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Enzyme Analysis of Oxidatively Modified Proteins Post-TBI

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DEDICATION

This thesis is dedicated to my mother, Ms. Patricia Rice, and grandmother, Mrs. Corine Stitt, who love me enough to selflessly abandon their dreams to nourish my own. Your struggles and sacrifices have not gone unnoticed and were not in vain.

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ABSTRACT

The brain is one of the most important organs in the body. It functions as a control center by regulating and coordinating actions and reactions, which is facilitated via signal transduction pathways. Its function is primarily dependent upon sufficient supply of glucose for energy metabolism. The dysfunction of the brain resulting from an external force is known as traumatic brain injury (TBI). Symptoms range from physical to psychological and effects can be mild, moderate, or severe depending on the extent of injury. TBI is associated with oxidative damage the overproduction of reactive oxygen/nitrogen species. Reduced energy metabolism is a consequence of traumatic brain injury, while reduced purine salvage is associated with deficient cell signaling. Previous studies have demonstrated that the administration of gamma-glutamylcysteine ethyl ester (GCEE) following TBI has protective effects against protein nitration. This study investigates the enzymatic activity of energy related and salvage related enzymes, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase, malate dehydrogenase, cytochrome C oxidase, ATP synthase, and hypoxanthine-guanine phosphoribosyltransferase, that have been identified as excessively nitrated following the administration of GCEE post-TBI. Adult male Wistar rats were divided equally into three groups: sham, saline, and GCEE. Rats in all groups (except sham) were subjected to a craniotomy and a moderate TBI via cortical contusion. Post-TBI rats in the saline group received an administration of saline

(150mg/kg), and rats in the GCEE treatment group received an administration of GCEE (same dosage). Upon sacrifice, brains were harvested and enzymatic activity was indirectly measured spectrophotometrically. Data demonstrates that the administration of GCEE following brain trauma increases enzymatic activity. Our results are promising and indicate potential therapeutic strategies to restore energy and salvage related enzymatic activity in the brain post-TBI.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-PGA	3-phosphoglyceric acid
3-PGK	3-phosphoglyceric phosphokinase
AMPA	a-amino-3-hydroxyl-5-methyl-4-isoxaloepropionate
AD	Alzheimer's Disease
ADP	Adeonsine diphosphate
AST	Aspartate amiontransferase
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CCI	Controlled Cortical Impact
Cyt-C	Cytochrome C oxidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCEE	Gamma-glutamylcysteine ethyl ester
GCS	Gamma-glutamylcysteine synthase
GCS	Glasgow Coma Scale
Glu	Glutamate
Gly	Glycine
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GPx	Glutathione peroxidase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Oxidized glutathione
HD	Huntington's Disease
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
IMP	Ionsine monophosphate

LDH	Lactate dehydrogenase
LP	Lipid peroxidation
MCI	Mild cognitive impairment
MDH	Malate dehydrogenase
MPT	Mitochondrial permeability transition pore
NMDA	N-methyl-D-asparate
PCS	Post-concussive syndrome
PD	Parkinson's Disease
PEP	Phosphoenolpyruvate
PK	Pyruvate Kinase
ROS	Reactive oxygen species
ROS/RNS	Reactive oxygen and nitrogen species
RNS	Reactive nitrogen species
SOD	Superoxide dimutase
TBI	Traumatic brain injury
γ-GluCys	Gamma-glutamylcysteine

CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) is a sudden event that results in the disruption of proper brain functionality and is caused by an external force, such as a blow to the head. Symptoms range from physical to psychological while effects can be mild, moderate, or severe depending on the extent of the injury experienced. Many people who experience TBI face progressive, lifelong physical disabilities and cognitive impairments. In the past, injury from head trauma was considered instantaneous and irreversible. Now it is clear that there are two phases of injury: primary and secondary. Upon insult of injury, the primary physical damage sustained by the brain alters biochemical and cellular pathways. Secondary injury then begins as a byproduct of the biochemical and cellular disruptions of primary injury, and is a progressive cytotoxic cascade causing damage accumulation, TBI progression, and neurodegeneration, over an extended period of time.

Membrane depolarization, a direct consequence of primary injury, initiates the progressive cytotoxic cascade of damage-accumulating events by causing the uncontrolled release of glutamate in the synaptic gap. The excessive amounts of glutamate cause the hyper-activation of glutamate receptors, which consequently cause a mass influx of calcium into the neuronal cells. As a result of this glutamate-induced excitotoxicity and calcium overload, reactive oxygen

(ROS) and reactive nitrogen species (RNS) are overproduced. Typically, the production of ROS is an aftereffect of oxygen metabolism; however, a host of studies investigating the molecular and cellular pathophysiological consequences of traumatic brain injury have found correlated associations with oxidative damage and protein nitration. Oxidative stress is characterized as an imbalance between free radicals and antioxidants that result from the body's inability to counteract the enhanced formation of ROS/RNS. The suppression of enhanced reactive oxygen species formation, in part, decreases the production of reactive nitrogen species, as RNS are derived from ROS interactions with nitric oxide, peroxynitrite, and peroxynitrite derivatives. Some proteins have an increased susceptibility to oxidative damage and undergo nitration, which results in structural and function loss. The culmination of effects from oxidative damage and protein nitration seen in persons with TBI leads to diminished antioxidant capacities and increased tissue loss (Shao et al. 2006).

Despite the various elucidations of TBI pathological consequences, no known federally approved therapeutic agent is available to disrupt its secondary injury processes. However, several studies demonstrate that therapeutic approaches utilizing antioxidants effectively prevent neurodegeneration by protecting neurons against oxidative stress. Innately, cells utilize a host of mechanisms for protection from ROS. These mechanisms include the implementations of superoxide dismutase, catalase, and antioxidants. Most notably, antioxidants, such as glutathione, inhibit oxidation by scavenging ROS/RNS. In circumstances of ROS/RNS overproduction, these neuroprotective

mechanisms are exhausted due to limited availability of substrates. Recent studies have investigated the administration of chemical substances that boost intracellular glutathione, as the administration of glutathione itself is ineffective. Gamma-glutamylcysteine ethyl ester (GCEE) is an ethyl ester moiety of gamma-glutamylcysteine, which is a glutathione precursor compound that exhibits antioxidant activity by increasing glutathione production (Reed et al. 2009). Previous studies demonstrated that the administration of GCEE following TBI has protective effects against protein nitration (Reed et al. 2009, Lok et al. 2011).

Mass spectral analysis previously performed by our collaborator, Dr. Sowell, indicates that energy-related and purine salvage enzymes are nitrated, thereby resulting in reduced energy metabolism and nucleotide salvage. Thus, the purpose of this study was to conduct a series of investigations that assessed the efficacy of GCEE as a neuroprotective therapeutic strategy for TBI by determining the optimum time point for treatment. The first study (Chapter 4) was conducted to assess the activity of energy related proteins post-TBI and determine if the administration of GCEE post-injury normalized enzymatic activity in experimental TBI brain through its ability to elevate intracellular glutathione levels and attenuate oxidative stress. The second study (Chapter 5) assessed and determined the activity of purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase with respect to GCEE administrative effects post-TBI.

CHAPTER 2

BACKGROUND

2.1 Traumatic Brain Injury

2.1.1 Overview

Traumatic brain injury is a serious medical issue and public health concern of significant proportions, as it is a leading cause of death and long-term disability in disproportionately affected heterogeneous populations worldwide, with an increasing burden of indirect and direct expenses associated with a lack of successful therapeutic strategies (Hyder et al. 2007, Bruns and Hauser 2003, Kabadi and Faden 2014). Higher incidences of traumatic brain injury are reported in individuals of minority descent and low socioeconomic status (Bruns and Hauser 2003). Studies confirm that this incidence and prevalence is increased in early childhood, late adolescence/early adulthood, and elderly populations (Bruns and Hauser 2003). The high incidence in these groups may be attributed to a culmination of factors, such as recreational sports, motor vehicle accidents, reduced motor skills, and physical abuse, according to the National Center for Injury Prevention and Control at the Centers for Disease Control (CDC). A collection of studies demonstrate the association of gender differences and TBI (Bazarian et al. 2010, Farace and Alves 2000, Ottochian et al. 2009). Females experience higher mortality and fatality rates from traumatic brain injury (Farace and Alves 2000, Ottochian et al. 2009). Ultimately, evidence suggests that TBI

can affect virtually everyone, considering risk factors and increasing incidence and prevalence rates.

2.1.2 Classification

Traumatic brain injury can be classified in numerous ways. These classifications are determined by characterizations of severity, type, skull exposure, damage, and injury. Clinically, the use of the Glasgow Coma Scale (GCS) is the instrument utilized to assess and classify the severity of TBI. Severity of brain injury can be categorized as mild, moderate, or severe (Table 1). The GCS is comprised of three parameters which evaluate one's responses of eye opening, verbal function, and motor function to various stimuli (Teasdale and Jennett 1974). Scores range from 3 to 15, where a score of 3 is considered the most severe (diminished capacity) and a score of 15 is considered least severe (normal ability). Persons receiving a total score of 13 to 15 is considered to have experienced a mild injury, scores of 9 to 12 are indicative of a moderate injury, and scores of 3 to 8 are indicative of severe TBI.

Table 2.1 Glasgow Coma Scale

Response Mechanism	Scale	Score
Eye Opening	Spontaneously	4
	To speech	3
	To pain only	2
	No response	1
Verbal Function	Oriented	5
	Confused	4
	Inappropriate words	3
	Incomprehensible sounds	2
	No response	1
Motor Function	Obeys commands	6
	Localizes to pain	5
	Withdraws from pain	4
	Abnormal flexion	3

Severity classification is contingent upon TBI type. The five types of traumatic brain injury are concussion, contusion, coup-contrecoup injury, diffuse axonal injury, and penetration (Chung and Khan 2013). The mildest and most common type of TBI experienced is a concussion. Although it is possible for anyone to sustain a concussion, recreational and professional athletes have an inherent risk, as participation in most sporting events involves high velocity collisional contact (Covassin, Stearne, and Elbin 2008, McKee 2014). Individuals with a concussion experience a host of medical manifestations, including headache, confusion, dizziness, blurred vision, tinnitus, hyposmia, hyperacusis, fatigue, and brief loss of consciousness. These are collectively referred to as post-concussive somatic symptoms (McAllister and Arciniegas 2002). These somatic symptoms along with cognitive and behavioral changes experienced post-concussion are commonly referred to as post-concussion syndrome (PCS),

and typically have a brief duration that resolves within weeks (Daneshvar et al. 2011). However, in persons who experience concussions more “routinely”, such as athletes, the deficits PCS presents can linger for months or longer. The persistence of PCS along with frequent and repeated incidences of concussions increase susceptibility of a person developing a more severe TBI.

Contusions and coup-contrecoup injury are moderate forms of TBI that exert post-concussive somatic symptoms with increased loss of consciousness. A contusion is characterized as bleeding on the brain that occurs at the site of impact, whereas in coup-contrecoup injury bleeding is experienced both at and away from the site of impact. Severe forms of traumatic brain injury include diffuse axonal injury and penetration. Tearing of brain structures occurs in diffuse axonal injury, when an object directly breaches through the skull into the brain in penetration. Persons who experience these severe types of TBI exhibit post-concussive symptoms with extreme loss of consciousness or coma.

Head trauma is classified by skull exposure, which can be referred to as an open or closed head trauma. This classification is contingent upon the lancing of the dura mater, one of the three protective layers of the brain. If a head injury pierces the dura mater, it is considered open; and if it does not it, is classified as a closed injury. Concussions, contusions, coup-contrecoup injuries, and diffuse axonal injuries are considered closed head injuries, whereas penetration is defined as an open head injury. Closed head trauma has inversely proportional prevalence, mortality, and fatality rates compared to open head trauma, as open head injury has a lower prevalence but higher mortality and fatality rates (Kazim

et al. 2011). Head injury is further categorized by damage distribution. The distribution of damage in terms of location is referred to as focal or diffuse. If TBI is focal, damage occurs at the site of impact, with damage localized to those areas. Alternatively, if TBI is diffuse, damage occurs over a more widespread area without solid structure impact. Open head trauma tends to be largely focal, while persons who experience closed head trauma experience focal and diffuse damage.

Previously, injury from head trauma was considered to be instantaneous and irreversible. Recent research shows that there are two phases of injury: primary and secondary (Werner and Engelhard 2007). Primary injury occurs at the instant the trauma occurs, and represents the immediate resultant physical damage. Secondary injury begins as a product of disruption from primary injury and is a progressive cytotoxic cascade causing damage accumulation, and TBI progression, over an extended period of time. Given that TBI is known to be an accumulative process, there is potential for improving therapeutic intervention.

2.1.3 Treatment

Currently, there are no FDA approved pharmaceutical interventions to interrupt or inhibit damage from secondary TBI, despite the attempts of preclinical studies to assess the efficacy of drugs for neuroprotection (Xiong, Mahmood, and Chopp 2009). Similarly, approaches to various treatments tested in clinical trials have all failed to show efficacy with the exception of induced hypothermia, as they target single factors (Doppenberg, Choi, and Bullock 2004, Peterson, Carson, and Carney 2008). Targeted temperature management via

induction of hypothermia is an approved non-pharmacological approach to TBI, as it promotes neuronal integrity by inhibiting ischemia inhibition. However, hypothermia as a treatment for TBI has been recommended for optional and cautious use, as its benefits are offset by an increased risk of developing pneumonia (Xiong, Mahmood, and Chopp 2009, Peterson, Carson, and Carney 2008). Given the complexity of primary and secondary injury mechanisms that exist in traumatic brain injury, neuroprotective strategies should reflect the cascade of interactive factors that contribute to damage accumulation. Current research demonstrates therapeutic promise as potential treatments strategically and simultaneously address the multiple potential effects of injury factors.

Recent investigations of the pleiotropic properties of statins and cyclosporin A as neuroprotective agents for TBI have resulted in significant improvement of outcomes after injury. Statins, a class of drugs that ultimately inhibit the biosynthesis of cholesterol, are typically prescribed to patients in an effort to prevent heart disease. However, the use of statins demonstrate beneficial effects unrelated to cholesterol in brain injury models, such as anti-inflammatory, antioxidant, anti-excitotoxic, anti-apoptotic, and neurogenic properties (Wible and Laskowitz 2010). These effects, respective to the wide therapeutic window for treatment (24 hours), are influential in targeting the multiple mechanisms of secondary injury by limiting production and expression of inflammatory mediators, decreasing lipid peroxidation, increasing neuronal survival, and improving cognitive function (Loane and Faden 2010). Similarly to statins, cyclosporin A exhibits neuroprotective effects in TBI models, although it

is FDA-approved for other clinical purposes and has a large therapeutic window. This immunosuppressant drug attenuates oxidative stress, lipid peroxidation, and mitochondrial failure often experienced in post-traumatic brain injury by stabilizing the mitochondrial permeability transition pore (Kabadi and Faden 2014, Okonkwo and Povlishock 1999).

