to prevent NO formation by inhibiting nitric oxide synthase (Nielsen 2008). When NO synthesis is inhibited, cerebral perfusion is reduced (Cherian 2000, Dokken 2015). NO plays an important role in maintaining blood vessels at a state of partial dilation of blood vessels. If DF-1 were reducing NO level or blocking NO synthase it would lead to vasoconstriction of blood vessels. This vasoconstriction would, in turn, reduce cerebral perfusion pressure (Cherian 2000).

In summary, these results demonstrate that DF-1 did not improve cerebral perfusion in a TBI + HH model. In fact, DF-1 reduced cerebral perfusion during and after the resuscitation period. While DF-1 did not improve cerebral vascular function, it still may be worth examining it's effects on neuropathological outcomes are TBI + HH. In a TBI model DF-1 also did not improve cerebral vascular function but it did preserve neurons in the hippocampus after injury. Neuropathologic outcomes of TBI + HH are more severe than when TBI occurs alone (Matsushita 2001). Thus, DF-1's effect on hippocampal neuronal injury may be worth investigating.

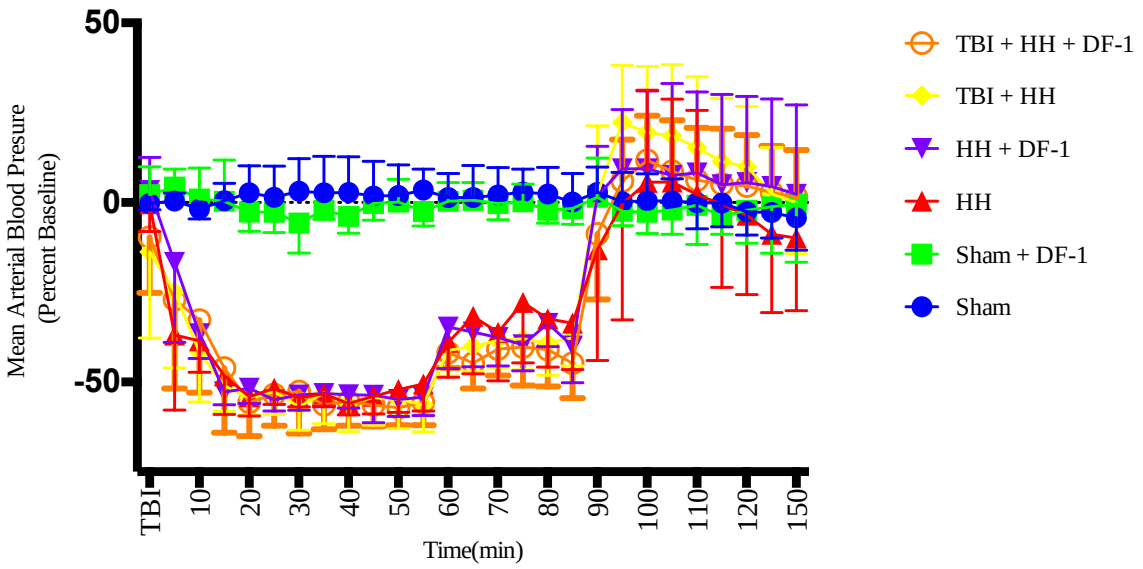

Figure 8 – Mean arterial blood pressure was significantly reduced in the HH, HH + DF-1, TBI + HH and TBI + HH + DF-1 compared to sham groups. These groups did not differ significantly from each other. Therefore hypotension was effectively achieved and uniform as desired.

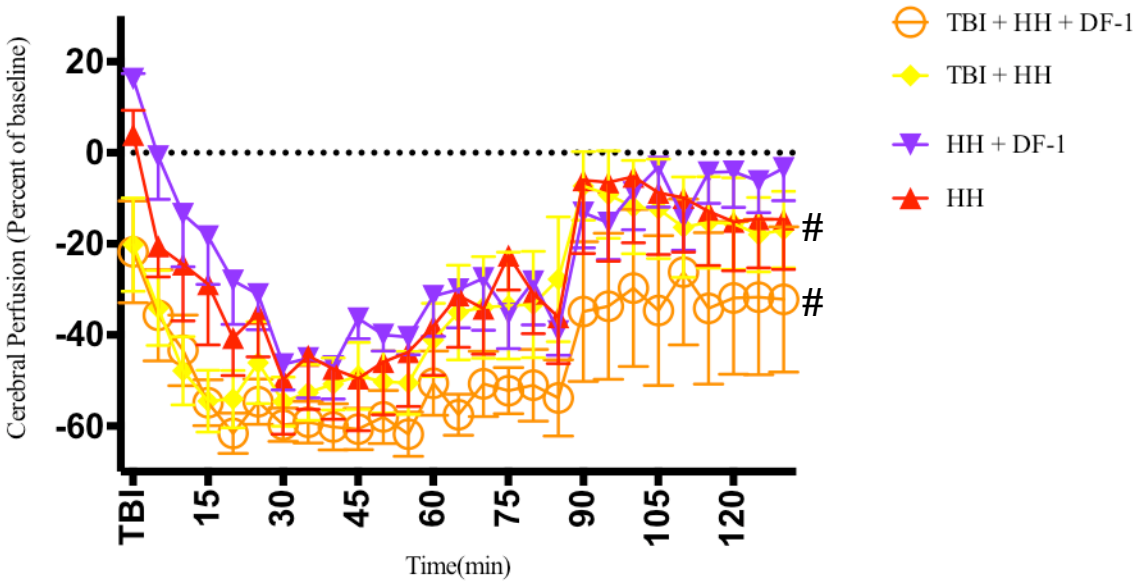

Figure 9 - Cerebral perfusion was significantly reduced in the TBI and hemorrhage groups both with and without DF-1 treatment compared to sham ($p<0.05$). TBI + HH and TBI +HH + DF-1 differed significantly from each other ($p<0.05$ indicated by # symbol).