2.2 Reactive Oxygen and Nitrogen Species

2.2.1 Overview

Reactive oxygen and nitrogen species (ROS/RNS), classified as either free radicals or as non-radicals, are generated in small amounts by cellular redox processes, such as the reduction of oxygen in the mitochondria to produce adenosine triphosphate (ATP) (Trachootham et al. 2008). When produced in excess ROS/RNS impair cellular homeostasis. Exogenous and endogenous defense mechanisms exist to combat, decrease, and eliminate excess ROS/RNS. Examples of these defense mechanisms that restore redox balance include the cellular antioxidant glutathione as well as a number of enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Apostolova and Victor 2015).

2.2.2 Free Radicals

Free radicals are species that possess at least one unpaired electron, whereas non-radicals, such as hydrogen peroxide and peroxynitrite, lack unpaired electron(s) (Vladimirov 1998). Aside from electron possession, radicals are characterized by their stability, in terms of reactivity and persistence

(Karogodina, Sergeeva, and Stass 2011). Radical stability and reactivity refers to internal energy level. The higher the energy level, the more unstable the radical is. Unstable radicals are considered highly reactive because they will attempt to reach a lower energy level. Hence, free radicals are considered extremely reactive as they have a great propensity to achieve stability via electron(s) donation and extraction, while non-radicals are not as reactive as they are more stable (Halliwell 2006). Persistence of a radical refers to their length of life. Non-radicals are said to have a longer life or persistence than that of a free radical, because of its stability and low reactivity. Despite differences in persistence, radicals are generally and collectively short lived.

2.2.3 Oxygen

In terms of ROS, oxygen (O_2) is increasingly susceptible to radical formation, as its lacks paired electrons. The consecutive reduction of oxygen via electron donation generates a number of ROS, which include, superoxide ($\bullet O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). Diatomic oxygen is also responsible for the formation of RNS, as peroxynitrite (PN: ONOO-) is produced by the reaction of nitric oxide (•ON), a reactive nitrogen species (RNS), and $\bullet O_2$. Thus, it can be said that O_2 is important in the derivation of ROS/RNS.

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} OH\cdot$$

Fig. 2.1 Formation of hydroxyl radical through electron transfer

2.2.4 Superoxide, Hydrogen Peroxide, and Hydroxyl Radical

As illustrated in Figure 2.1, superoxide is produced by the reduction of one molecule of oxygen with an electron. This transfer of an electron to O_2 is conducted by cytochromes within the mitochondria. Given the high reactivity of $\bullet O_2$, the enzyme superoxide dismutase (SOD) acts as a catalyst to rapidly facilitate the neutralization of $\bullet O_2$. This neutralization of reactivity involves the reduction of $\bullet O_2$ to H_2O_2. Superoxide dismutase is a protein that is commonly oxidatively modified in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Choi et al. 2005).

In excess accumulation, superoxide and hydrogen peroxide have the capacity to induce profound effects on mitochondrial function via production of other ROS (Hall, Vaishnav, and Mustafa 2010). Thus the elimination of H_2O_2 by catalase and glutathione proves pivotal in reducing the damaging effects of ROS (Baud et al. 2004). Catalase and glutathione peroxidase are responsible for the decomposition of H_2O_2 into water (H_2O) and O_2. Hydroxyl radicals are considered the most toxic ROS, because their ability to react with any molecule to balance their unpaired electrons is limitless. Secondly, their inability to be eliminated through enzymatic means, unlike $\bullet O_2$ and H_2O_2, contribute to their increased toxicity. Hydroxyl radicals are generated from H_2O_2 in the presence of high "catalytic acting" metals. Reactions such as the Fenton reaction demonstrate the cyclic production of $\bullet OH$ from H_2O_2 with the aid of metals.

In the Fenton reaction (Fig.2.2), ferrous iron (Fe^{2+}) is oxidized into ferric

iron (Fe^{3+}), and then reduced by superoxide (Haber and Weiss 1934). The reduction results in the production of ferrous iron, which can be cycled into another Fenton reaction (Haber and Weiss 1934). High concentrations of iron result in an acceleration of radical reactions. Iron-containing proteins, such as transferrin and hemoglobin, have an overwhelming presence and high affinity for iron at physiological pH (Halliwell and Gutteridge 1988). Hence, free ionic iron is almost nonexistent. At conditions in which pH is lowered, the affinity for iron is reduced within iron storing proteins (Halliwell and Gutteridge 1986). Therefore this reduction results in the mobilization of iron, which in turn increases the production of •OH (Halliwell and Gutteridge 1986).

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$$

Fig.2.2 Fenton Reaction

2.2.5 Nitric Oxide and Peroxynitrite

Though moderately toxic, nitric oxide is involved in many cellular activities within the body. Uniquely, high concentrations of •ON allow it to surpass superoxide dismutase in competition for $\bullet O_2$ (Beckman and Koppenol 1996). The coupling of •ON and $\bullet O_2$ produce peroxynititrite. Peroxynitrite is considered a selective oxidant as its reactivity with biological molecules is gradual, because it lacks an unpaired electron (Beckman and Koppenol 1996).

Despite the limited reactivity of PN, its derivatives, such as nitrite radical ($\bullet NO_2$) and carbonate radical ($\bullet CO_3$), are extremely reactive and very toxic.

2.3 Molecular and Cellular Consequences of Traumatic Brain Injury

2.3.1 Overview

Aside from primary (mechanical) damage, TBI induces a host of secondary insults that collectively contribute to oxidative damage. Excitotoxicity, calcium (Ca^{2+}) overload, and reactive oxygen species are the primary participants in mitochondria-centered cell damage. The culmination of these sequential contributors ultimately result in lipid peroxidation and protein nitration, both oxidative protein modifications. Oxidative damage experienced post-traumatic brain injury is irreversible and influential in neurodegeneration.

2.3.2 Glutamate-induced Excitotoxicity

A neurotransmitter is defined as a chemical messenger that facilitates the transmission of signals between neurons, and can be classified as excitatory or inhibitory. Excitatory neurotransmitters “excite” or stimulate other neurons resulting in firing of action potentials, while inhibitory neurotransmitters prevent the generation of action potentials. Among the host of excitatory neurotransmitters found in the brain, glutamate is the most abundant. Typically low concentrations of glutamate are found in the extracellular space. However, membrane depolarization, a consequence of primary traumatic brain injury, increases the concentration of glutamate post-TBI due to the lack of inhibition

regarding glutamate release (Yi and Hazell 2006, Choi 1994, Hartings et al. 2009).

Glutamate excitotoxicity refers to the excessive activation of glutamate receptors, N-methyl-D-aspartate (NMDA), a-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) and kainite (Mark et al. 2001). These receptors are collectively responsible for the activation of ion channels that promote the influx of Ca^{2+} (Mark et al. 2001). The binding of extreme levels of glutamate to these receptors result in an enormous influx of Ca^{2+} into neurons, which consequentially leads to the overproduction of ROS/RNS, excitotoxicity, and possibly death (Weber 2012).

2.3.3 Overproduction of Reactive Oxygen Species

Mitochondrial uptake of Ca^{2+} results in the stimulation of ROS overproduction via mitochondrial permeability transition pore (MPT) activation (Cheng 2012, Starkov, Cinopoulous, and Fiskum 2004). This activation results in the release of cytochrome c and loss of intra-mitochondrial glutathione (Starkov, Cinopoulous, and Fiskum 2004). The release of cytochrome C results in stimulated $\bullet O_2$ production (Cai, Yang, and Jones 1998). The consequential loss of intra-mitochondrial glutathione upon MPT activation results in the inability to detoxify H_2O_2 and stop the ongoing cascade of ROS production. Although there are numerous mitochondrial sources that contribute to the overproduction of ROS, it is important to consider aftereffects of MPT activation, as the electron transport chain is the primary location of hydroxyl radical production post-TBI.

2.3.4 Lipid Peroxidation

The brain, being largely composed of polyunsaturated fatty acids, is extremely susceptible to lipid peroxidation (LP), as the overproduction of ROS occurs post-TBI (Lok et al. 2011). Lipid peroxidation is defined as the oxidation of lipids via ROS, and can be divided into three stages: initiation, propagation, and termination (Fig.2.3). The chain reaction of LP is initiated by the formation of a lipid radical (•L) via •OH extraction of a hydrogen atom. During lipid peroxidation propagation, •L readily reacts with $•O_2$ to form a lipid peroxyl radical (•LOO). The instability and high reactivity of •LOO with lipid hydroperoxide formation perpetuates the production of other lipid peroxyl radical, until termination. Termination occurs once lipid availability depletes or stability is achieved via non-radical species production and/or antioxidant detoxification.

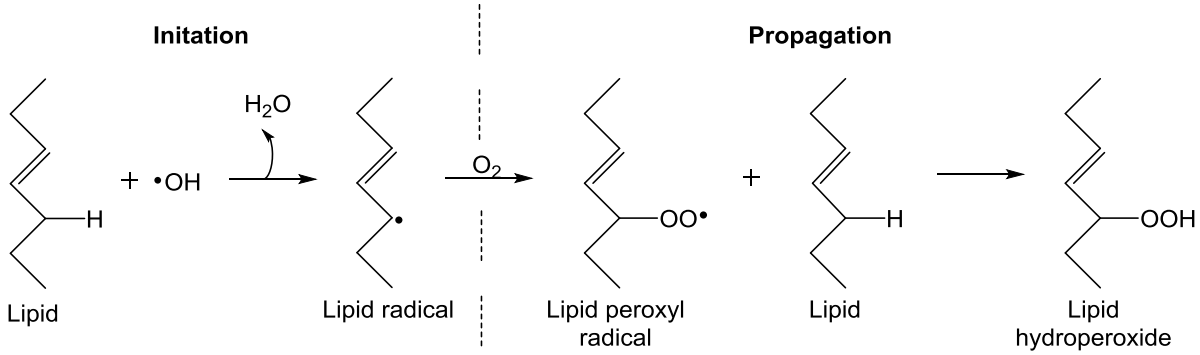

Fig.2.3 Illustration of lipid peroxidation

2.3.5 Protein Nitration

The nitration of proteins leads to a wide variety of structural and functional consequences, and is induced by the introduction of oxygen and nitrogen compounds, such as the LP derivatives $\bullet CO_3$ and $\bullet NO_2$. ROS/RNS specifically target amino acids that contain hydroxyl or amine groups, such as tyrosine and proline (Corpas et al. 2009). Thus it can be said the level of protein susceptibility to oxidative and nitrosative damage is contingent upon functional group possession. In all common forms of TBI, protein dysfunction has been demonstrated as a consequence of protein nitration (Deng et al. 2007, Orihara et al. 2001).

2.4 Glutathione

2.4.1 Overview

The ubiquitous tripeptide glutathione, composed of glutamate (Glu), cysteine (Cys), and glycine (Gly), is an important component of antioxidant defense, as it behaves as both a substrate for glutathione peroxidase in the removal of H_2O_2, and as a free radical scavenger (Winterbourn and Metodiewa 1994). The ability of glutathione to act as an antioxidant is primarily governed by its active thiol group, which is in the form of a cysteine residue. The antioxidant stability of glutathione is enhanced by the atypical γ-peptide linkage between Glu and Cys, which protects glutathione from hydrolysis (Wu et al. 2004). Glutathione exists in reduced and oxidized forms, GSH and GSSG, respectively. The

recycling of glutathione, interconversion of GSH to GSSG, is critical for maintaining redox balance.

2.4.2 Biosynthesis

Glutathione biosynthesis consists of two sequential ATP-dependent reactions catalyzed by gamma-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS), and is tightly regulated by GCS, cysteine availability, and GSH feedback inhibition (Noctor et al. 1997). The initial reaction consists of the formation of gamma-glutamylcysteine (γ-GluCys) via γ-peptide linkage between Glu and Cys. Glutathione is then produced via catalysis of γ-GluCys coupling to Gly.

2.4.3 Metabolism

Glutathione is critical in terms of cellular defense against ROS, as it functions as an antioxidant. Glutathione effectively participates as a ROS/RNS scavenger directly by detoxification and indirectly through enzymatic reactions. In such reactions, •OH, •ON, and $ONOO^-$ are removed directly via reduction with GSH, while enzymatic catalysis by glutathione peroxidase removes H_2O_2. Both reactions lead to the generation of oxidized glutathione, which is recycled via reduction to form GSH using NADPH and glutathione reductase (Dringen 2000) (Fig.2.4).

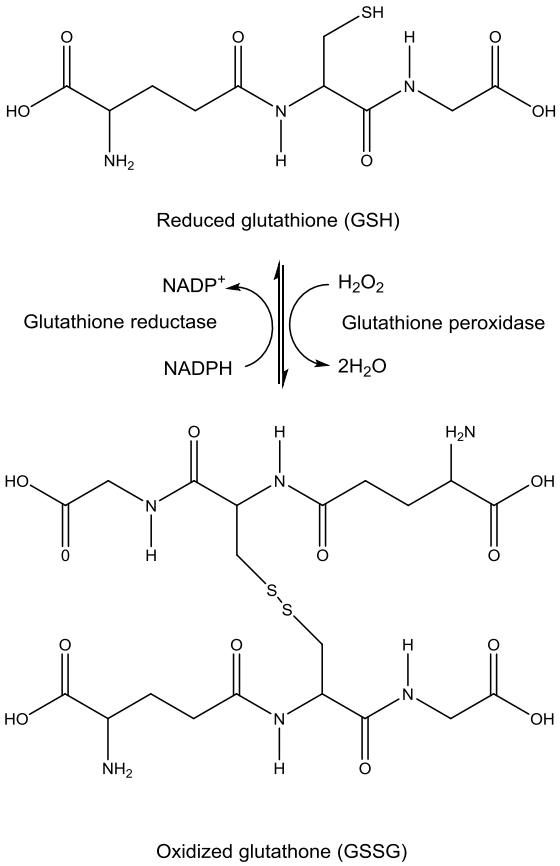

Fig.2.4 Illustration of glutathione recycling

2.4.4 Upregulation of Glutathione

The role of glutathione in maintaining redox equilibrium is immense, as the loss and eventual depletion of glutathione decreases the reducing capacity of the cell and increases the levels of ROS/RNS (Martin and Teismann 2009). The high levels of ROS/RNS associated with glutathione depletion causes oxidative stress-mediated neuronal dysfunction and death. To ameliorate the deleterious effects of glutathione depletion and oxidative stress, glutathione levels must be elevated.

Strategies to increase glutathione levels have been investigated, as the administration of crude glutathione has been deemed ineffective (Lash and Jones 1985, Ammon, Melien, and Verspohl 1986, Wendel and Jaeschke 1982). Much of the research investigating the upregulation of glutathione biosynthesis has been aimed at providing cysteine to the cell, as this is the limiting amino acid in glutathione biosynthesis and exists in the brain at lower concentrations than Glu and Gly (Drake et al. 2002). Among the glutathione precursors that have been demonstrated to increase cellular glutathione levels, gamma-glutamylcysteine (GCEE) uniquely circumvents the feedback inhibition (Drake et al. 2002). GCEE, an analog compound of γ-glutamylcysteine with an ethyl ester moiety addition, has been demonstrated to have neuroprotective effects against protein nitration and oxidative stress through glutathione elevation (Reed et al. 2009, Drake et al. 2002, Lok et al. 2011).

CHAPTER 3

EXPERIMENTAL METHODS

3.1 Controlled Cortical Impact

TBI is a disease of great complexity, as there is no known drug to interrupt and inhibit its pathological consequences. However, the utilization of animal models in TBI research aids in the identification and assessment of potential neuroprotective therapeutics. Given the varying heterogeneities of TBI, such as physical, behavioral, and histopathological changes, the use of animal models are preferred as researchers are able to investigate single factors in the pathobiology through experimental design. Whereas the constraints of experimental control in human subjects TBI research is limited as the injury type, severity, and condition change dynamically.

In lieu of the heterogeneous nature of TBI, experimental designs to induce TBI vary, and include fluid percussion injury model, controlled cortical impact (CCI) injury model, penetrating ballistic-like injury model, weight drop model, and blast model, (Xiong, Mahmood, and Chopp 2013). The selection of an injury model to implement in experimental design is based upon its varying strengths and weaknesses in regards to the research question. In this study, CCI was selected as the injury model of use for a number of reasons. Our primary reason for this choice was the ease in manipulating mechanical factors, such as time, velocity, and impact depth, allowing for the selection of injury severity induced. CCI also can focally localize injury, decreasing the risk of rebound injury.

Upon approval of animal surgical procedures by the University of Kentucky Institutional Animal Care and Use Committee, animals were housed in the Division of Laboratory Animal Resources at the University of Kentucky in a 12 hour light/dark cycle with ad libitum access to feed. Eighteen adult male Wistar rats (Harlan Laboratories, Indianapolis, IN, USA) were equally divided into three experimental groups: sham, saline, and GCEE. Animals were shaved to remove hair on the scalp and anesthetized using isoflurane (3.0%) prior to being placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgery. Surgery was completed and conducted in the same fashion as previously described by Sullivan (Sullivan et al. 2002). Upon placement in the frame, rats underwent a craniotomy, a surgical process that allows access to the brain via skull cap removal. A metal ball was dropped from the impactor on the cortex of the exposed brains of the vehicle and treatment groups, saline and GCEE respectively, at a 1.5 mm depth to simulate a moderate TBI, in the form of contusion. A 4 mm disk made from dental cement was placed over the site of the surgery and/or injury and adhered to the skull with cyanoacrylate. The skull cap was then returned and the skin was sutured. Rats were placed on a heating pad until consciousness and mobility were regained.

3.2 Spectrophotometric Enzyme Assays

Proteins have a host of functions within cells. Proteins termed enzymes are biological catalysts that work by lowering activation energy. A large number of studies indicate that posttranslational modifications of proteins, such as nitration and oxidation, alter protein function. Reductions in energy metabolism

and protein catabolism have been reported post-TBI (Weinzierl et al. 2002, Yao, Liu, and McCabe 2007). In an effort to assess the activity of energy related and purine salvage enzymes post-TBI, various spectrophotometric enzyme assays were conducted in this research study.

The use of spectrophotometric assays allowed the detection of changes in substrate absorbance values. This change was measured by the appearance of product or the disappearance of substrate. In circumstances in which substrates or products were incapable of absorbing light at wavelengths detectable by the spectrophotometer, reactions were coupled. In coupling reactions, the product of one reaction is used as a substrate for another reaction.

All reagents utilized in the assay reaction mixtures were prepared in deionized water or buffer specific to the enzyme of interest. Appropriate volumes of reaction reagents and enzymes were added to each respective microplate to reach a final volume of 200 µL per well. Microplate wells serving as negative controls (“blank”) lack test samples and enzyme, and have an increased volume of buffer in comparison to wells housing enzyme and samples. Wells containing the enzyme of interest or samples are respectively referred to as positive controls and analytes. The addition of equivalent volumes of enzyme or samples in wells respective to their designation initiates the reactions. Upon initiation, the reactions were incubated and monitored at specific wavelengths and temperatures for 15 minutes using a plate reader. All reactions in each assay are performed in triplicate and incubated at room temperature unless noted otherwise.

3.2.1 Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the oxidoreductase responsible for many processes in glycolysis, such as producing ATP through substrate level phosphorylation and reducing NAD+ to NADH. Apart from its role in glucose metabolism, GAPDH acts as an intracellular sensor of oxidative stress in the initiation of apoptosis, and is involved in membrane trafficking and nuclear translocation (Chuang, Hough, and Senatorov 2005, Ventura et al. 2010, Tristan et al. 2011). Given its wide array of participation in cellular events, aberrant activity is suggestive of pathological consequences, such as abnormal cell proliferation and carcinogenesis. Thus the quantitation of glyceraldehyde-3-phosphate dehydrogenase provides insight into normal cellular physiology and the detection of disease.

The GAPDH assay is a coupled spectrophotometric assay used in indirectly measuring the activity of GAPDH via determination of the number of moles of glycerate-1,3 bisphosphate converted to glyceraldehyde-3-phosphate at 340 nm (Fig.3.1). In this assay, the substrate, glycerate-1,3 bisphosphate is produced by the ATP-dependent phosphorylation of 3-phosphoglyceric acid (3-PGA) catalyzed by 3-phosphoglyceric phosphokinase (3-PGK) (Fig.3.1, step 1). Then glycerate-1,3 bisphosphate is reduced by β-nicotinamide adenine dinucleotide (β-NADH) to glyceraldehyde-3-phosphate (G-3-P) and inorganic phosphate in the presence of glyceraldehyde-3-phosphate dehydrogenase (Fig.3.1, step 2).

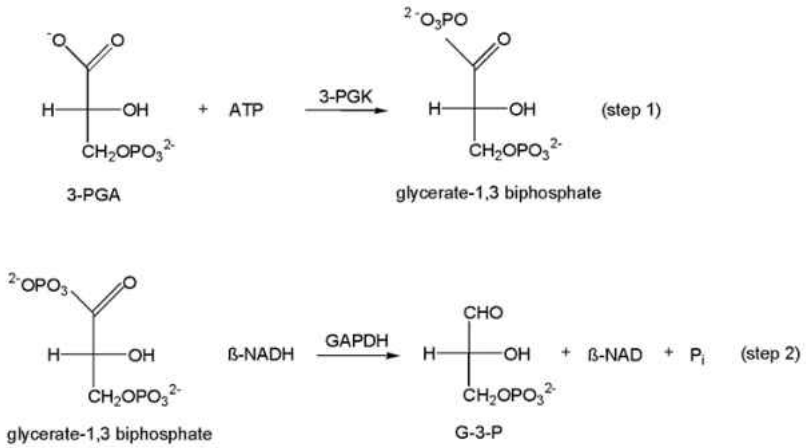

Fig.3.1 Enzymatic reaction of glyceraldehyde-3-phosphate dehydrogenase assay

All reagents were prepared using deionized water, with the exception of the GAPDH enzyme solution, which was prepared using 100 mM triethanolamine buffer (pH 7.6). Reaction reagents, as found in Table 3.1, were added to the microplate and mixed prior to insertion into the plate reader.

Table 3.1 Glyceraldehyde-3-phosphate dehydrogenase assay reagents (volume per well)

Reagent	Blank	Positive Control
100 mM Triethanolamine hydrochloride	166.8 µL	160 µL
100 mM D(-) 3-phosphoglyceric acid	13.3 µL	13.3 µL
200 mM L-cysteine hydrochloride	3.3 µL	3.3 µL
7 mM β-NADH	3.3 µL	3.3 µL
100 mM $MgSO_4$	3.3 µL	3.3 µL
34 mM ATP	6.7 µL	6.7 µL
200 U/mL 3-phosphoglyceric phosphokinase	3.3 µL	3.3 µL
0.6 U/mL GAPDH enzyme solution	-	6.8 µL

3.2.2 Pyruvate kinase

Pyruvate kinase (PK) is an essential enzyme in glucose metabolism as it catalyzes the conversion of phosphoenolpyruvate (PEP) into pyruvate, and assists in the production of ATP via phosphoryl group transfer from PEP to adenosine diphosphate (ADP), in the final irreversible step of glycolysis (Fig.3.2). Pyruvate produced from this reaction serves as an intermediate in connecting glycolysis and the TCA cycle. Thus the strict regulation of PK activity is of immense importance in overall cellular metabolism. The pyruvate kinase assay, a coupled enzyme assay, is often implemented to assess the enzymatic activity of PK, as defects in activity impede glycolysis, while the absence of PK is causal to the development of hemolytic anemia. This two-step reaction assay is monitored at 340 nm upon initiation, and results in the generation of pyruvate and ATP (Fig.3.2, step 1). Pyruvate produced in the initial reaction is used as a substrate to produce lactate in the second reaction via coupling with β-NADH in the presence of lactic dehydrogenase (LDH) (Fig.3.2, step 2).

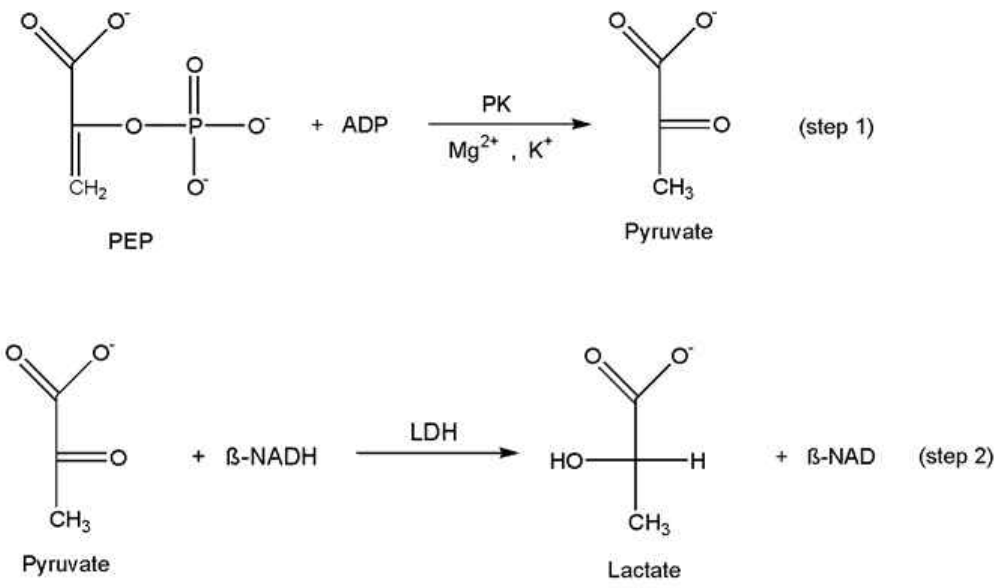

Fig.3.2 Enzymatic reaction of pyruvate kinase assay

All reagents found in Table 3.2 were prepared using deionized water, with the exception of L-lactic dehydrogenase and pyruvate kinase enzyme solutions, which were prepared using 100 mM KH_2PO_4 buffer (pH 7.6 at 37°C). The reaction mixture was mixed and equilibrated to 37°C. Pyruvate kinase enzyme solution or sample was then added to initiate the reaction.

Table 3.2 Pyruvate kinase assay reagents (volume per well)

Reagent	Blank	Positive Control
dH_2O	93.3 µL	93.3 µL
100 mM KH_2PO_4	53.3 µL	60 µL
8 mM PEP	10.7 µL	10.7 µL
3 mM β-NADH	13.3 µL	13.3 µL
100 mM $MgSO_4$	13.3 µL	13.3 µL
40 mM ADP	6.7 µL	6.7 µL
500 U/mL LDH	2.7 µL	2.7 µL
0.6 U/mL PK	-	6.7 µL

3.2.3 Lactate dehydrogenase

Lactate dehydrogenase (LDH), an oxidoreductase responsible for the reduction of pyruvate to lactate (Fig 3.3), is present in a wide variety of tissues within the body. LDH levels are elevated and released into the bloodstream upon injury, disease, or exposure to toxic material. Thus, clinical quantification of this enzyme is used to investigate pathological conditions and assess tissue damage and toxicity.

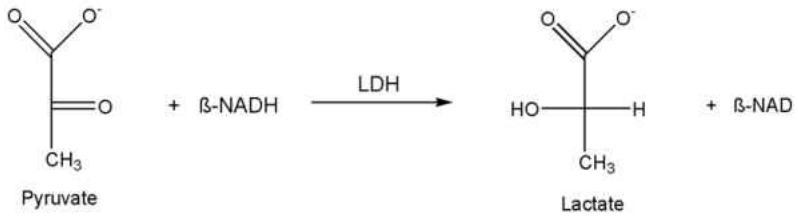

Fig 3.3 Enzymatic reaction of lactate dehydrogenase

In assaying the activity of LDH post-TBI, all reaction reagents were prepared using 100 Mm NaH_2PO_4 (pH 7.5 at 37°C), with the exception of the enzyme which was prepared in 1.0% (w/v) BSA solution. Upon preparation, reaction reagents were added to a microplate in specific volumes (Table 3.3), where they were mixed and incubated at 37°C for 5 minutes prior to being placed and monitored in a plate reader at 340 nm.

Table 3.3 Lactate dehydrogenase assay reagents (volume per well)

Reagent	Blank	Positive Control
0.13 mM β-NADH	186.6 µL	186.6 µL
34 mM Sodium Pyruvate	6.7 µL	6.7 µL
1.0 % BSA	6.7 µL	-
0.5 U/mL LDH	-	6.7 µL

3.2.4 Aspartate aminotransferase

Aspartate aminotransferase (AST), also known as serum glutamic oxaloacetic transaminase, facilitates and accelerates the reversible conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate (Figure 3.4). AST is not organ specific and can primarily be found in significant amounts in the liver and heart, with minimal amounts in the kidneys and brain. Damage experienced by these organs result in the elevation of AST levels in serum. Serum levels typically correlate with the extent of damage experienced. Measurement of AST activity following brain injury via coupled kinetic photometric analysis offers insight regarding the role of amino acid metabolism in the TCA cycle.