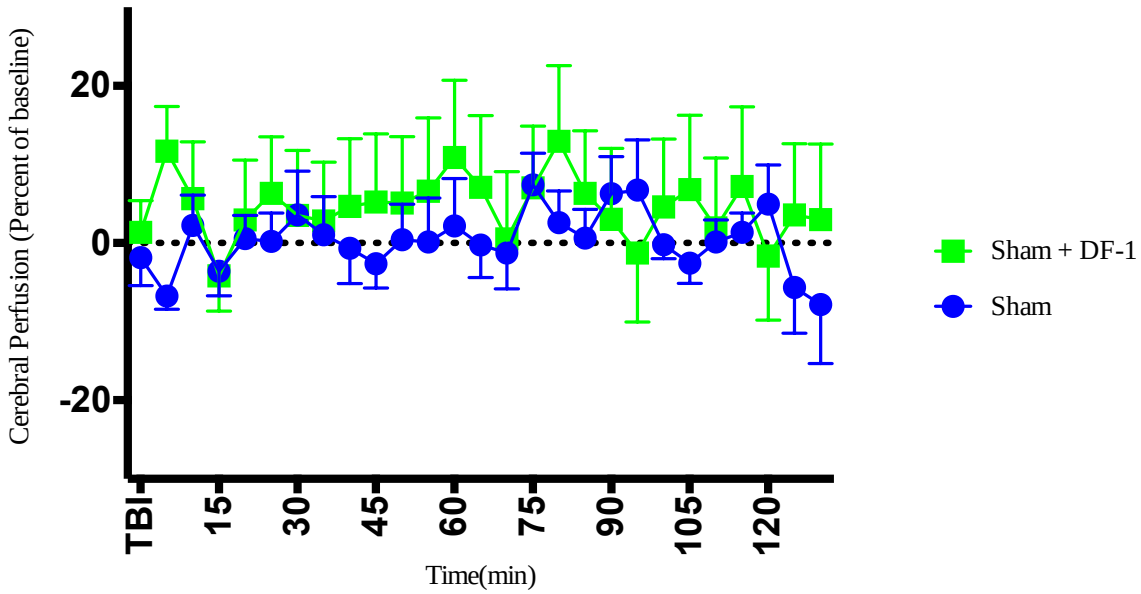

Figure 10 - Cerebral perfusion was not altered by DF-1 treatment in the sham groups.

Chapter 5: Dendro[60]Fullerene Improves Behavioral Outcomes After Traumatic Brain Injury

Abstract

Traumatic brain injury (TBI) causes an increase in reactive oxygen species (ROS) levels that cause cerebral vascular dysfunction and neuronal cell death. The use of antioxidants to reduce ROS can improve outcome after TBI. In these studies we examined the effects of Dendro[60]fullerene (DF-1) an antioxidant nanoparticle, administration on vestibulomotor function and spatial learning and memory after TBI. Male Sprague-Dawley rats were subjected to moderate fluid percussion injury or sham FPI and DF-1 (doses, i.v.) was administered one hour later. Animals were tested using beam balance and beam walking tasks on post-injury days (PID) 0-4 and the Morris water maze on PID 11 to 15. Although DF-1 administration did not affect performance on the BB task, the average times to the goal box on the BW task and the hidden platform on the MWM were reduced significantly ($p < 0.05$ TBI vs. TBI + DF-1). These results demonstrate that DF-1 treatment improved vestibulomotor and memory function after TBI.

Introduction

Traumatic brain injury affects 2.5 million people yearly and causes over 50,000 deaths in the United States alone (Cecil 2010, Center for Disease Control 2014, Ling 2010). Worldwide, TBI is the leading cause of morbidity and mortality in people under 45 (Marshall 1990, Werner 2007). Annual treatment costs amount to over 50 billion dollars annually, making TBI a serious public health concern (Laskowitz 2007). The neurological damage individuals sustain as a result of TBI is typically classified into two categories: primary injury is the initial damage due to mechanical force, and aside from the use of preventative measures there is little that can be done to treat this type of damage and secondary injury consists of all of the pathological damage resulting from the primary injury (Gennarelli 1998, Golding 2002, Graham 1996, Werner 2007). The majority of neural damage sustained from TBI is generally the result of the secondary injury (Hall 2010, Werner 2007), and the underlying processes can last for months to years after injury (Masel 2010). For both of these reasons, secondary injury is the primary target for therapeutic interventions (Ghajar 2000, Hall 2010).

Increased levels of ROS are components of the secondary injury process that contribute to vascular damage and neuronal death after TBI (Kontos 1989, Madamanchi 2005, Smith 1994, Werner 2007). This oxidative stress that occurs after TBI contributes to neuronal cell death, cognitive and motor impairments (Kontos 1989, Laskowitz 2007). Antioxidant administration improved cerebral vascular function and neurological, histopathological, and behavior outcomes in experimental TBI models (Beni 2004, Hall 2010, Kelso 2011) and antioxidant therapies may improve outcomes for TBI patients (Gilgun-Sherki 2002, Slemmer 2008)

Carboxyfullerene nanoparticles have been described as “radical sponges” and experiments in biological systems have demonstrated their strong antioxidant properties (Beuerle 2008, Brown 2010). DF-1 is a monoadduct carboxyfullerene (Da Ros 2008). Functional attachments added to a fullerene make them less stable, while reducing their antioxidant properties. Since DF-1, has only one attachment, it has greater affinity for ROS and is potentially less toxic than other carboxyfullernes with multiple attachments (Beuerle 2008). DF-1 is one of the most soluble fullerene derivatives that has been studied thus far (Da Ros 2008, Brown 2010). While carboxyfullerenes have yet to be investigated in relation to TBI, they have been studied in other neurological disorders. Carboxyfullerene nanoparticles have been shown to reduce neuronal cell death induced by excitatory neurotransmitters (e.g. NMDA, AMPA), while other antioxidants are ineffective in reducing NMDA induced excitotoxic cell death (Dugan 1996, Dugan 2001). Carboxyfullerene nanoparticles reduced cognitive deficits in MWM testing in aging mice and extended life span when administered orally in mice (Quick 2006).

In the present studies, we determine whether treatment with DF-1 improved performance in vestibulomotor function and working memory after FPI. The effect of DF-1 on vestibulomotor function (BB and BW), and working memory performance by MWM testing were tested. We utilized a MWM paradigm that specifically examines the effect of FPI on the rat’s working memory. The animal’s performance in this MWM test is dependent on the hippocampus, which is a particularly susceptible to injury after TBI (Hamm 1996). DF-1 treatment has been demonstrated to reduce neuronal cell death in the hippocampus and we hypothesize that this will lead to improved performance in BB, BW and MWM.

Methods

Animals

All animal experiments adhered to the National Institutes of Health's guidelines for ethical animal care and use and were approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee. Adult (3-6 month, 350-425 g) male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed two per cage at constant temperatures (21°–23°C) and humidity levels (45–50%) in a 12-hour light and dark cycle with food and water provided *ad libitum*.

Animal Surgical Preparation

Rats were anesthetized with 4% isoflurane in an anesthetic chamber, intubated, and mechanically ventilated (1.5-2.0% isoflurane in O_2: room air (30:70) using a volume ventilator (EDCO Scientific, Chapel Hill, NC)). All survival surgeries were performed under aseptic conditions (clean surgical gowns, sterile gloves and head covers). Instruments were autoclaved prior to use and drapes were used during the procedures. Incision sites were shaved, scrubbed, rinsed with alcohol and painted with iodine prior to procedures. Throughout the procedure, rectal and temporalis temperatures were monitored and maintained at 37.0±0.5 °C with an overhead lamp and water blanket (Gaymar, Orchard Park, NY). Rats were prepared for parasagittal fluid percussion TBI as described by Dixon, et al. (Dixon 1987). Briefly, anesthetized rats were placed in a stereotaxic head holder and the skin over the dorsal surface of the skull was incised at the midline and reflected along with the temporalis muscles bilaterally. A 3 mm craniotomy was trephined 2.5 mm from the midline between the lambda and bregma and the dura was left intact. A modified plastic Luer Lock needle hub was cemented over the craniotomy using cyanoacrylic and dental cement (Hawkins 2013a).

Parasagittal Fluid Percussion Injury

Following the surgical preparation, rats were connected to the fluid percussion device at the craniotomy site and subjected to fluid percussion TBI. Immediately after injury, rats were disconnected from the FPI device. Post-injury, the trauma adapter was removed and the scalp was sutured. In the case of survival experiments, rats were placed in a cage and monitored hourly for at least four hours. For non-survival experiments, animals were euthanized at the conclusion of the experiment by decapitation while deeply anesthetized (4.0% isoflurane).

Beam Balance

Vestibulomotor function was assessed using BB and BW tasks. Animals were trained on the BB on the day prior to injury. For training animals were placed on a narrow wooden beam that is 60 cm in length, 1.75 cm in width, 4.0 cm in height and elevated 1 m from the floor. The time the animal remained on the beam out of the 60-second trial was recorded (Dixon 1987). If the animal reached the sixty seconds, the trial was ended. If it was unable to balance it was allowed to fall into a safety box placed below the beam. Three trials were performed per day with 15 seconds of rest given between each trial (Kline 2000). A pre-assessment was done on the day of injury and testing was conducted on PIDs 1 through 4. Each trial was also scored with a rating from 1 to 6. The scoring was: 1 = balances with steady posture (grooms, climbs barrier); 2 = grasps sides of beam and/or has shaky movements; 3 = hugs beam or slips or spins on beam; 4 = attempts to balance, but falls off after 10 s; 5 = drapes over beam or hangs from beam and falls off in less than 10 s; or 6 = falls off, makes no attempt to balance or hang from beam (Sell 2008).

Beam Walk

The beam was 100 cm in length by 2.5 cm in width by 4.0 cm in height and elevated 1 m above the floor. The beam also had four equally spaced pegs 2 cm in height and 25 cm apart. The goal box used was 28 cm in length, 18 cm in height and 18 cm in width (Dixon 1987, Feeney 1982). To begin animals were first placed in the goal box with no light or noise stimulus for two minutes. Next the animal was placed at one end of the narrow beam, opposite from the goal box (Kline 2000). At the start of the trial a bright light and white noise source near the starting position of the task was turned on. Time to reach the escape chamber was recorded. Once the animal reached the goal box the light and white noise were discontinued (Dixon 1987). Three trials were performed each day with thirty seconds between each trial, during which time the animal remained in the darkened goal box (Dixon 1987, Kline 2000, Sell 2008). Animals were trained on the BW test the day prior to injury. A pre-assessment was done on the day of injury and testing was conduct on PIDs 1 through 4 (Sell 2008).

Morris Water Maze

A variation of the MWM was used that examines working memory after TBI in a FPI model (Hamm 1996). The water maze was a tank approximately 180 cm in diameter and 75 cm deep that was filled with water to a depth of 28 cm. A Plexiglas goal platform (10 cm diameter) was submerged 2.5 cm below the surface of the water. Distal clues were present on the 4 walls of the room surrounding the water maze. The testing room had a video camera on the ceiling to track the rat's movement in the maze. A platform (10cm diameter) was placed in water 2.5 cm below the surface. Testing began 11 days post-surgery and continued until day 15. Each day consisted of four paired two-minute trials for

a total of eight trials per rat per day. For a pair of trials the location of the platform and start location was both randomized to one of four quadrants. Rats performed two tests with the platform and start location in the same spot. For the following three pairs the location of the platform and the start location where again randomized. Time to reach the platform was measured (Hamm 1996).

Statistical Analysis

For the BB experiments, data were expressed as mean ± SEM. A Friedman's test was performed and Dunn's multiple comparison tests were performed with an α level of significance of 0.05. For BW experiments, data were expressed as mean ± SEM and were analyzed using a two-way ANOVA. For post hoc comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05. MWM latencies were expressed as mean ± SEM and was analyzed using two-way ANOVA in Prism®. Each set of measurements from the same animal was considered a correlated cluster of observations. For *post hoc* comparisons, Tukey's multiple comparison tests were performed with an α level of significance of 0.05 (Sell 2008).

Results

BB and BW were used to test vestibular motor function after FPI. There were no significant differences in BB performance among the groups across trial days (Figure 10). In BW testing, DF-1 significantly ($p<0.05$ TBI + 10 mg/kg DF-1 vs. TBI) reduced the time to reach the goal box (Figure 11). When comparing latency to hidden platform in the MWM across all trials TBI significantly increased swim times compared to sham ($p<0.05$) (Figure 13). DF-1 treatment significantly reduced latency to hidden platform in the TBI + 10-mg/kg DF-1 group compared to the TBI group across all trials ($p<0.05$). The TBI + 10-mg/kg

DF-1 group did not differ significantly from the sham group. When we examined the effect of DF-1 treatment on individual trial days, DF-1 did not significantly reduce the latency to hidden platform (Figure 12).

It is also important to analyze the difference between trial one and trial two in each group to see if working memory is impaired (Hamm 1996). In all groups latencies to hidden platform in trial one were significantly greater than in trial two (Figure 14). This indicates that working memory was not impaired in the TBI group or the TBI + 10-mg/kg DF-1 group.

Discussion & Conclusion

We observed that DF-1 administration one hour after moderate fluid percussion TBI in rats significantly improved vestibulomotor and cognitive performance. Carboxyfullerenes reduced apoptosis and excitotoxic necrosis in cultured cortical neurons (Partha 2009; Dugan 1997) delayed the onset of symptoms and significantly delayed cell death in an *in vitro* mouse model ALS (Dugan 2001) and reduced aging-induced MWM (Quick 2008), this is the first report of improved vestibulomotor and cognitive function with carboxyfullerene nanoparticle administration after TBI in rats.

Traumatic brain injury was associated with a significant increase in the times to the goal box in the BW task. The administration of 10 mg/kg DF-1 improved the time to goal box to levels not significantly different from those of the sham-injured rats. In contrast, BB scores were not significantly different between the TBI and TBI + DF-1 groups. The BB task is less sensitive than other measures of motor or vestibulomotor tasks, particularly in mild and moderate injury (Hamm 1994). However, since we observed no trend towards

improved BB scores in the DF-1-treated rats (Figure 10), the absence of a therapeutic effect of DF-1 in the BB task is unlikely to have been related to the insensitivity of the task.

We also analyzed the differences between latencies to find the platform in trial one and trial two within groups to determine if working memory was impaired (Hamm 1996). In all groups goal latencies in trial one were significantly greater than in trial two ($p<0.05$). This indicates that TBI animals do not have impaired working memory (Hamm 1996). Therefore we cannot asses if DF-1 treatment improved working memory as moderate TBI did not result in severe enough working memory deficits. Our results demonstrated that DF-1 significantly improved MWM performance after TBI but did not improve working memory, as working memory was not significantly impaired in injured animals. Unlike other designs that assess both learning and memory (Hamm 1996), the procedure for the MWM task used in the present study only evaluates working memory. Therefore, we did not assess the effects of DF-1 administration on learning.