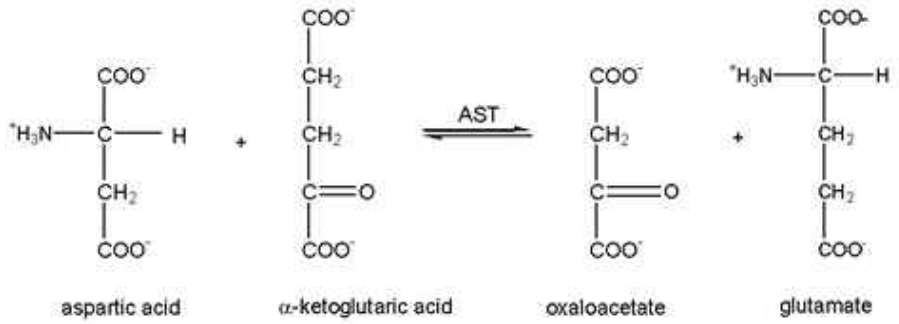

Fig.3.4 Enzymatic reaction of aspartate aminotransferase

To assay the activity of aspartate aminotransferase, the reagent cocktail was prepared as described in Table 3.4. AST enzyme solution (0.25 U/mL) was prepared in a 100 Mm KH_2PO_4 (pH 7.4). The reaction was monitored at 340 nm, loading the volumes of reagents and enzyme according to Table 3.5.

Table 3.4 Aspartate aminotransferase assay cocktail mixture

Reagent	Concentration
L-aspartic acid	134 mM
α-ketoglutaric acid	6.64 mM
β-NADH	0.24 mM
LDH	5 U/mL
malate dehydrogenase	1.25 U/mL
KH_2PO_4 buffer	50 mM

Table 3.5 Aspartate aminotransferase assay reagents (volume per well)

Reagents	Blank	Positive Control
AST assay cocktail mixture	200 µL	195 µL
AST enzyme solution	-	5 µL

3.2.5 Malate dehydrogenase

Malate dehydrogenase (MDH) is an oxidoreductase enzyme that participates in the reversible catalysis of the conversion of L-malate to oxaloacetate using NAD^+ (Fig.3.5). Research indicates increased MDH activity in Alzheimer's disease is consequential of oxidative stress (Shi and Gibson 2011). Thus, quantifying the activity of MDH post-TBI provides insight into the correlation of oxidative stress and neurodegenerative diseases. Prior to loading wells (Fig.3.5), assay reagents and enzymes were prepared in 100 mM KH_2PO_4 buffer (pH 7.4). This assay was monitored at 340 nm in a plate reader.

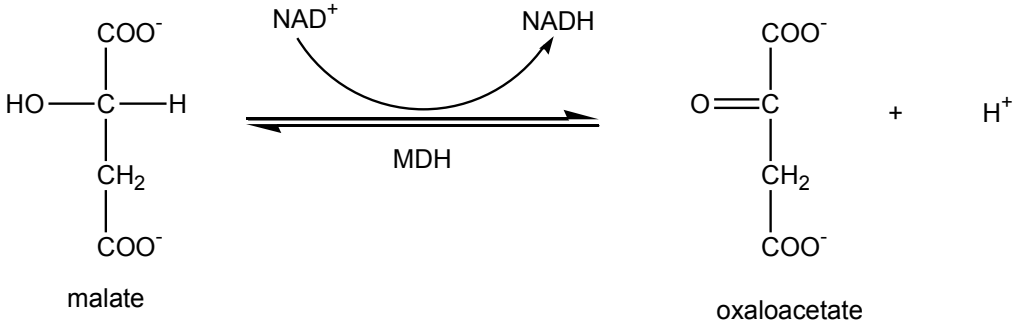

Fig.3.5 Enzymatic reaction of malate dehydrogenase

Table 3.6 Malate dehydrogenase assay reagents (volume per well)

Reagents	Blank	Positive Control
100 mM KH_2PO_4	180 µL	175 µL
3.75 mM β-NADH	13.3 µL	13.3 µL
6 mM Oxaloacetate	6.7 µL	6.7 µL
5 U/mL MDH enzyme solution	-	5 µL

3.2.6 Cytochrome C oxidase

Cytochrome C oxidase (Cyt-c) is a mitochondrial enzyme that catalyzes the conversion of molecular oxygen to water (Fig.3.6), in Complex IV of the respiratory chain, and functions in providing energy to the cell via coupling electron transport and oxidative phosphorylation. Cyt-c activity is based upon the photometric assessment of the decrease in absorbance of reduced cytochrome c at 550 nm, which occurs subsequently after oxidation by Cyt-c (Fig.3.6).

$$\text{Cytochrome c (reduced)} + 2H^+ + \frac{1}{2}O_2 \xrightarrow{\text{Cyt-c}} \text{Cytochrome c (oxidized)} + H_2O$$

Fig.3.6 Enzymatic reaction of cytochrome C oxidase

All reagents utilized in this assay were prepared in 100 mM KH_2PO_4 buffer (pH 7.4 at 37°C), with the exception of reduced cytochrome c and cytochrome C oxidase enzyme. Cytochrome c was prepared in 10 mM KH_2PO_4 buffer and reduced using 5 mg of ascorbic acid. The cytochrome C and ascorbic acid solution was then placed in a cellophane membrane, and was dialyzed in 10 mM KH_2PO_4 buffer for 24 hours in efforts to remove excess ascorbic acid from the

dialysate (cytochrome C and ascorbic acid solution). The cytochrome C oxidase enzyme was prepared in 250 mM sucrose solution with 1% Tween 80 (v/v). Reagents and enzyme were added to the microplate as described in Table 3.7, with the addition of test samples, which were added in equivalent volumes to the enzyme. Reactions in the microplate were equilibrated to 37°C prior to spectrophotometric analyses.

Table 3.7 Cytochrome C oxidase assay reagents (volume per well)

Reagents	Blank	Positive Control
10 mM KH_2PO_4	180 µL	180 µL
1.0 % (w/v) reduced cytochrome c	13.3 µL	13.3 µL
100 mM potassium ferricyanide	6.7 µL	-
0.02 mg/mL Cyt-c enzyme solution	-	6.7 µL

3.2.7 ATP synthase

ATP synthase is a membrane protein that plays a pivotal role in energy metabolism, as it is responsible for catalyzing the phosphorylation of ADP to ATP (Fig 3.7). A deficiency or decrease in ATP synthase activity results in impaired ATP production. To determine ATP synthase activity, assay reagents were prepared in Tris buffer (pH 8.0) and loaded into microplate wells in volumes described in Table 3.8. This assay was monitored spectrophotometrically at 340 nm via the indirect reactions of LDH and PK.

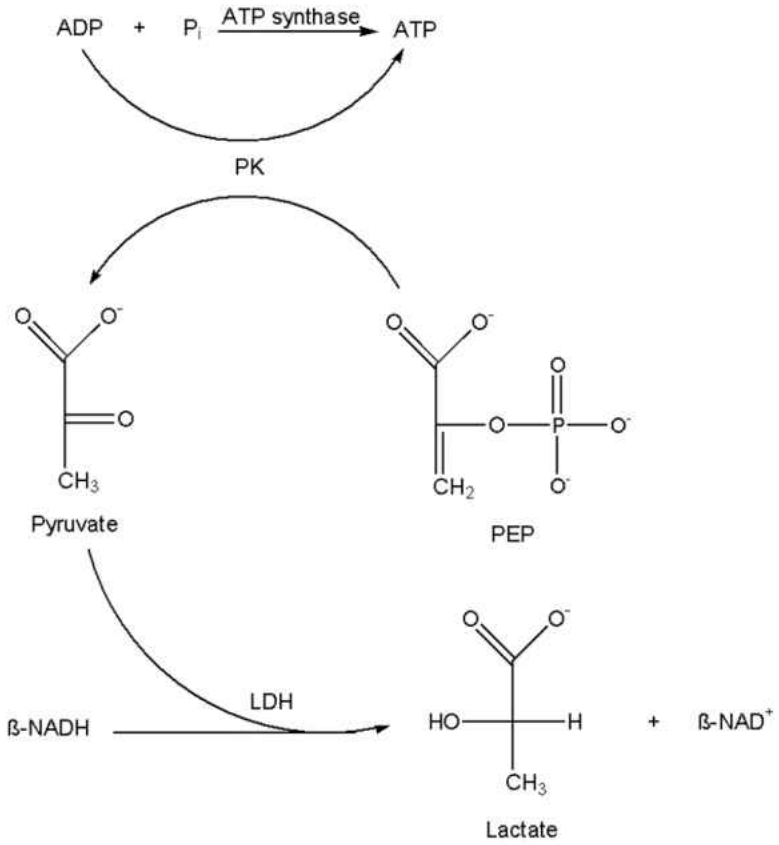

Fig.3.7 Enzymatic reaction of ATP synthase assay

Table 3.8 ATP synthase reagents (volume per well)

Reagent	Blank	Positive Control	Test Sample
100 mM Tris base	6 µL	5 µL	1 µL
2 mM $MgCl_2$	5 µL	5 µL	5 µL
50 mM KCl	5 µL	5 µL	5 µL
0.2 mM EDTA	5 µL	5 µL	5 µL
0.23 mM β-NADH	170 µL	170 µL	170 µL
1 mM PEP	9 µL	9 µL	9 µL
1.4 U/mL PK/LDH	-	1 µL	-

3.2.8 Hypoxanthine-guanine phosphoribosyltransferase

Purines are nitrogenous-based precursors in DNA/RNA synthesis that can be synthesized de novo or recycled by salvage pathways. Purine salvage is said to save the energetic cost of de novo synthesis, as it forms purine nucleoside monophosphates by attaching purine bases derived from the degradation of nucleic acids and nucleotides to 5-phosphoribosyl-1-pyrophosphate (PRPP). Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a salvage enzyme responsible for converting hypoxanthine to inosine monophosphate (IMP) (Fig.3.8 reaction 1) and guanine to guanosine monophosphate (GMP) (Fig.3.8 reaction 2). Deficiency in HGPRT activity clinically manifests as Lesch-Nyhan syndrome, a severe neurological disorder characterized by uncontrollable self-mutilation and the overproduction of uric acid.

In assaying the activity of HGPRT, reaction reagents were prepared according to the PRECICE® HPRT Assay Kit protocol obtained from Novocib (http://www.novocib.com/HPRT_Assay_Kit.html). Briefly, reaction mixture 1x without PRPP was prepared by dissolving dithiothreitol, nicotinamide adenine dinucleotide, bacterial inosine-5'-monophosphate dehydrogenase in 1 mL of reaction buffer 10x containing hypoxanthine. Reaction mixture 1x with PRPP was prepared similarly, with the addition of 2 mM PRPP. Human recombinant HPRT enzyme solution (75 mU/mL) was prepared using deionized water. Loaded volume of assay reagents per well are displayed in Table 3.9. This assay was monitored at 340 nm.

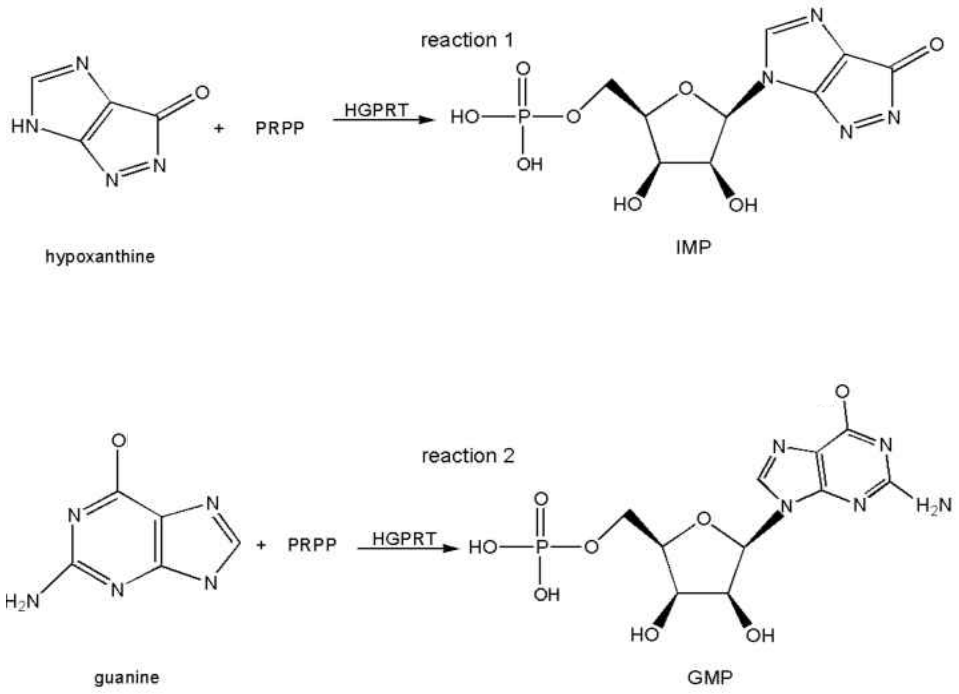

Fig.3.8 Enzymatic reactions of hypoxanthine-guanine phosphoribosyltransferase

Table 3.9 Hypoxanthine-guanine phosphoribosyltransferase reagents (volume per well)

Reagent	Blank	Positive Control
Reaction 1x without PRPP	200 µL	-
Reaction 1x with 2 mM PRPP	-	195 µL
75 mU/mL HGPRT enzyme	-	5 µL

CHAPTER 4

ENZYME ANALYSIS OF SPECIFIC ENERGY RELATED ENZYMES NITRATED BY MODERATE TRAUMATIC BRAIN INJURY

4.1 Overview

Proper brain functionality is dependent upon a sufficient supply of glucose for energy metabolism. Thus, biochemical processes involved in cellular respiration are important in producing ATP for the brain. Reduced energy metabolism and mitochondrial dysfunction are associated with traumatic brain injury, as studies have demonstrated that protein nitration is a consequence of TBI through the production of ROS/RNS (Deng et al. 2007, Orihara et al. 2001). Antioxidants, such as glutathione, combat the deleterious effects of oxidation by scavenging ROS/RNS, inhibiting propagation of lipid peroxyl radicals, and removing neurotoxic byproducts such as 4-hydroxynonenal, acrolein, and malondialdehyde. GCEE, an ethyl ester moiety of gamma-glutamylcysteine, exhibits antioxidant activity by increasing glutathione production. Previous studies have demonstrated that the administration of GCEE following TBI has protective effects against protein nitration through the elevation of glutathione (Reed et al. 2009, Drake et al. 2002, Lok et al. 2011). This study used controlled cortical impact on Wistar rats to induce TBI. This choice in experimental TBI model permitted the observation of neurological and pathological changes that contribute to secondary injury based upon severity of injury induced (Potts et al 2009, Dixon et al 1991) From brain homogenate, enzymatic activity of energy related proteins that have been identified as nitrated in moderate TBI were

studied. Results demonstrate an increase in enzymatic activity upon GSH elevation via GCEE administration in several key enzymes including glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase, malate dehydrogenase, cytochrome C oxidase, and ATP synthase, thereby indicating GCEE is a potential therapeutic strategy to restore energy related proteins in the brain post-TBI via GSH elevation.