Cognitive and motor deficits are hallmark long-term disabilities of clinical TBI (Kline 2000, Zasler 2007). Memory is frequently impaired after TBI and the hippocampus is one key structure that is linked to these memory impairments that occurs after TBI (Smith 1991). Damage to the hippocampus has been demonstrated in both clinical TBI patients, as well as in animal models (Hicks 1993, Smith 1991). Both behavioral tests and histological studies have demonstrated impairment of the hippocampus after TBI. The CA1 and CA 3 regions of the hippocampus have high levels of neuronal damage after injury (Cortez 1989, Smith 1991). The working memory MWM paradigm specifically examines the effect of FPI on the rat's working memory. The animal's performance in this MWM

test is dependent on the hippocampus, which is a particularly susceptible to injury after TBI (Hamm 1996).

In previous experiments, discussed in chapter 3, we demonstrated that DF-1 is capable of reducing neuronal cell death in the CA1/2 and CA3 regions of the hippocampus. This preservation of neurons may contribute to the improvements in MWM. Patients frequently suffer from impaired memory, perception and information processing after injury (Masel 2010). Memory function has been associated with the hippocampus, a structure that is particularly vulnerable to brain injury (Hamm 1996, Smith 1991). This preservation of neurons that we say in our FluoroJade studies may contribute to the improvements we saw in MWM and BW performance.

In summary, these results demonstrate that DF-1 treatment improved vestibular motor function in BW testing. DF-1 also improved latencies to find the platform across all trials but not on individual days in MWM testing. However, we were not able to determine if DF-1 improved working memory, as we did not see significant impairment after TBI. Our results indicated that DF-1 does have benefits to cognitive function after FPI and is worth further investigation as a therapeutics option for TBI.

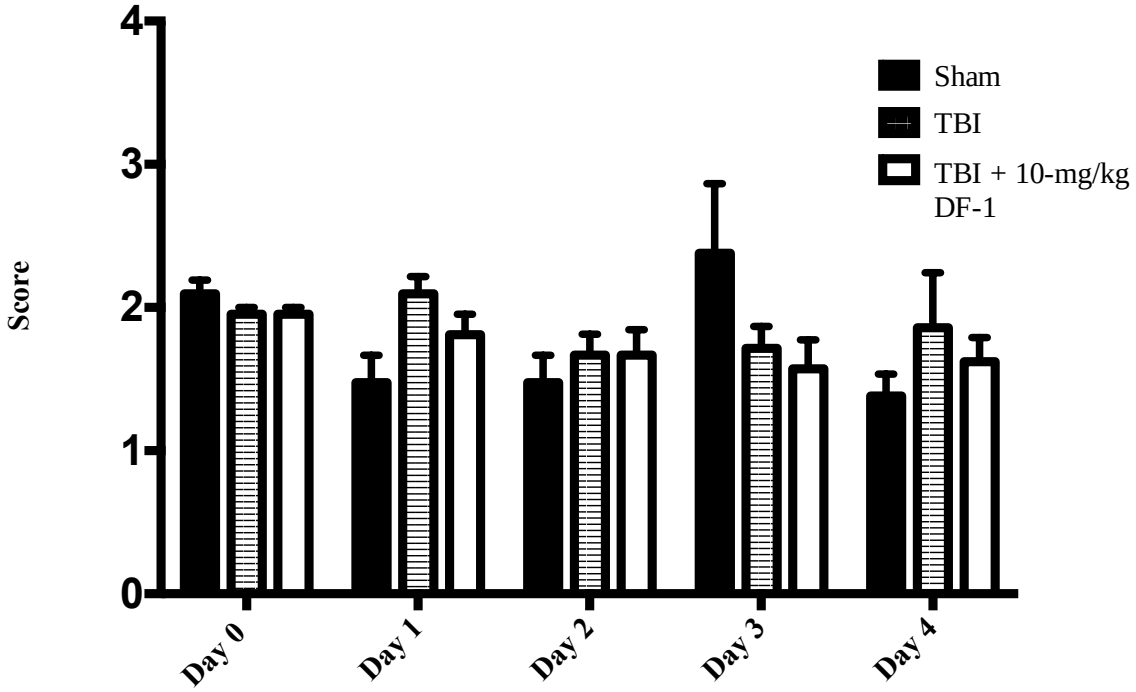

Figure 11 – Average scores on BB testing. There were no significant differences among the groups. All values in the text and figures are stated as mean score SEM.

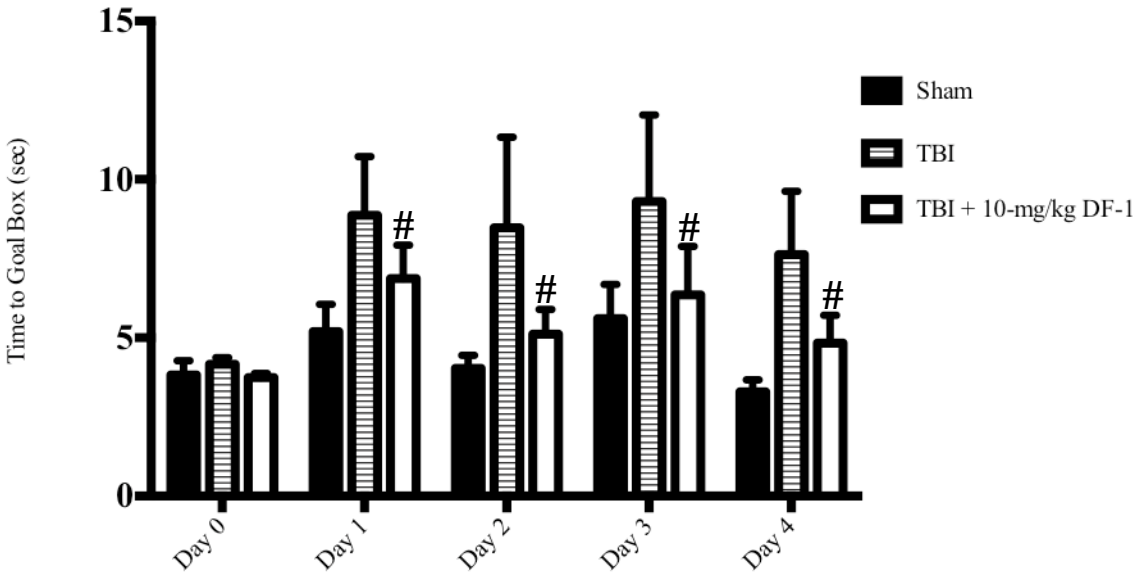

Figure 12 – Average times to complete the BW test. DF-1 treatment significant reduced the time to traverse the beam (# indicates $p<0.05$ TBI vs. TBI + DF-1).