4.2 Introduction

Traumatic brain injury is a sudden spontaneous event in which brain dysfunction occurs as a result of an external force. The dysfunction experienced is a result of the external physical impact occurring at the site of injury, and is termed primary damage. Primary damage subsequently initiates immediate or delayed physiological disturbances, known as secondary damage, which collectively result in mitochondrial dysfunction. After TBI, the mitochondria experience an affluence of toxic radical species that decrease ATP production.

Mitochondrial ATP production typically occurs through a cascade of reactions, in which electrons carried by NADH and $FADH_2$ interact with O_2 via a respiratory proton pump (Pieczenik and Neustadt 2007). In the pathological circumstances of traumatic brain injury, ATP production is often limited due to nitration of enzymes responsible for facilitating the biochemical reactions of cellular respiration. The scavenging of ROS/RNS is protective against oxidative stress; however, the antioxidant glutathione exists in the brain at lower

concentrations than other organs and is easily depleted post-TBI (Lok et al. 2011, Dringen 2000). Strategies that increase glutathione concentrations post brain injury, such as GCEE, show therapeutic promise in protecting proteins against nitration and neurons against oxidative stress.

In this study, we assessed the efficacy of GCEE as a therapeutic strategy in the overall restoration of brain energy metabolism via glutathione elevation following the onset of injury. Energy-related enzymes were assayed at various time points to determine the optimum time point for treatment.

4.3 Materials and Methods

4.3.1 Chemicals and Materials

All chemicals utilized were obtained from Sigma-Aldrich (St Louis, MO, USA) with the exception of GCEE, purchased from Bachem (Torence, CA, USA), β-NADH and Tris Base, which was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

4.3.2 Surgical, Treatment, and Sample Preparation

Please refer to Chapter 3, sections 3.1.1, for information regarding surgical procedures. Sham animals served as the control, and were not injured or treated. Saline and GCEE animals received a moderate CCI and an intraperitoneal injection of saline or GCEE (150 mg/kg) according to their corresponding experimental groupings, at either 30 or 60 minutes post-injury. All rats were sacrificed via decapitation 24 hours post-injury. Upon sacrifice, whole

brain was harvested, suspended, sonicated, and homogenized in 10 mM HEPES buffer (pH 7.4), containing 137 mM NaCl, 4.6 mM $MgSO_4$, as well as proteinase inhibitors leupeptin (0.5 mg/mL), pepstatin (0.7 ug/Ml), type II soybean trypsin inhibitor (0.5 µg/mL), and PMSF (40 µg/mL). Brain homogenates were centrifuged to remove debris at 14,000 x g for 10 minutes, and supernatant was collected.

4.3.3 Glyceraldehyde-3-phosphate dehydrogenase assay

The enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase assay (GAPDH) was determined through the glycerate-1,3 biphosphate coupled assay at 340 nm. In the assay, the 0.2 mL reaction mixture contained 100 mM triethanolamine hydrochloride pH 7.6, 100 mM D(-)3-phosphoglyceric acid (cyclohexylammonium) salt, 200 mM L-cysteine hydrochloride monohydrate, 100 mM $MgSO_4$, 7 mM β-NADH, 34 mM ATP, and 200 units 3-phosphoglyceric phosophokinase. The addition of 6.8 µL of GAPDH or brain sample initiated the reaction. Upon initiation, the reaction was monitored at room temperature for 15 minutes in a microplate reader. One unit of GAPDH will reduce one micromole of 3-phosphoglycerate to D-glyceraldehyde-3-phosphate per minute under the conditions previously mentioned.

4.3.4 Pyruvate kinase assay

The spectrophotometric determination of pyruvate kinase (PK) enzymatic activity coupled the production of pyruvate to the oxidation of β-NADH via PK and lactate dehydrogenase (LDH) reactions, which were equilibrated to 37^{o} C and monitored at 340 nm for 15 minutes in a microplate reader. The 0.2

mL reaction mixture contained 100 mM KH_2PO_4 (pH 7.6), 8 mM phosphoenolpyruvate (PEP), 3 mM β-NADH, 100 mM $MgSO_4$, 40 mM ADP, and 500 units LDH. The reaction was initiated by the addition of 6.7 µL of enzyme or brain sample. One unit of activity is the amount of PK catalyzing the conversion of PEP to pyruvate and one unit of LDH reducing 1.0 µmole of pyruvate to L-lactate per minute.

4.3.5 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was quantified by the change in absorption at 340 nm resulting from the oxidation of β-NADH in a microplate reader. The reaction mixture, containing 0.13 mM β-NADH, 34 mM sodium pyruvate, and 1.0% (w/v) BSA, was started upon the addition of 6.7 µL of LDH enzyme or brain sample and equilibrated to 37°C. One unit of LDH activity will reduce 1.0 µmole of pyruvate to l-lactate per minute at pH 7.5 at 37^{o} C. This assay was conducted for 15 minutes.

4.3.6 Aspartate aminotransferase assay

The activity of aspartate aminotransferase (AST) was measured spectrophotometrically at 340 nm for 15 minutes by MDH coupled spectrophotometric assay. The standard reaction mixture contained 134 mM L-aspartic acid (pH 7.4), 6.64 mM of α-ketoglutaric acid, 240 mM β-NADH, 5 units LDH, 1.25 units MDH, and 50 mM Na_3PO_4. The addition of five microliters of AST enzyme or brain sample at room temperature initiated the reaction. This assay was carried out using a microplate reader. Under the conditions mentioned above, one unit oxidizes one micromole of β-NADH per minute.

4.3.7 Malate dehydrogenase assay

MDH activity was determined spectrophotometrically at room temperature using a microplate reader to quantify the decrease in absorbance at 340 nm for 15 minutes. The reaction mixture contained 100 mM of KH_2PO_4 pH 7.4, 6 mM oxaloacetic acid, and 3.75 Mm β-NADH. The addition of five microliters of MDH or brain sample to reaction mixture initiated the assay. One unit of malate dehydrogenase catalyzes the conversion of one micromole of oxaloacetic acid and β-NADH to L-malate and β-NAD^+ per minute.

4.3.8 Cytochrome c oxidase assay

The enzymatic activity of cytochrome c oxidase was assayed at 550 nm for 15 minutes using a microplate reader. Prior to performing this assay, a stock solution containing ten milligrams per milliliter of Cyt-c was reduced with excess sodium L-ascorbate, and then dialyzed before use. The reaction mixture contained 2 µg of reduced cytochrome C and 10 mM KH_2PO_4 (pH 7). A complete reaction mixture along with the addition of 100 mM $K_3Fe(CN)_6$ served as the blank. The reaction was started by the addition of 6.7 µL of Cyt-c or brain sample and equilibrated at 37^{o}C. One unit of cytochrome c oxidase oxidizes one micromole of reduced Cyt-c to oxidized Cyt-c per minute.

4.3.9 ATP Synthase assay

The activity of ATP synthase was determined via PK-LDH coupled assay. Standard reaction mixture contained 100 mM Tris pH 8, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.23 β-NADH, 1 mM PEP. The addition of 1.4 units PK and 1.4 units LDH to the reaction mixture served as the enzyme control. This

reaction was initiated upon the addition of 1 microliter of PK/LDH enzyme or brain sample and was assayed at 30^{o}C using a microplate reader.

4.3.10 Statistical Analysis

All data were analyzed using ANOVA (two-way) and statistical significance was set at $p < 0.05$. Tukey's adjustments were made for ANOVA to determine which treatments were significant.

4.4 Results

4.4.1 Glyceraldehyde-3-phosphate dehydrogenase activity

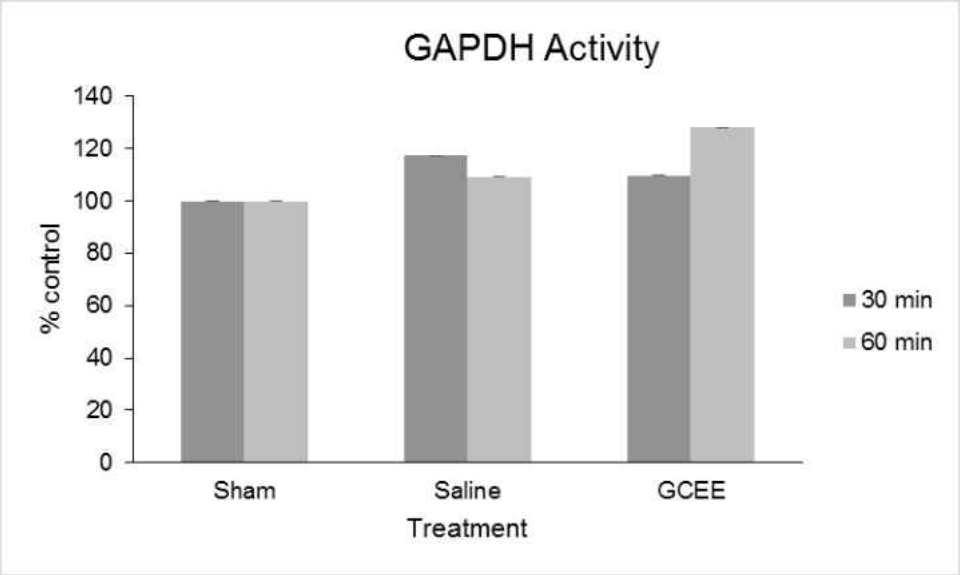

Fig.4.1 Activity of glyceraldehyde-3-phosphate dehydrogenase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group.

The glyceraldehyde-3-phosphate dehydrogenase assay was performed to assess the enzyme activity of GAPDH upon traumatic brain injury in rats and the efficacy of GCEE in restoring GAPDH enzyme activity. The administration of saline and GCEE following TBI shows an increase in the enzyme activity of

glyceraldehyde-3-phosphate dehydrogenase compared to sham, but was not statistically significant ($p > 0.05$). The administration of saline 30 minutes post injury had a greater effect on the rats in comparison to those treated with saline 60 minutes post-TBI; whereas, the administration of GCEE 60 minutes post-TBI had a greater effect on the rats in comparison to those treated with GCEE 30 minutes post-injury. However, the differences in treatment time were marginally statistically significant compared to controls ($p = 0.0593$).

4.4.2 Pyruvate kinase activity

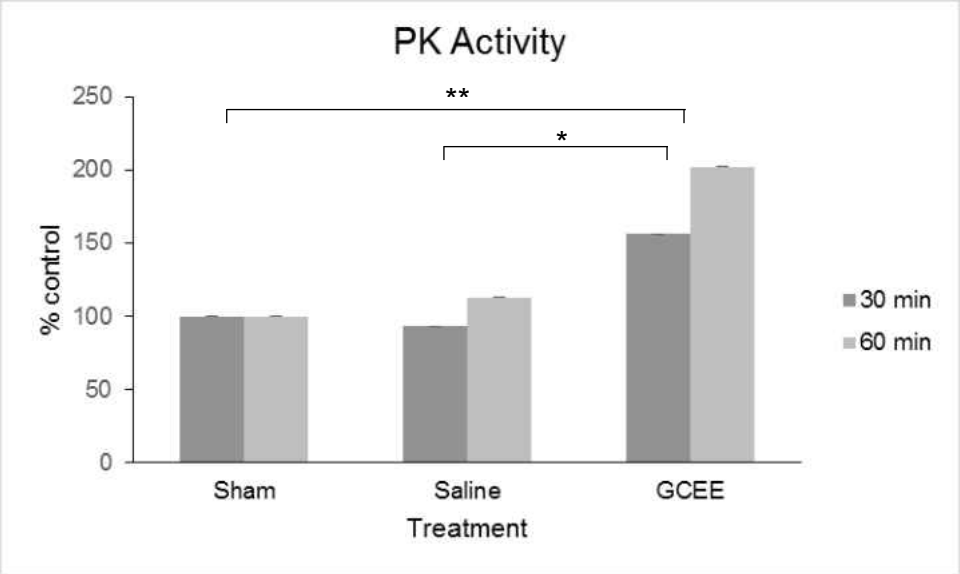

Fig.4.2 Activity of pyruvate kinase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group, *p = 0.0254, **p = 0.0226.

The catalytic activity of PK using the pyruvate kinase assay was determined. A non-significant reduction in activity was observed following the administration of saline 30 minutes after injury compared to sham, but increased within 60 minutes (Fig.4.2). PK activity significantly increased post injury

following the administration of GCEE 30 and 60 minutes post-TBI compared to sham and saline treated animals ($p = 0.0129$), with an elevation in activity at 60 minutes.

4.4.3 Lactate dehydrogenase activity

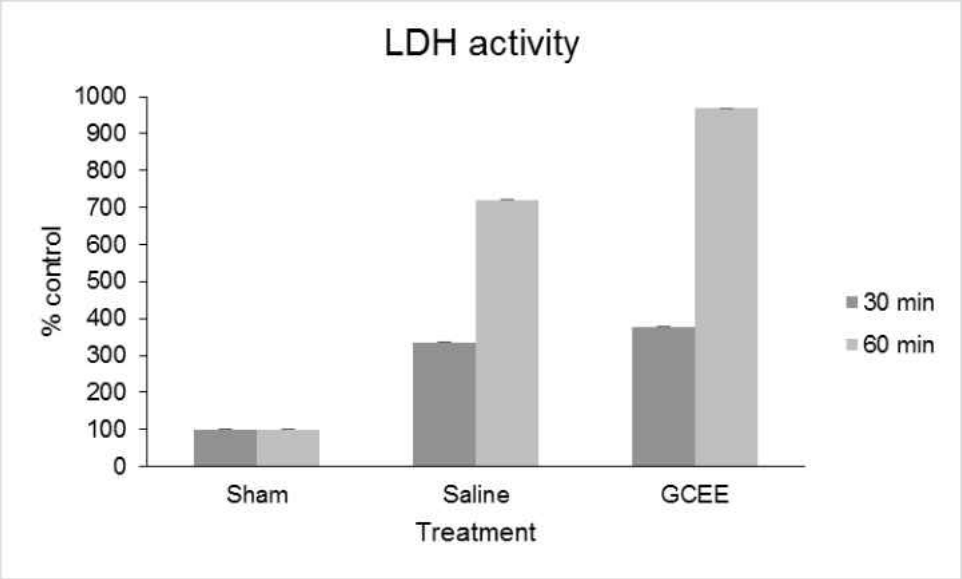

Fig.4.3 Activity of lactate dehydrogenase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group.

The utilization of the lactate dehydrogenase assay quantified the activity of LDH. The administration of saline and GCEE at 30 and 60 minutes post-injury increased LDH activity compared to the control. However, this was not a significant increase ($p > 0.05$). Although the administration of both saline and GCEE 60 minutes post-injury had a more pronounced effect on rats in comparison to those treated at 30 minutes post-injury, the elevation in activity was non-significant ($p > 0.05$).

4.4.4 Aspartate aminotransferase activity

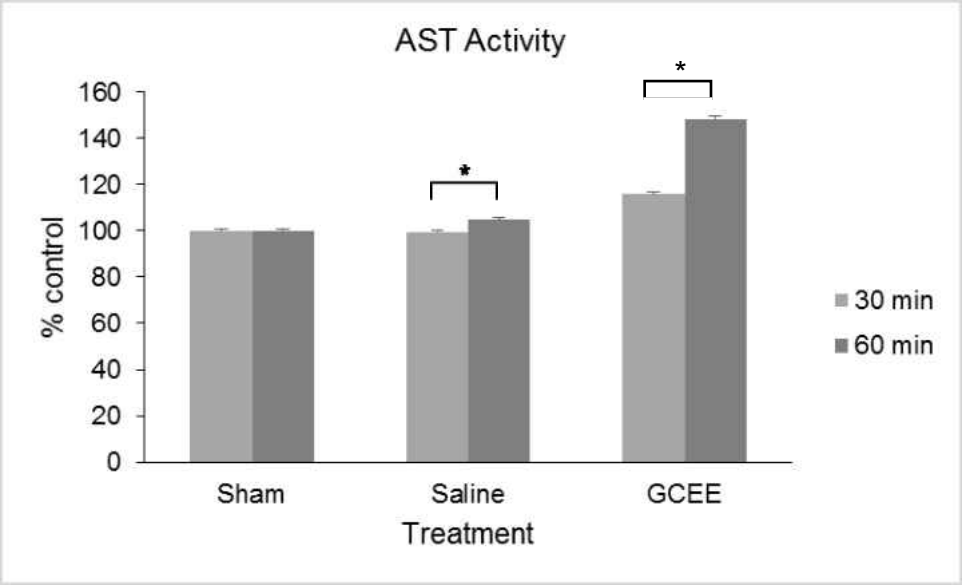

Fig.4.4 Activity of aspartate aminotransferase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group, *p = 0.0013.

The activity of aspartate aminotransferase corresponding to the administrative effect of treatment was quantified using the AST assay. Measurements of aspartate aminotransferase activity in traumatically brain injured rats demonstrated a slight reduction following saline administration 30 minutes after injury compared to sham, but significantly increased within 60 minutes compared to saline administered 30 minutes following traumatic brain injury ($p = 0.0013$). The administration of GCEE post-TBI increased activity compared to sham and saline treated animals sans significance ($p > 0.05$); however, the administration of GCEE 60 increased activity compared to GCEE administered 30 minutes post injury was statistically significant ($p = 0.0013$). Observations of the effect of time of treatment demonstrated significance, where

administration of saline and GCEE 60 minutes upon injury increased enzyme activity compared to administration at 30 minutes (Fig.4.4).

4.4.5 Malate dehydrogenase activity

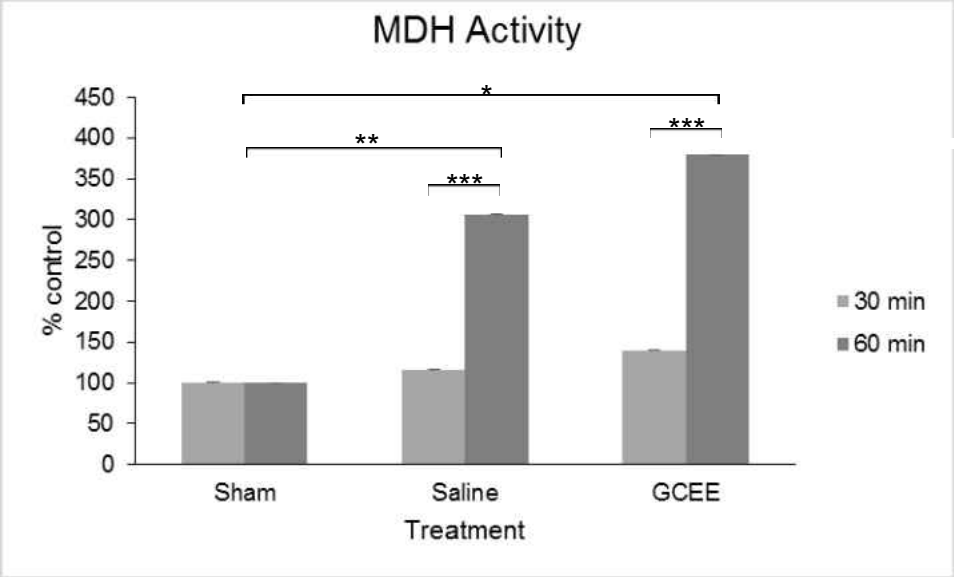

Fig.4.5 Activity of malate dehydrogenase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group, *p = 0.0024, **p = 0.0200, ***p < 0.001.

Using the MDH assay, enzyme activity of MDH was measured post incident administration of treatment. Results indicate the administration of vehicle (saline) and drug significantly increased activity compared to sham ($p = 0.0025$). A more robust significant increase in activity was observed following the administration of saline and GCEE 60 minutes post-TBI, in comparison to administration at 30 minutes ($p < 0.001$). Statistical analyses determined a two-way interaction between treatment and time for malate dehydrogenase.

4.4.6 Cytochrome C oxidase activity

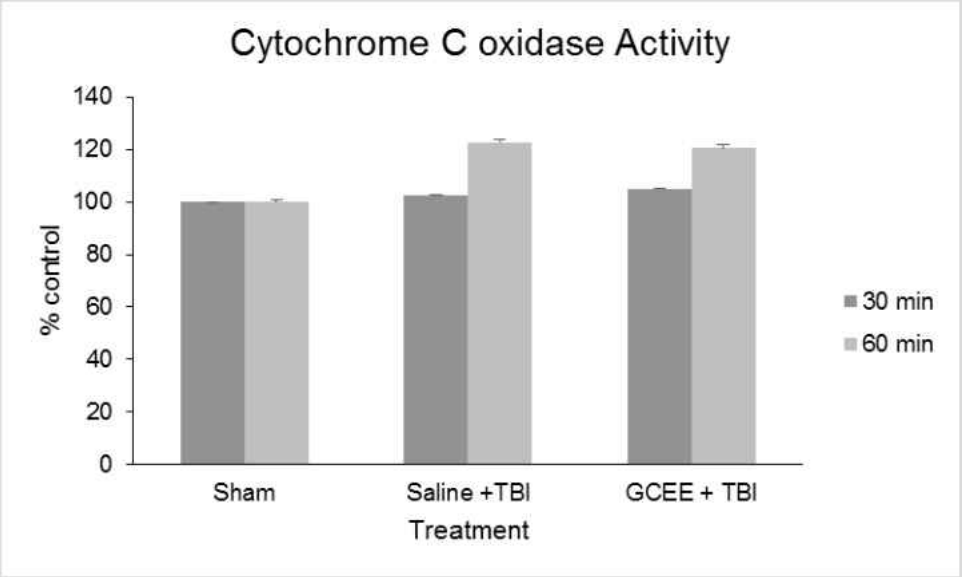

Fig.4.6 Activity of cytochrome C oxidase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group.

The investigation of the catalytic activity of cytochrome C oxidase was performed using the Cyt-c assay. Activity upon administration of saline and GCEE in traumatically brain injured rats was determined to be not statistically significant ($p > 0.05$), with the greatest increase in activity observed 60 minutes post injury.

4.4.7 ATP synthase activity

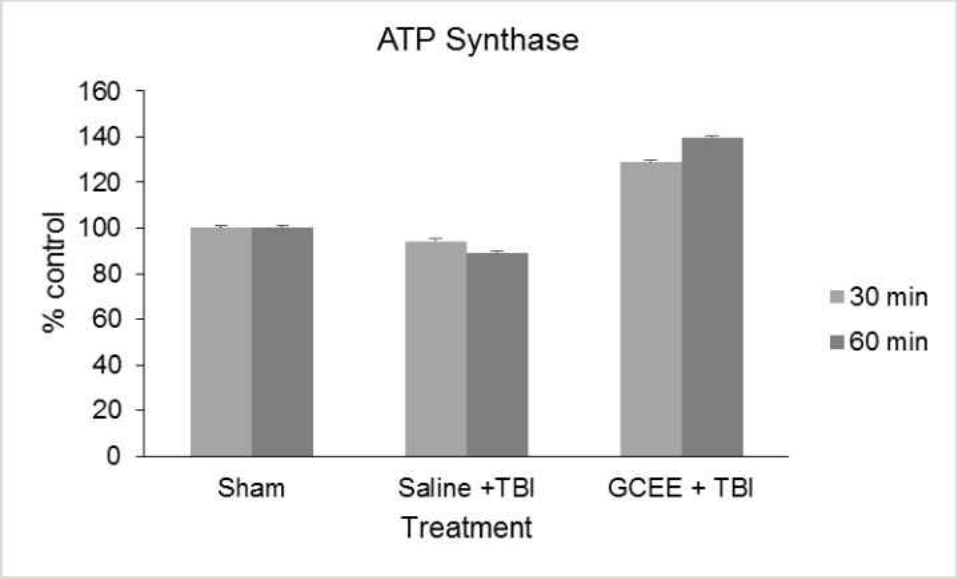

Fig.4.7 Activity of ATP synthase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group.

In determining the activity of ATP synthase post-TBI, the ATP synthase assay was used. Reductions in activity were observed upon the administration of saline compared to control (Fig.4.7). Despite the increase in activity observed after the administration of GCEE at both time points compared to saline treated and sham animals, differences did not achieve statistical significance ($p > 0.05$).

4.5 Discussion

The pathogenic role of oxidative stress in neurodegenerative disease in relation to glutathione depletion has been well documented. Research demonstrates the contribution of oxidative stress to the energy crisis observed post-TBI. Studies show the attenuation of oxidative stress experienced upon

traumatic brain injury impedes the disruption of redox homeostasis, which can improve cellular and functional outcomes after injury. Given the complexity and heterogeneity of TBI, therapeutic approaches to combat the deleterious effects are still non-existent. However, the administration of chemical substances, such as GCEE, in experimental settings have been shown to increase intracellular glutathione levels, which in turn alleviates neurotoxic byproducts accumulated upon injury, and offers therapeutic promise. Here we investigated the enzymatic activity of the energy related proteins glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase, malate dehydrogenase, cytochrome C oxidase, and ATP synthase, which were found to be excessively nitrated in moderate TBI, and assessed the efficacy of GCEE as a potential therapeutic strategy in improving brain energy metabolism following moderate TBI.

Glyceraldehyde-3-phosphate dehydrogenase is a multifunctional protein with glycolytic, sensory, and apoptotic activities expressed ubiquitously in tissues. Studies demonstrate the functional role of glyceraldehyde-3-phosphate dehydrogenase in the progression of neurodegenerative disease, as impaired glycolytic function and increased pro-apoptotic function is observed in Alzheimer's and Huntington's disease (AD and HD, respectively) (Butterfield et al. 2010, Hara et al. 2006, Nicholls, Li, and Liu 2012). In glycolysis, this cytosolic enzyme catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1, 3-biphosphate glycerate by converting NAD^+ to NADH. The catalysis of this reaction also produces ATP through substrate level phosphorylation. Impairment

of GAPDH glycolytic activity in persons who have experienced TBI may contribute to an increased concentration of glyceraldehyde-3-phosphate and dihydroxyacetone. The accumulation of both compounds give rise to methylgloxal, a highly oxidative metabolite associated with DNA and protein modification (Murata-Kamiya and Kamiya 2001). During pathologic conditions of oxidative stress, such as those experienced in the brain through TBI, glyceraldehyde-3-phosphate dehydrogenase inactivation triggers cell death signaling pathways through aggregation, translocation, and binding (Schuppe-Koistinen et al 1994, Tarze et al 2007). Specifically, the accumulation and translocation of cytosolic GAPDH to the nucleus increases levels of DNA damage and inhibits DNA repair, which may in turn induce transcription of cell death mediated genes (Butterfield et al 2010). The mitochondrial binding of GAPDH is associated with apoptosis via membrane depolarization, which respectively leads to calcium uptake, matrix swelling, and the release of cytochrome C (Butterfield et al 2010). Attenuation of oxidative stress via administration of GCEE following traumatic brain injury may improve maintenance of glycolytic function while simultaneously reducing neuronal cell death commonly observed in TBI.

In the final step of glycolysis, pyruvate kinase converts phosphoenolpyruvate (PEP) to pyruvate, which produces ATP through substrate level phosphorylation. Pyruvate kinase exists in four isoforms: M1, M2, R, and L. Pyruvate kinase M1 is typically expressed in the brain. Evidence indicates reduced enzyme activity of PK in individuals with amnestic mild cognitive

impairment (MCI), a predecessor condition frequently experienced upon development of Alzheimer's disease, and posits that protein function is impaired by oxidative modification (Reed et al 2008). Given its glycolytic nature, pyruvate kinase is vital to the production of energy in the brain, as pyruvate produced by its catalytic activity acts as an intermediate to continue cellular respiration. Oxidative modifications of pyruvate kinase observed in individuals with MCI have been suggested to result in limited ATP production and altered ATP-dependent cellular activities, such as signal transduction and cell membrane potential maintenance (Reed et al 2008). Similar manifestations may be observed in persons with TBI, as pyruvate kinase is also found to be oxidatively modified. The significantly increased enzyme activity of PK upon the administration of GCEE 30 and 60 minutes compared to sham and saline treated animals following traumatic brain injury suggests potential improvements of redox homeostasis and ATP production outcomes following TBI.

Lactate dehydrogenase, a cytoplasmic enzyme, exists in five isoforms. Although the isoforms have similar catalytic functions, their tissue distribution varies. LDH-1 is expressed within the brain. Damage to tissues results in increased serum levels of LDH, respective of isozyme expression in tissue type. Thus, the utilization of serum LDH levels as a biomarker are of clinical interest, as it reflects pathological conditions of specific tissues. Under glycolytic conditions, the reduction of pyruvate to lactate is catalyzed by lactate dehydrogenase using NADH. The utilization of lactate as a substrate for gluconeogenesis ensures the perpetuation of glucose and energy metabolism, in

circumstances of limited glucose availability. Lowered glucose metabolism attributed to LDH dysfunction has been observed in AD, Parkinson's disease (PD), and MCI (Reed et al 2008, Hoyer 2004). Impairment in the catalytic activity of lactate dehydrogenase via oxidative modifications could result in reduced gluconeogenesis and the overproduction and/or accumulation of pyruvate (Reed et al 2008). Inhibition of protein oxidative damage associated with TBI via administration of GCEE may increase LDH enzymatic activity, which may successively result in increased brain lactate levels, glucose production, NAD^+ production, and energy metabolism.