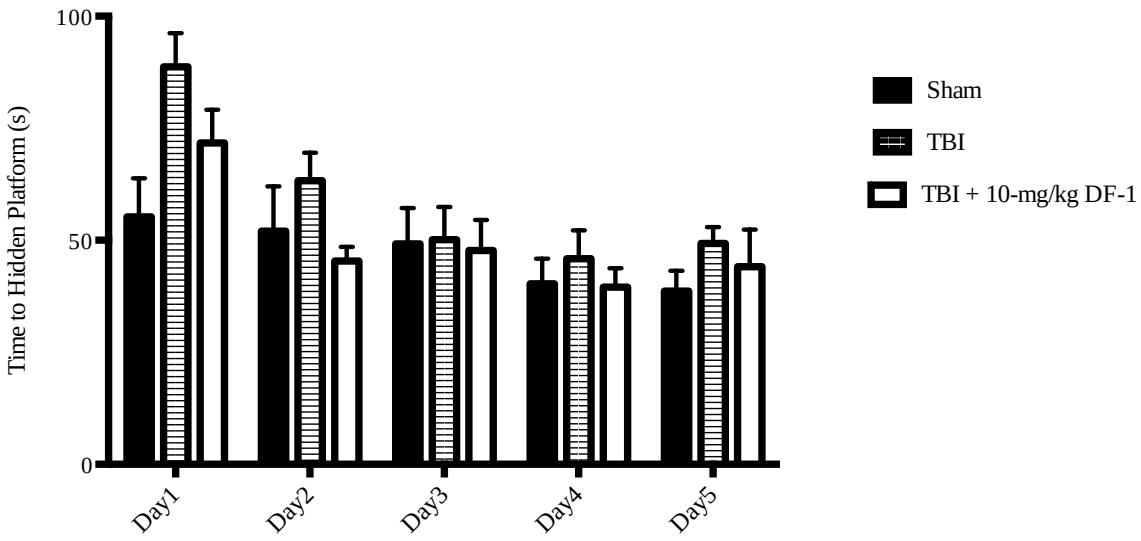

Figure 13 – This graph shows the time average time to locate the hidden platform in MWM testing by trial day.

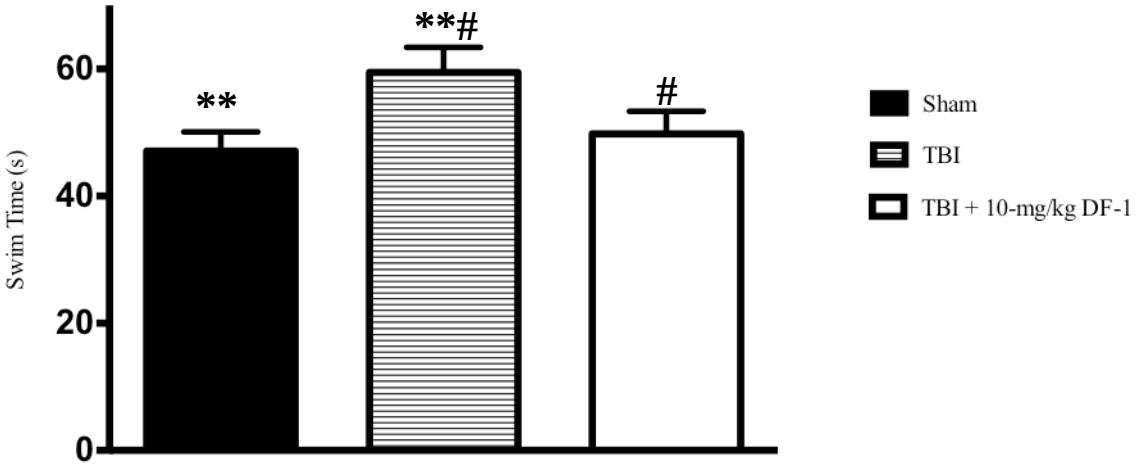

Figure 14 – When the time to reach the hidden platform across all trials was analyzed, DF-1 treatment significantly reduced swim time compared to the untreated injured group. (** $p<0.05$ Sham vs. TBI) (# - $p<0.05$ TBI vs. TBI + 10-mg/kg DF-1).

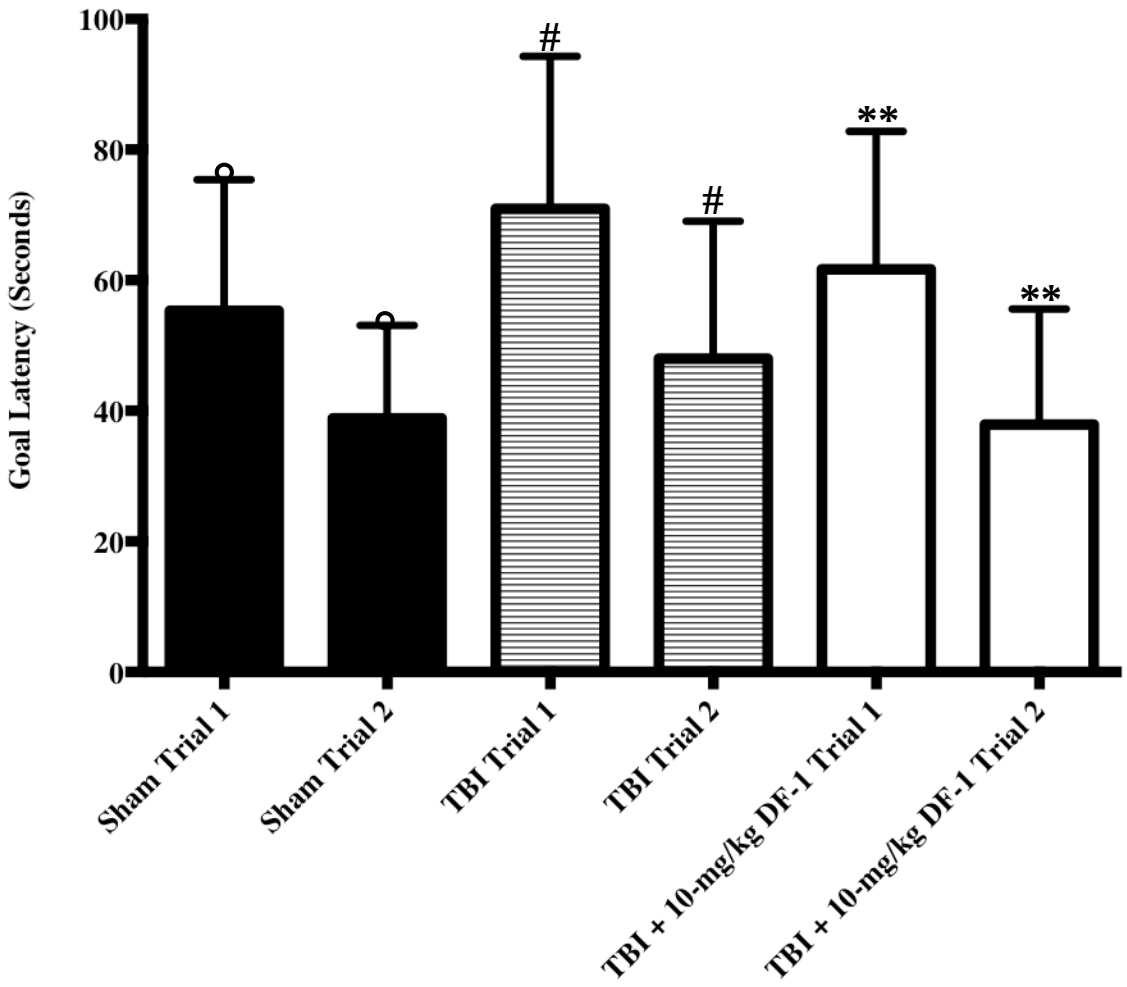

Figure 15 – In order to determine if working memory is impaired, this graph shows the differences in goal latency in MWM testing between the first and second trials. In sham, TBI and TBI + 10-mg/kg DF-1 the first trial was significantly longer than the second trial. This indicates that working memory was not impaired after TBI. If working memory was not impaired the two trials would no be significantly different. (Circle - $p<0.05$ Sham trial 1 vs. sham trial 2) (#- $p<0.05$ TBI trial 1 vs. TBI trial 2) (** - $p<0.05$ TBI + 10-mg/kg DF-1 trial 1 vs. TBI + 10-mg/kg DF-1 trial 2)

Chapter 6: Discussion and Conclusions

The central hypothesis of this dissertation work was that DF-1, a powerful antioxidant nanoparticle, would preserve vascular function after TBI, reduce hippocampal cell death and improve behavioral outcomes after TBI with and without posttraumatic HH. We predicted the following results, for aim one we hypothesized that DF-1 would improve cerebral perfusion, cerebral vascular reactivity and the vasodilatory response. We also predicted that if DF-1 were capable of preserving the vasculature, which would result in preservation of neurons in the hippocampus. The purpose of aim two was to determine if DF-1 would improve cerebral perfusion after TBI + HH. In aim three, we hypothesized that if DF-1 was able to preserve the vasculature and reduce neuronal cell death, it would improve behavioral outcome in Morris Water Maze (MWM) testing.

While some work has been done with nanoparticles for drug delivery, diagnostics and other application in TBI research, the use of antioxidant nanoparticle as a therapeutic treatment for brain injury is relatively unexplored (Cho 2012, Hockey 2013, Weinstein 2010). PEG-HCCs improved cerebral perfusion after cortical contusion injury and HH model (Bitner 2012). Hicks, et al., reported that cerium oxide nanoparticles improved behavioral outcome in MWM testing after FPI (Hicks 2013). Our studies were the first to use the carboxyfullerene nanoparticle DF-1, as a therapeutic agent. Compared to other carboxyfullernes, DF-1 has advantages in stability and ROS scavenging capabilities (Beuerle 2008). Also, to our knowledge, this is the first use of DF-1 in any model of central nervous system injury.

Aim One Discussion

In aim one we examined DF-1's effect on cerebral perfusion, MAP and CVR after moderate, parasagittal FPI. Vasodilatory responses to reduced intravascular pressure were measured in isolated, pressurized MCAs harvested from rats after FPI, with and without DF-1 treatment. We also determined the numbers of FJC-positive hippocampal neurons after FPI with or without DF-1. Our results indicated that DF-1 treatment did not improve CVR, MAP, cerebral perfusion or cerebral dilatory responses to reduced intravascular pressure compared to untreated TBI animals, as we had initially hypothesized. CVR, MAP and cerebral perfusion were all either unaffected or worsened, depending on the dose of DF-1. Trauma-induced reductions in vasodilation in response to reduced intravascular pressure in isolated, pressurized MCA segments were not reversed by DF-1 administration.

There are several possible explanations for the absence of a vasoprotective effect by DF-1 after TBI. One explanation may be related to the time point of administration at one-hour post-TBI. Superoxide anion radical levels increased within minutes of TBI and continued to increase for at least 45 min post-injury (Fabian 1998, Kontos 1986b). Hydroxyl radicals had an initial peak in release at five minutes after injury (Smith 1994). This was accompanied by an increase in lipid peroxidation (Abdul-Muneer 2014, Smith 1994). Because ROS peaked early after TBI, it is possible that ROS-mediated cerebral vascular injury was too far advanced by the time DF-1 was administered. The one-hour time point of DF-1 administration may have been too late for DF-1 to effect cerebral vascular injury.

While we did not see an effect on CVR or cerebral perfusion through treatment with DF-1, studies by Bitner et al., did see improvement through antioxidant nanoparticle

treatment. PEG-HCCs were effective at improving cerebral perfusion after TBI + HH (Bitner 2012). Unlike our study that used only one dose of DF-1, these studies used multiple doses of PEG-HCCs at different time points. Animals were also given PEG-HCCs at a time point that coincided with blood reinfusion. Blood reinfusion is known to cause additional spikes in ROS levels (Bitner 2012, Fabian 1995). Administering the nanoparticle at the same time as reinfusion might have been an important factor in why PEG-HCCs were able to have an effect on cerebral perfusion in this model. The use of multiple doses may have also played a role in the effects of nanoparticle administration on CVR (Bitner 2012).

Another explanation for our results is that the increased CVR and reduced cerebral perfusion that we observed after DF-1 administration may have to due to the scavenging of nitric oxide (NO) by DF-1. Nielson, et al., reported that DF-1 scavenged NO and reduced NO formation by inhibiting NO synthase (Nielsen 2008). NO contributes to the maintenance of resting vasodilation in cerebral arteries and the inhibition of NO synthesis reduced cerebral perfusion (DeWitt 2003, Cherian 2000). Scavenging NO would result in vasoconstriction of arteries and reduced cerebral perfusion (Cherian 2000, Förstermann 2012). This is supported by the observation that PEG-HCCs increased brain tissue levels of NO (Bitner 2012).

While DF-1 administration did not improve the CVR, cerebral perfusion or cerebral dilatory responses to reduced intravascular pressure, we did observed a significant reduction in the numbers of FJC-positive neurons in the hippocampus. This indicates that DF-1 treatment is preserving neurons after FPI and suggests that it may have a potential therapeutic benefit after TBI. While the time of administration in this study may not have

been early enough to protect the cerebral vasculature, it may have reduced subsequent oxidative stress in the brain. ROS are elevated in the parenchyma for several hours up to a few days after injury (Hall 2010, Tyurin 2000), which would permit DF-1 administered one-hour post-TBI to exert a neuroprotective effect.

Although ROS scavengers may exert neuroprotective effects without traversing the BBB (Hall 2010), DF-1 would be more likely to have neuroprotective activity if it is capable of traversing the BBB. There is abundant evidence that the BBB is permeable to water soluble carboxyfullerenes such as DF-1 (Baati 2012, Beuerle 2007, Cagle 1999, Ji 2006, Qingnuan 2002, Quick 2008, Riviere 2009, Yamago 1995). DF-1's ability to cross the BBB would aid the nanoparticle's ability to reduce ROS levels in the brain and preserve neurons. Published research also indicates that carboxyl-functionalized fullerenes cross the blood brain barrier (Quick 2008, Riviere 2009). Two studies reported that all water-soluble fullerenes such as DF-1 are able to cross the BBB (Baati 2012, Yamago 1995). Other reports indicated that functionalized fullerenes (e.g. DF-1) readily cross the blood brain barrier (Beuerle 2007, Cagle 1999, Ji 2006, Qingnuan 2002). We believe that these reports support our research indicating that while DF-1 does not improve cerebral vascular function after TBI; it is likely able to cross the BBB to provide neuroprotection through local anti-oxidant activity.

These experiments contributed to a better understanding of the most effective dose of DF-1 for TBI treatment. We measured cerebral perfusion, MAP and FJC levels in the hippocampus after TBI and administration of 10, 25 or 50-mg/kg doses of DF-1. Neither 25 nor 50 mg/kg DF-1 were more effective at improving MAP or cerebral perfusion than

10 mg/kg DF-1 and the 10-mg/kg dose was most effective at reducing the numbers of FJC positive hippocampal neurons.