Aspartate aminotransferase is a transaminase that exists as two forms, cytosolic and mitochondrial. Its expression can be observed in multiple tissues, including the brain, liver, heart, skeletal muscle, kidneys, and red blood cells. Similar to LDH, serum levels of AST are used as a biomarker to assess extent of injury in pathological conditions. Aspartate aminotransferase catalytic function is vital to amino acid metabolism, as it is responsible for the interconversion of oxaloacetate and glutamate to aspartate and α-ketoglutarate. α-ketoglutarate generated from the anaplerotic transamination of glutamate serves as a key intermediate in the TCA cycle via cyclization continuation and production of NADH. Impairments in AST activity may contribute to the increased concentration of glutamate observed following primary TBI. Here we report increased enzymatic activity following the administration of GCEE 60 minutes post-TBI compared to saline treated and sham. The ability of GCEE to impede the cascade of ROS production resulting from glutamate-induced excitotoxicity

and increased calcium influx may alleviate AST inactivation due to protein oxidation.

Malate dehydrogenase is an oxidoreductase that exists within the cell as either a mitochondrial or cytoplasmic isozyme. Mitochondrial MDH participates in the oxidation of malate to oxaloacetate using NAD^+, in the final step of the TCA cycle before regeneration, while cytoplasmic MDH participates in the malate-aspartate shuttle which permits the passage of malate through the mitochondria membrane for conversion into oxaloacetate via exchange of reducing equivalents. Malate dehydrogenase has been shown to be oxidatively modified in persons with MCI (Reed et al 2008), and its activity has been demonstrated to be elevated in persons dying of AD (Shi and Gibson 2011, Bubber et al 2005, Oop den Velde and Stam 1976). Impairments in the activity of MDH may result in reduced oxaloacetate and ATP production in persons with TBI. Increases in MDH activity may elevate oxaloacetate levels, which may in turn decrease the increasingly high levels of glutamate (and excitotoxicity) experienced after brain injury, as oxaloacetate has been demonstrated to scavenge glutamate (Zlotnik et al 2010). Increases in MDH activity may also increase oxaloacetate levels and passage of reducing equivalents to ETC, which may profoundly increase the reduced ATP production exhibited in TBI affected individuals. In this current study, we have demonstrated that the administration of GCEE significantly increases MDH activity in TBI rats, where treatment administered 60 minutes post injury significantly increases activity compared to treatment administered at 30 minutes. The ability of GCEE to increase activity suggest its efficacy as a

therapy to attenuate oxidative stress and increased MDH susceptibility to oxidative modification.

Cytochrome C oxidase, also known as Complex IV of the electron transport chain, catalyzes the transfer of electrons from ferrocytochrome c to molecular oxygen, and the conversion of molecular oxygen to water. In mammals this enzyme consists of 13 subunits responsible for regulatory, assembly, and catalytic functions. Three of the subunits have catalytic function, while the other 10 subunits have regulation and assembly function. The activity of Cyt-C is said to be an indicator of the oxidative capacity of cells under normal physiological conditions (Srinivasan and Avadhani 2012). Under normal physiological conditions, the mitochondria produce ROS in small amounts. As a result of brain injury, the opening of the mitochondrial permeability transition pore initiates the overproduction of ROS and apoptosis via cytochrome C release. Observations of deficient cyt-C activity via protein dysfunction has been correlated with clinical and experimental manifestations of mitochondrial dysfunction resulting in Leigh syndrome, stroke, AD, PD, and TBI (Nelson and Silverstein 1994, Hovda et al 1991). Reduced alterations in cytochrome C oxidase activity may directly affect cellular ATP levels and calcium homeostasis. The attenuation of oxidative stress via administration of GCEE may indirectly increase ATP levels in individuals suffering from traumatic brain injury by improving mitochondrial function, decreasing calcium influx, and decreasing susceptibility of Cyt-C to oxidative modifications.

ATP synthase, also known as Complex V of the electron transport chain, plays a key role in energy production and transduction in cells. ATP synthase is an enzyme whose catalytic mechanism involves the utilization of energy from the proton gradient established by the ETC to synthesize ATP via phosphorylation of ADP. Reduced energy metabolism as well as mitochondrial dysfunction has been subsequently observed in TBI, as well as other neurodegenerative disease, as a consequence of oxidative stress and damage. Here we report reduced activity of ATP synthase upon brain injury compared to the control, which is not statistically significant. Reductions in ATP synthase activity are indicative of oxidative modifications incurred by protein via increased ROS/RNS production after TBI, possibly resulting in depressions in ETC and impaired ATP production. The administration of GCEE following injury resulted in non-significant increases in ATP synthase activity. This suggests that the GCEE is protective in reducing the oxidative modifications of ATP synthase experienced post traumatic brain injury.

In this study, we assessed the enzymatic activity of energy related proteins identified as being excessively nitrated. Oxidative modification of proteins contribute to the alterations of enzyme activities found in many neurodegenerative disorders. Slight reductions in the activities of some energy related proteins were observed upon the administration of the vehicle following TBI, where pyruvate kinase and aspartate aminotransferase activity were reduced upon administration at 30 minutes, while ATP synthase activity was reduced at 30 and 60 minutes. Elevations in activities of glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, aspartate

aminotransferase, malate dehydrogenase, and cytochrome C oxidase upon administration of saline are suggestive of saline providing protection from protein oxidation incurred post injury. The administration of saline solutions containing hydrogen, pyruvate, and lactate have been shown to diminish the effects of secondary brain injury by attenuating increased ROS/RNS production, enhancing bioenergetics, and improving neurological recovery, respectively (Ichai et al 2009). All proteins in this current investigation have been found to be upregulated, with significant elevations observed in the activities of pyruvate kinase and malate dehydrogenase. Given that the administration of GCEE attenuates the overproduction of oxidative stress observed after TBI, elevations in the activities of proteins upon administration of drug in vehicle compared to only vehicle suggests saline's ability to enhance attenuation of oxidative stress. Trends in activities indicated that administration of GCEE 60 minutes post-TBI demonstrated the highest level of activity compared to treatment at 30 minutes, with significant observations of increased activities in pyruvate kinase, aspartate aminotransferase, and malate dehydrogenase. Taken together, these results suggests that GCEE is a promising potential therapeutic that increases enzyme activity of several key energy related proteins upon administration at one hour following traumatic brain injury.

CHAPTER 5

SALVAGE ENZYME ANALYSIS MODIFIED BY PROTEIN NITRATION VIA MODERATE TRUAMATIC BRAIN INJURY

5.1 Overview

Protein dysfunction via protein modification has been observed in all common forms of TBI, and is a result of cellular redox homeostatic perturbations. Oxidative modifications in neurodegenerative diseases have been demonstrated to decrease the enzymatic activity of an enzyme, in comparison to the controls, as studies have reported an inverse correlation between oxidative modifications and enzyme functionality, where functional activity decreases as oxidative modifications increase (Reed et al. 2008, Aksenov et al. 2000, Butterfield et al. 2006, Poon et al. 2005, Reed et al. 2009). Our data suggests that cerebral oxidative stress experienced after traumatic brain injury ultimately leads to reduced purine recycling, as salvage related enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) has been demonstrated to be oxidatively modified. The administration of GCEE post-TBI upregulates cellular glutathione levels (Reed et al 2009, Drake et al 2002, Lok et al 2011), which in turn normalizes redox homeostasis via attenuation of ROS/RNS. In an exploratory effort to assess the efficacy of GCEE as a potential therapeutic strategy for TBI for the restoration of salvage related enzyme HGPRT, traumatically brain injured Wistar rats were administered saline or GCEE, with the exception of sham animals, 30 minutes post injury. The enzymatic activity of hypoxanthine-guanine phosphoribosyltransferase was then determined

spectrophotometrically using brain homogenate. A non-significant increase in the activity of HGPRT was observed in animals treated with GCEE compared to saline treated animals ($p > 0.05$). Results suggest the correlation of elevated antioxidant capacity with increased enzyme activity. Thus, the elevation of glutathione via GCEE administration post injury indicates the therapeutic promise of GCEE as a potential strategy in the restoration of hypoxanthine-guanine phosphoribosyltransferase following traumatic brain injury.

5.2 Introduction

Purine nucleotides are active participants in a wide variety of biochemical processes, such as cell signaling. The strict regulation of their biosynthesis and metabolism are therefore important. Purine nucleotides may be assembled from "scratch" via de novo synthesis, or "recycled" from preformed bases via salvage pathway. The brain depends on the latter mechanism in preserving purine pools, as it has a limited capacity for de novo synthesis.

Our findings are the first to report purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase as being oxidatively modified upon TBI. Oxidative stress, a hallmark of TBI, is characterized by an imbalance between prooxidants and antioxidants. Failure to attenuate oxidative stress is the result of the body's inability to counteract the enhanced formation of toxic radical species. Depletion of glutathione levels upon brain trauma increases cellular components susceptibility to oxidative modifications, such as structural and functional alterations, which ultimately contributes to cellular dysfunction and death. GCEE,

a potential therapeutic strategy for TBI, exhibits antioxidant activity by increasing glutathione production. Elevations in intracellular glutathione levels restore redox homeostasis, which improves cell functional outcomes post-TBI. In this present study, we assessed the enzymatic activity of HGPRT after experimental TBI and the administration of treatment by conducting a spectrophotometric assay.

5.3 Materials and Methods

5.3.1 Chemicals and Materials

All chemicals utilized were obtained from Novocib (Lyon, France) with the exception of GCEE, purchased from Bachem (Torence, CA, USA), PRPP, which was purchased from Thermo Fisher Sigma Aldrich (St. Louis, MO, USA).

5.3.2 Surgical, Treatment, and Sample Preparation

Please refer to Chapter 3, sections 3.1.1, for information regarding surgical procedures. For information regarding to treatment and sample preparation, please refer to Chapter 4, section 4.3.2.

5.3.3 Hypoxanthine-guanine phosphoribosyltransferase assay

Please refer to Chapter 3, section 3.1.3.8, for more information regarding the quantification of HGPRT activity using the PRECICE ® HPRT Assay (http://www.novocib.com/HPRT_Assay_Kit).

5.3.4 Statistical Analysis

Data was analyzed using ANOVA. Statistical significance was set at $p < 0.05$.

5.4 Results

The catalytic activity of HGPRT was determined using the hypoxanthine-guanine phosphoribosyltransferase assay. A non-significant reduction in activity was observed upon the treatment of saline 30 minutes post brain injury compared to sham animals (Fig.5.1). The administration of GCEE non-significantly increased activity compared to saline ($p = 0.0963$); however, it did not restore activity to levels seen in the control, as a slight non-significant reduction in activity was observed ($p < 0.05$).

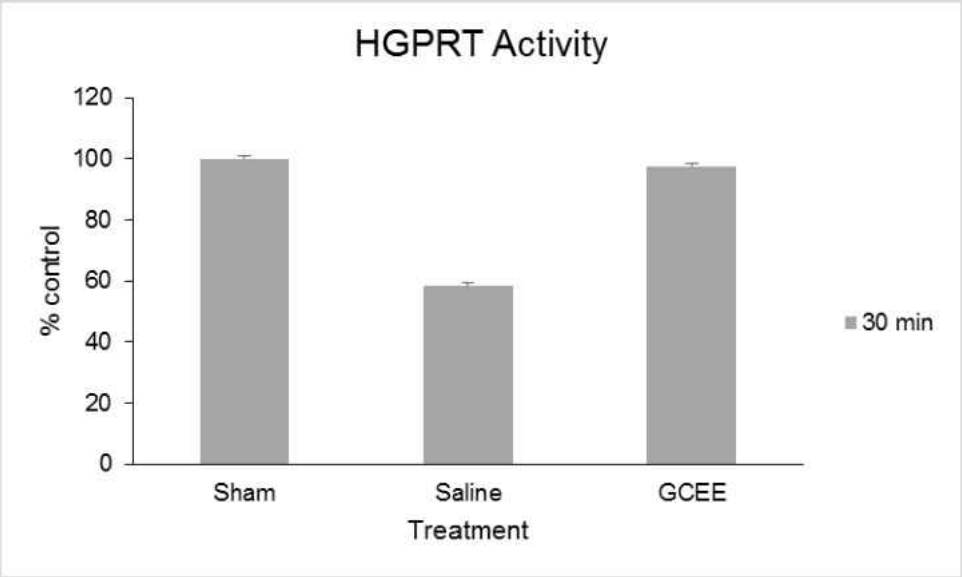

Fig.5.1 Activity of hypoxanthine-guanine phosphoribosyltransferase in traumatically brain injured GCEE treated animals compared to saline treated and sham animals. Bars are representative of mean ± S.E.M., n = 3 for each group.

5.5 Discussion

Traumatic brain injury is associated with oxidative stress through the increased production of reactive oxidants. Oxidative modifications contribute to protein nitration and dysfunction exhibited in all common forms of TBI. The administration of GCEE following brain trauma elevates glutathione levels and decreases oxidative damage.

In this study, we investigated whether the administration of GCEE in traumatically brain injured rats could be utilized as a potential therapeutic strategy in the restoration of HGPRT via glutathione elevation following the onset of injury.

Hypoxanthine-guanine phosphoribosyltransferase is a cytosolic salvage enzyme that is expressed in all tissues, and is very abundant in the brain. Individuals suffering from Lesch-Nyhan syndrome have deficient hypoxanthine-guanine phosphoribosyl activity, which results in severe neurological disorders characterized by the overproduction of uric acid and self-injurious behavior. Specifically, HGPRT catalyzes the conversion of preformed purine bases, hypoxanthine and guanine, to inosine monophosphate and guanosine monophosphate, respectively. Here we report reduced activity of HGPRT upon traumatic brain injury in animals receiving saline compared to control, which is not statistically significant. The role of reductions in hypoxanthine-guanine phosphoribosyltransferase activity may cause a deficit in intracellular GTP and GTP depletion, despite its indirect participation in the metabolism of guanosine. Thus, brains of individuals who have brain trauma may experience impaired G-protein-mediated signal transduction. During signal transduction, a stimulus is

converted into a cellular response. This conversion is stimulated by the attachment of a signaling molecule to cell membrane protein receptor, which elicits changes in cell function via transmission of signal into cell via second messenger. Glucagon and epinephrine are hormones that distinctly control glycogen metabolism via response mechanisms. Epinephrine release assists in the body's response to a stressor, while glucagon release is to maintain homeostasis by elevating blood sugar. Glycogen may act as an endogenous source of metabolic energy, in circumstances when energy metabolism is reduced, such as TBI. In this work, we demonstrated that the administration of GCEE 30 minutes following brain trauma non-significantly increased HGPRT activity to levels almost of normality, referring to activity levels exhibited in control animals. Our data suggests GCEE may indirectly improve or increase the metabolism of glycogen upon TBI via attenuation of oxidative damage experienced by HGPRT upon injury.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

Oxidative stress, a hallmark of traumatic brain injury, can cause perturbations in protein structural and functional activity via oxidative damage. The body's innate ability to combat oxidative stress upon TBI is ineffective, as glutathione is depleted. Research demonstrates the ability of gamma-glutamylcysteine to elevate cellular levels of glutathione in the brain (Drake et al 2002). The studies presented in this thesis have investigated the antioxidant capabilities of GCEE to increase enzymatic activity of proteins identified as excessively nitrated following TBI.