In summary, these results demonstrated that DF-1 treatment did not improve cerebral perfusion, MAP or CVR after in a TBI. The 25-mg/kg dose of DF-1 reduced cerebral perfusion, and all doses increased MAP. DF-1 also did not have a significant effect on cerebral dilator responses to reduced intravascular pressure in MCA segments. It did, however, effectively preserve neurons in the hippocampus. So while DF-1 did not have a therapeutic effect on the cerebral vasculature, DF-1 may be an effective neuroprotective therapy after TBI and further studies with DF-1 are warranted.

Aim Two Discussion

Our studies showed that cerebral perfusion was decreased after TBI + HH, which is consistent with previous literature (DeWitt 1992, Matsushita 2001, Navarro 2012, Schmoker 1992). Because autoregulation is impaired after TBI, the vasculature is unable to maintain normal cerebral perfusion when systemic arterial blood pressure is reduced. Cerebral perfusion may be decreased after head injury due to arterial hypotension or elevated intracranial pressure (Lewelt 1980, Oddo 2011). HH further impacts cerebral vascular reactivity (Hawkins 2013b, Kong 1991). Although we had hypothesized that DF-1 would improve cerebral perfusion after TBI + HH, we found that cerebral perfusion was lower in the TBI+HH+10-mg/kg DF-1 group than in the untreated TBI+HH group during the resuscitation period. Since autoregulation is needed to maintain proper cerebral perfusion (Chan 1992, DeWitt 2003), our results suggest that autoregulation is impaired after TBI and these impairments are worsened by DF-1 treatment.

It is notable that for the groups that received HH alone, cerebral perfusion increased to baseline and was not significantly different from sham in the final resuscitation phase. In the TBI + HH and TBI + HH + 10-mg/kg DF-1 groups, cerebral perfusion remained significantly reduced during the resuscitation phase. Cerebral perfusion in the TBI + HH + DF-1 group was significantly reduced compared to the TBI + HH. Reduced cerebral perfusion in the resuscitation phase is correlated with poor restoration of MAP (Hawkins 2013), and reductions in MAP are associated with higher rates of cerebral ischemia (Dandapani 1995, Zhu 1995).

These results may be explained by the fact that DF-1 is capable of acting as a pro-oxidant (Foley 2002). Due to this property, fullerenes can inhibit vasodilation that is NO dependent by producing superoxide radicals. If DF-1 is acting as pro-oxidant in our model of TBI + HH, then it may be preventing vasodilation (Satoh 1997). It is also possible that DF-1 is in fact acting as an antioxidant and that the impaired vasodilatory response may be caused by DF-1 scavenging NO. Fullerene nanoparticles has been demonstrated to be capable of scavenging NO. It is also able to prevent NO formation by inhibiting nitric oxide synthase (Nielsen 2008). When NO synthesis is inhibited, cerebral perfusion is reduced (Cherian 2000, Dokken 2015). NO plays an important role in maintaining blood vessels at a state of partial dilation of blood vessels. If DF-1 were reducing NO level or blocking NO synthase it would lead to vasoconstriction of blood vessels. This vasoconstriction would, in turn, reduce cerebral perfusion (Cherian 2000).

The results of aim two demonstrate that DF-1 did not improve cerebral perfusion in a TBI + HH model. In fact, DF-1 reduced cerebral perfusion both during and after the resuscitation period. While DF-1 did not improve cerebral vascular function in this model,

it still may be worth examining it's effects on neuropathological outcomes in the TBI + HH model. Our work in aim one showed that in a FPI TBI model, DF-1 also did not improve cerebral vascular function but it did preserve neurons in the hippocampus after injury. Neuropathologic outcomes of TBI + HH are more severe than when TBI occurs alone (Matsushita 2001). Thus, DF-1's effect on hippocampal neuronal injury may be particularly advantageous.

Aim Three Discussion

In aim three we observed that DF-1 administration one hour after moderate fluid percussion TBI in rats significantly improved vestibulomotor in the BW test and cognitive performance in the MWM. Carboxyfullerenes reduced apoptosis and excitotoxic necrosis in cultured cortical neurons (Partha 2009; Dugan 1997), delayed the onset of symptoms, significantly delayed cell death in an *in vitro* mouse model ALS (Dugan 2001) and reduced aging-induced deficits in the MWM (Quick 2008). The results from aim three are the first report of improved vestibulomotor and cognitive function with carboxyfullerene nanoparticle administration after TBI in rats.

Traumatic brain injury was associated with a significant increase in the times to reach the goal box in the BW task. The administration of 10-mg/kg of DF-1 improved the time to goal box to levels not significantly different from those of the sham-injured rats. In contrast, BB scores were not significantly different between the TBI and TBI + 10-mg/kg DF-1 groups. The BB task is less sensitive than other measures of motor or vestibulomotor tasks, particularly in mild and moderate injury (Hamm 1994). However, since we observed no trend towards improved BB scores in the DF-1-treated rats, the

absence of a therapeutic effect of DF-1 in the BB task is unlikely to have been related to the insensitivity of the task.

We also analyzed the differences between latencies to find the platform in trial one and trial two within groups to determine if working memory was impaired (Hamm 1996). In all groups, goal latencies in trial one were significantly greater than in trial two ($p<0.05$). This indicates that TBI animals do not have impaired working memory (Hamm 1996). Therefore, we cannot asses if DF-1 treatment improved working memory as moderate TBI did not result in severe enough working memory deficits. Our results demonstrated that DF-1 significantly improved MWM performance after TBI as a whole, but we do not know if it impacted working memory. Unlike other designs that assess both learning and memory (Hamm 1996), the procedure for the MWM task used in the present study only evaluates working memory. Therefore, we did not assess the effects of DF-1 administration on learning.

Cognitive and motor deficits are hallmark long-term disabilities of clinical TBI (Kline 2000, Zasler 2007). Memory is frequently impaired after TBI and the hippocampus is one key structure that is linked to these memory impairments that occurs after TBI (Smith 1991). Damage to the hippocampus has been demonstrated in both clinical TBI patients, as well as in animal models (Hicks 1993, Smith 1991). Both behavioral tests and histological studies have demonstrated impairment of the hippocampus after TBI. The CA1 and CA 3 regions of the hippocampus have high levels of neuronal damage after injury (Cortez 1989, Smith 1991). The working memory MWM paradigm specifically examines the effect of FPI on the rat's working memory. The animal's performance in this MWM

test is dependent on the hippocampus, which is a particularly susceptible to injury after TBI (Hamm 1996).

In previous experiments, discussed in chapter three, we demonstrated that DF-1 is capable of reducing neuronal cell death in the CA1/2 and CA3 regions of the hippocampus. This preservation of neurons may contribute to the improvements in MWM. Patients frequently suffer from impaired memory, perception and information processing after injury (Masel 2010). Memory function has been associated with the hippocampus, a structure that is particularly vulnerable to brain injury (Hamm 1996, Smith 1991). This preservation of neurons that we say in our FJC studies may contribute to the improvements we saw in MWM and BW performance.

In summary, these results demonstrate that DF-1 treatment improved vestibular motor function in BW testing. DF-1 also improved latencies to find the platform across all trials but not on individual days in MWM testing. However, we were not able to determine if DF-1 improved working memory, as we did not see significant impairment after TBI. Our results indicated that DF-1 does have benefits to cognitive function after FPI.

Conclusion

This dissertation work has demonstrated that DF-1 does not improve cerebral perfusion, MAP or CVR following FPI; nor was it able to improve vasodilation as measured in MCA experiments. When DF-1 was tested in a TBI + HH model, it did not improve cerebral perfusion. However, it was demonstrated that DF-1 was able to preserve hippocampal neurons after FPI as tested by FJC. Treatment with DF-1 also improved results in MWM and BW. Based on all of this data, we have concluded that DF-1 improves neuropathological and behavioral outcome after moderate FPI, but does not improve

cerebral vascular outcome as we had hypothesized. Following these behavioral and neuropathological results we believe that DF-1 deserves further examination as a therapeutic option for TBI.

Based on the working in this dissertation there are many directions to explore with future research. We have proposed several explanations for how ROS/RNS might be negatively affecting the vascularature by acting as a pro-oxidant or by scavenging NO. Experiments to determine how DF-1 impacts ROS/RNS levels would therefore be valuable. Since DF-1 positively impacted neuronal survival in the hippocampus and cognitive endpoints in MWM testing, it is worth exploring how DF-1 is impacting these endpoints since they cannot be explained by improvements in the cerebral vasculature. One explanation for these outcomes is that DF-1 is promoting synaptic function after brain injury. The hippocampus is an area that is particularly susceptible to oxidative stress and particularly susceptible after TBI. ROS play a role in long-term potentiation in this region, and maintaining proper levels is important to synaptic function (Serrano 2004). Improved synaptic function has resulted in treatment with other nanoparticles such as zinc oxide nanoparticles. These nanoparticles increased neuronal excitability, as well as the release of glutamate (Xie 2012). It is also possible that DF-1's is protecting glial cells, and this might potentially explanation how the nanoparticle is impacting neurons in the hippocampus and performance in behavioral tests. Brain injury produces structural damages to glia, as well as neurons (Chan 1984). It has been demonstrated that high levels of ROS can result in the death of oligodendrocytes. Glial cells are important for neurons to function properly. (Juurlink1997). Therefore DF-1 may be acting as an antioxidant to preserve these cells,

and improving cognitive function after brain injury. We believe that experiments in these areas would be while for further investigation of DF-1's impact on TBI.

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Vita

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Publications

1) Petrov, A., **Wynne, K.E.,** Parsley, M.A., Petrov, I.Y., Petrov, Y., Ruppert, K.A., Prough, D.S., DeWitt, D.S., Esenaliev, R.O. (2014) "Optoacoustic detection of intra- and extracranial hematomas in rats after blast injury." Photoacoustics 2:75-80.

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3) Petrov, A., **Wynne, K.E.,** Prough, D.S., DeWitt D.S., Petrov, Y., Parsley, M.A., Esenaliev, R.O. Optoacoustic detection and monitoring of blast-induced intracranial hematomas in rats. International Society for Optics and Photonics (SPIE) Photonics West 2015, San Francisco, California, February 1-6, 2014.

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5) D.K. Weiss, N.S. Levine, E.K. Beutel, N. De Munster, L.G. Barajas, **K.E. Wynne**, A. Stein, C. Runyon, "Mapping Rover Routes and Hydrous Soil Location

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7) D. Tucker, A. Manivannan, D. Haynes, H. Abernathy, N. Miller**, K. Wynne**, A. Matos, "Evaluating Methods for Infiltration of LSCF Cathodes With Mixed Electronic/Ionic Conductors for Improved Oxygen Exchange," ASME 2010 8th International Conference on Fuel Cell Science, Brooklyn, New York, June 14 -16 2010.

Abstracts

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9) **K.E. Wynne**, Y.P., Zeng, D.S. Prough, D.S. DeWitt, "Effects of Dendro[60]fullerene on Neurobehavioral Outcome and Cerebral Vascular Function After Traumatic Brain Injury." Mission Connect Symposium 2014, Houston, Texas, December 2014.

10) **Wynne, K.E.,** Prough, D.S., DeWitt, D.S. "Investigation of the effects of Carboxyfullerene Nanoparticles on the cerebral vasculature after fluid percussion injury." National Neurotrauma Symposium 2014, San Francisco, California June 29 – July 2, 2014.

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15) **K.E. Wynne**, Y. Zeng, D.S. Prough, D.S. DeWitt, "Carboxyfullerene Nanoparticles Reduce Oxidative Stress After Rapid Stretch Injury In Vascular Smooth Muscle Cells, in vitro" Mission Connect Symposium 2012, Houston, Texas, December 7, 2012.

16) **K.E. Wynne**, D.S. Dewitt, Y. Petrov, I.Y. Petrov, A. Petrov, M.A. Parsley, K.A. Ruppert, R.O. Esenaliev, D.S. Prough, "The Use of Optoacoustic Monitoring to Measure Cerebral Oxygen Saturation in Rats Subjected to Blast-Induced Neurotrauma," Mission Connect Symposium 2012, Houston, Texas, December 7, 2012.

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19) **K.E. Wynne**, D.S. Dewitt, Y.Y. Petrov, I.Y. Petrov, M.A. Parsley, R.O. Esenaliev, D.S. Prough, "Optoacoustic Monitoring of Cerebral Venous Blood Oxygenation in Rats With Traumatic Brain Injury." (Oral Presentation) International Society for Optics and Photonics (SPIE) Photonics West 2012, San Francisco, California, January 21 – 26, 2012

20) I.Y. Petrov, **K.E. Wynne**, Y.Y. Petrov, R.O. Esenaliev, C.J. Richardson, D.S. Prough, "Noninvasive Optoacoustic Monitoring of Cerebral Venous Blood Oxygenation in Newborns." International Society for Optics and Photonics (SPIE) Photonics West 2012, San Francisco, California, January 21 – 26, 2012

21) **K.E. Wynne**, J. Richardson, R. Esenaliev, I. Petrov, Y. Petrov, D.S. Prough, "Optoacoustic Monitoring of Oxygen Saturation in the Superior Sagittal Sinus of Neonates," (Oral Presentation) Mission Connect Symposium 2011, Houston, Texas, December, 2011

22) **K.E. Wynne**, J. Richardson, R. Esenaliev, I. Petrov, Y. Petrov, D.S. Prough, "Optoacoustic Monitoring of Oxygen Saturation in the Superior Sagittal Sinus of Neonates," American Society of Anesthesiology 2011, Chicago, Illinois, October 15 – 19, 2011

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24) D. Tucker, A. Manivannan, D. Haynes, H. Abernathy, N. Miller**, K. Wynne**, A. Matos, "Evaluating Methods for Infiltration of LSCF Cathodes With Mixed Electronic/Ionic Conductors for Improved Oxygen Exchange," ASME 2010 8th International Conference on Fuel Cell Science, Brooklyn, New York, June 14 -16 2010

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