The utilization of enzyme assays determined the enzymatic activity of several proteins identified as being excessively nitrated following moderate traumatic brain injury using GCEE treatment 30 and 60 minutes post injury. These enzymes include glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase, malate dehydrogenase, cytochrome C oxidase, ATP synthase, and hypoxanthine-guanine phosphoribosyltransferase. The goal of the first study was to test our hypothesis that GCEE could increase the activity of oxidatively modified energy-related proteins. Glyceraldehyde-3-phosphate dehydrogenase is responsible for the conversion of glyceraldehyde-3-phosphate to 1, 3-biphosphate glycerate in the sixth step of glycolysis (see Fig.3.1) and the production of ATP via substrate

level phosphorylation. Inactive and impaired GAPDH activity may contribute to increased oxidative susceptibility of DNA and protein damage via methylgloxal production and nuclear translocation. The administration of GCEE post injury increased the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (Fig.4.1, page 43). Administration 60 minutes post injury had a greater effect on the rats in comparison to those treated with saline 30 minutes following trauma (Fig.4.1). In the final step of glycolysis, pyruvate kinase assists in the catalysis of phosphoenolpyruvate (Fig.3.2, page 27), and in the production of ATP through substrate level phosphorylation. Our findings demonstrate a slight reduction in PK activity following the administration of saline 30 minutes after TBI compared to sham; however, activity increased within 60 minutes (Fig.4.2) Reductions in the activity of PK may result in limited ATP and altered signal transduction and cell membrane potential maintenance (Reed et al 2008). We also observed a significant elevation of PK catalytic activity post injury following the administration of GCEE compared to sham and saline treated animals, where a more pronounced increase in activity was observed at 60 minutes (Fig.4.2).

Lactate dehydrogenase catalyzes the reduction of pyruvate to lactate using NADH (Fig.3.3, page 28). Deficient LDH activity could result in reduced glucose production and the accumulation of pyruvate (Reed et al. 2008). Upon the administration of the drug, LDH activity increased compared to control and vehicle animals, where the administration of GCEE 60 minutes post injury had a greater elevation in activity compared to 30 minutes (Fig.4.3, page 45). Aspartate aminotransferase converts oxaloacetate and glutamate to aspartate and α-

ketoglutarate (Fig.3.4, page 46). Reduced AST activity may contribute to increased concentration of glutamate. Our data demonstrates a slight reduction of activity following the administration of saline 30 minutes after injury compared to sham, but activity significantly increased within 60 minutes (Fig.4.4, page 62). We report elevated activity following the administration of GCEE post-TBI compared to saline treated and sham animals, where activity significantly increased within 60 minutes (Fig.4.4). Malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate (Fig.3.5, page 31). Impaired MDH activity may result in reduced oxaloacetate and ATP production. Administration of GCEE post-TBI significantly increases MDH activity compared to the control, where drug administered 60 minutes after trauma significantly increases activity compared to administration at 30 minutes (Fig.4.5, page 47).

Cytochrome C oxidase catalysis transfers the electrons from ferrocytochrome c to molecular oxygen, while converting molecular oxygen to water (Fig.3.6, page 32). Reductions in Cyt-C activity may directly affect ATP levels and calcium homeostasis. The administration of GCEE upon TBI slightly increased the enzyme activity of Cyt-C compared to sham animals, where a greater increase in activity was observed at 60 minutes administration compared to administration of drug at 30 minutes (Fig.4.6, page 48). ATP synthase catalyzes the phosphorylation of ADP. Here, we report a reduction in activity of ATP synthase upon injury compared to control (Fig.4.7, page 34). The administration of GCEE following trauma increased activity compared to control

and vehicle groups, where administration of drug 60 minutes had a greater increase in activity than that of 30 minutes (Fig.4.7).

Hypoxanthine-guanine phosphoribosyltransferase catalysis converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate (Fig.3.8, page 36). A reduction in HGPRT activity was observed following TBI (Fig.5.1, page 61). Impaired activity may cause a deficit in GTP and GTP depletion. Activity increased upon treatment with GCEE compared to sham and saline treated animals (Fig.5.1).

Reductions in enzyme activity upon traumatic brain injury suggest enzyme susceptibility to oxidative damage induced by oxidative stress. Protein oxidative modifications in our study bolsters suggestions of the functional role of oxidative stress in the progression of neurodegenerative diseases. The loss of enzyme activity attributed to protein oxidative modifications potentially contributes to abnormal energy and nucleotide metabolism. The administration of GCEE demonstrates elevations in enzymatic activity of energy and salvage related proteins in the brain post-TBI, with more pronounced effects observed with administration at 60 minutes. This suggests that GCEE may prevent brain damage against toxic radical species that cause oxidative stress and protein nitration in traumatic brain injury.

6.2 Future Studies

1. (Re-analysis) In our experiments, there are three experimental groups: sham, saline, and GCEE. All groups receive a craniotomy; however, saline and GCEE groups were further subjected to a moderate TBI via cortical contusion. To assess the efficacy of GCEE as a potential therapeutic strategy in restoring enzymatic activity of proteins identified as nitrated post-TBI, we compared the measurements of activity of enzymes in our treatment group, GCEE, to the activities of our control and vehicle groups, sham and saline respectively. In most cases, the administration of saline 30 and 60 minutes post injury exhibited an increase in enzyme activities compared to sham animals. Given that research indicates that hydrogen-rich saline is protective against oxidative damage observed in traumatic brain injury (Hou et al 2012), the addition of a TBI only experimental group should be included to confirm whether the enzymatic activities of the saline group are truly representative of enzyme activities experienced post-TBI.
2. (Re-analysis) An exploratory effort to investigate the effect of administration of drug on activity, in a time dependent manner, should be performed. Our findings indicate the administration of GCEE 60 minutes post-TBI demonstrate a more pronounced elevation in activity of enzymes excessively nitrated upon TBI. Here, we only assessed the enzymatic activity of hypoxanthine-guanine phosphoribosyltransferase upon

treatment with saline and GCEE 30 minutes following traumatic brain injury.

3. Although results from both studies in this work are encouraging, indication of their therapeutic promise should be further examined. Both studies demonstrated increasing activity of proteins nitrated post-TBI within experimental animals 30 and/or 60 minutes after injury; however, realistically speaking, an individual may not have access to treatment 30 or 60 minutes following injury. The delay of treatment upon injury should be considered, as an individual suffering a mild to moderate TBI may not exhibit prolonged secondary effects of injury. Thus, they may not feel inclined to seek treatment. The proposal of potential studies investigating the effect of GCEE should include administration of treatment at more realistic time points such as 90 minutes, 120 minutes, and so forth.

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APPENDIX

DATA

Table 6.1 Supporting data for assessment of glyceraldehyde-3-phosphate dehydrogenase activity in experimental TBI groups

Groups (n = 3)	Δ Abs	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.201222	-0.1499	0.052655	0.0304	100
	-0.096				
	-0.152333				
Saline 30	-0.241333	-0.1758	0.073069	0.042187	117.3
	-0.189				
	-0.097				
GCEE 30	-0.132556	-0.1646	0.029626	0.017105	109.84
	-0.170222				
	-0.191				
Sham 60	-0.2105	-0.1879	0.059939	0.034606	100
	-0.12				
	-0.233333				
Saline 60	-0.151167	-0.2052	0.053671	0.030987	109.19
	-0.206				
	-0.2585				
GCEE 60	-0.27	-0.2406	0.026879	0.015519	127.99
	-0.217333				
	-0.234333				

ANOVA	P value
Treatment	0.5063
Time	0.0593

Table 6.2 Supporting data for assessment of pyruvate kinase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.082889	-0.077	0.0152	0.0088	100
	-0.059778				
	-0.088333				
Saline 30	-0.065667	-0.072	0.006568803	0.037925	93.482
	-0.0715				
	-0.078778				
GCEE 30	-0.151	-0.1205	0.0467	0.027	156.45
	-0.066667				
	-0.143722				
Sham 60	-0.041556	-0.0541	0.013	0.0075	100
	-0.067444				
	-0.053444				
Saline 60	-0.053167	-0.0611	0.007757314	0.004478688	112.86
	-0.0615				
	-0.068667				
GCEE 60	-0.1695	-0.1096	0.053	0.0306	202.33
	-0.069				
	-0.090167				

ANOVA	P value
Treatment	0.0129
Time	0.2818

Adjustment for Multiple Comparisons: Tukey	
Group	Least Square Mean
Sham	-0.06557407
Saline	-0.06654630
GCEE	-0.11500926

Least Squares Means for Treatment			
	Sham	Saline	GCEE
Sham	-	0.9980	0.0226
Saline	0.9980	-	0.0254
GCEE	0.0226	0.0254	-

Table 6.3 Supporting data for assessment of lactate dehydrogenase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.17844	-0.041	0.2989	0.1725	100
	-0.24711				
	0.301444				
Saline 30	-0.14522	-0.139	0.0068	0.0039	335.36
	-0.13933				
	-0.13167				
GCEE 30	-0.13689	-0.156	0.0199	0.0115	376.9
	-0.17667				
	-0.15422				
Sham 60	-0.18489	-0.019	0.3232	0.1866	100
	-0.22489				
	0.353778				
Saline 60	-0.12383	-0.134	0.014	0.0081	719.94
	-0.12906				
	-0.15028				
GCEE 60	-0.18067	-0.181	0.007	0.004	967.76
	-0.18761				
	-0.17367				
ANOVA			P value		
Treatment			0.3513		
Time			0.9923		

Table 6.4 Supporting data for assessment of aspartate aminotransferase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.44078	-0.36663	0.083332	0.048112	100
	-0.27644				
	-0.38267				
Saline 30	-0.45556	-0.36478	0.082149	0.047429	99.495
	-0.34322				
	-0.29556				
GCEE 30	-0.411	-0.42552	0.058479	0.033763	116.06
	-0.37567				
	-0.48989				
Sham 60	-0.29711	-0.21541	0.105065	0.06066	100
	-0.09689				
	-0.25222				
Saline 60	-0.18567	-0.22581	0.057308	0.033087	104.83
	-0.20033				
	-0.29144				
GCEE 60	-0.37156	-0.31996	0.046675	0.026948	148.54
	-0.28067				
	-0.30767				
ANOVA			P value		
Treatment			0.1113		
Time			0.0013		

Table 6.5 Supporting data for assessment of malate dehydrogenase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.00996	-0.0163	0.0129	0.0074	100
	-0.00788				
	-0.03112				
Saline 30	-0.01325	-0.018889	0.0092663	0.0005499	115.74
	-0.02958				
	-0.01383				
GCEE 30	-0.01498	-0.022813	0.0069807	0.0040303	139.79
	-0.02508				
	-0.02838				
Sham 60	-0.03092	-0.0647	0.0429	0.0248	100
	-0.05017				
	-0.113				
Saline 60	-0.18342	-0.1981	0.0128	0.0074	306.18
	-0.20375				
	-0.20708				
GCEE 60	-0.16467	-0.2455	0.0768	0.0444	379.48
	-0.25425				
	-0.31758				

ANOVA	P value
Treatment	0.0025
Time	< 0.0001
Treatment*Time	0.0041

Adjustment for Multiple Comparisons: Tukey	
Group	Least Square Mean
Sham	-0.04050694
Saline	-0.10848611
GCEE	-0.131415625

Least Squares Means for Treatment			
	Sham	Saline	GCEE
Sham	-	0.0200	0.0024
Saline	0.0200	-	0.4744
GCEE	0.004	0.4744	-

Table 6.6 Supporting data for assessment of cytochrome C oxidase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.046	-0.041685	0.005913558	0.003414194	100
	-0.03494				
	-0.04411				
Saline 30	-0.04239	-0.042814	0.00642726	0.00371078	102.71
	-0.03661				
	-0.04944				
GCEE 30	-0.03794	-0.043814	0.008186547	0.004726505	105.11
	-0.05317				
	-0.04033				
Sham 60	-0.05583	-0.04333	0.026	0.015011	100
	-0.06072				
	-0.01344				
Saline 60	-0.06722	-0.05317	0.013346	0.007705	122.69
	-0.05161				
	-0.04067				
GCEE 60	-0.04822	-0.05226	0.014862	0.00858	120.6
	-0.06872				
	-0.03983				
ANOVA			P value		
Treatment			0.7178		
Time			0.2976		

Table 6.7 Supporting data for assessment of ATP synthase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.18433	-0.13504	0.049899	0.028809	100
	-0.08456				
	-0.13622				
Saline 30	-0.15367	-0.012737	0.029166	0.016839	94.323
	-0.13244				
	-0.096				
GCEE 30	-0.15978	-0.17411	0.037293	0.021531	128.94
	-0.14611				
	-0.21644				
Sham 60	-0.233	-0.13678	0.102007	0.058894	100
	-0.1475				
	-0.02983				
Saline 60	-0.08667	-0.12161	0.049826	0.028767	88.911
	-0.0995				
	-0.17867				
GCEE 60	-0.17067	-0.19083	0.023969	0.01839	139.52
	-0.1845				
	-0.21733				
ANOVA			P value		
Treatment			0.1528		
Time			0.8634		

Table 6.8 Supporting data for assessment of hypoxanthine-guanine phosphoribosyltransferase activity in experimental TBI groups

Groups (n = 3)	Δ Abs.	Mean	Std. Deviation	Std. Error of Mean	% of control
Sham 30	-0.15983	-0.20667	0.041754	0.024106	100
	-0.24				
	-0.22017				
Saline 30	-0.156	-0.12044	0.032698	0.018878	58.28
	-0.11367				
	-0.09167				
GCEE 30	-0.16183	-0.20161	0.041985	0.02424	97.554
	-0.1975				
	-0.2455				
ANOVA			P value		
Treatment			0.0614		

VITA

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