2007

OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN TRAUMATIC BRAIN INJURY IN AGING

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By

Changxing Shao

Lexington, Kentucky

Director: Dr. Mark A. Lovell, Associate Professor of Chemistry

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

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Traumatic brain injury (TBI) is a prominent disease in developed countries, and age is an important factor in functional outcome. Although aged patients typically show diminished recovery compared to young patients, and have higher mortality and morbidity following TBI, the mechanism is not well understood. To date, there is no effective therapeutic for TBI.

Previous studies indicate a secondary injury in TBI begins immediately after impact, and is likely the major contribution to delayed neuron dysfunction and loss. Studies also suggest mitochondrial dysfunction and increased free radical species (ROS) production following TBI may play a key role in the process. To evaluate oxidative damage following TBI, especially in aging, young (3 months), middle aged (12 months) and aged (22 months) Fisher-344 rats were subjected to a unilateral controlled cortical impact (CCI) injury, and tissue sparing, 4-hydroxynonenal (HNE) and acrolein levels, and antioxidant enzyme activities, and DNA oxidative damage were measured. In order to evaluate changes in mitochondria following TBI, mitochondrial protein levels were investigated using young adult animals. To evaluate a potential therapeutic for TBI, the effect of creatine on oxidative damage was evaluated.

These studies show an age dependent increase of oxidative damage following TBI, demonstrated by increased levels of 4-HNE, acrolein and 8-hydroxyguanine. Middle aged and aged animals showed increased tissue loss compared to young animals 7 days post injury. Mitochondrial proteins involved in the respiratory chain, carrier proteins and channel proteins were significantly decreased 24 h post injury in ipsilateral cortex, but increased in both ipsilateral and contralateral hippocampus. To study potentially protective compounds in TBI, animals were fed with creatine two weeks before TBI and showed less oxidative damage and increased antioxidant capacity, which suggests creatine may be a potential drug for clinical treatment of TBI.

The work described in this dissertation is the first to show increased oxidative damage and diminished antioxidant capacity in TBI in aging. The study of mitochondria following TBI using quantitative proteomics is also the first time to show multiple mitochondrial proteins change following TBI. These data are also the first to show

creatine can increase antioxidant defenses. These studies contribute to our understanding the mechanisms of secondary injury in TBI in aging.

KEYWORDS: Antioxidant Enzyme, Creatine, Oxidative Damage, Mitochondrial Dysfunction, Traumatic Brain Injury

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May 30, 2007

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

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CHAPTER ONE

Introduction and Background

1.1 Traumatic Brain Injury and Physiological Consequences

Traumatic brain injury (TBI), also called acquired brain injury or head trauma, occurs when a sudden impact results in damage to the brain. TBI can result when the head violently hits an object, or when an object pierces the skull and enters the brain. The injury can be short term or long term. As a short term injury, patients with severe head trauma may die immediately or experience coma for hours and days. Patients with moderate or mild injury may experience a loss of consciousness for a few seconds or hours. As a long term injury, TBI leads to neuron damage or death at the site of injury and adjacent regions of brain, and can be a factor for such neurodegenerative disorder as Alzheimer's disease (AD) (Guo et al., 2000). Clinical studies reveal that survivors of TBI may experience trouble in one or multiple areas, including thought and reasoning, understanding words, remembering things, paying attention, solving problems, thinking abstractly, talking, behaving, walking and other physical activities, seeing and/or hearing, and learning (McAllister et al., 2006; Tombaugh et al., 2006; O'Keeffe et al., 2007).

TBI is the leading cause of injury-related death in developed countries (Das-Gupta and Turner-Stokes, 2002), accounting for 26% of all trauma-associated deaths in the United States (Sosin et al., 1989; Sosin et al., 1995). TBI is also a major cause of persistent functional disability and psychological impairment (Harvey, 1986). Recent statistics show that motor vehicle accidents (~60%), falls, assaults and various sports-related injuries (40%) result in TBI that affects ~ 7 million individuals each year in North America (Tibbs et al., 1998; McNair, 1999). Approximately 70,000 to 90,000 of those injured suffer long-term disability. The cost to treat these individuals is estimated at ~ $20 to $25 billion annually (McIntosh et al., 1996). In the last 30 years, our understanding of the physiopathology of TBI has greatly improved, but the mechanism of injury, particularly delayed injury, is still unclear. The improved outcome of patients with severe TBI has reached what looks like an unmodifiable plateau resulting from limited understanding of the neurological and neurochemical cascades triggered by primary mechanical injury. There is still no specific pharmaceutical therapy for TBI and further studies are needed for more clues in clinical therapy.

1.2 Factors Affecting the Outcome of TBI

Several factors are believed to be important to the pathogenesis of TBI. Age is one of the most widely investigated factors. Age-specific TBI associated death rates show a bimodal distribution with peaks at 15- to 24-years of age and >75 years of age (Figure 1.1) (Sosin et al., 1989). Studies of TBI in young adulthood and elderly patients show that very young and old patients are more severely damaged and less likely to recover (Hoane et al., 2004; Kirkness et al., 2004; Testa et al., 2005; LeBlanc et al., 2006; Thompson et al., 2006). Gender is believed to be another factor that may play a role in TBI (Kovacs, 2005; Robertson et al., 2006). Males are 2 to 3 times as likely as females to sustain TBI, especially during high-risk years (Thurman, 2001). The reason why age and gender affect the outcome of TBI is still under debate. Genetic factors may also impact TBI, particularly the presence of apolipoprotein E4 alleles (Diaz-Arrastia et al., 2003; Nathoo et al., 2003; Diaz-Arrastia and Baxter, 2006). Additionally, preventive methods are also important to limit TBI. For example, helmet and airbags can greatly reduce brain injury.

1.3 Animal Models of Traumatic Brain Injury

Because of the limited availability of human TBI specimens, several animal models that simulate human TBI have been developed in last several decades with new models still under development.

For over a century, various *in vivo* models of TBI have been employed to systematically produce pathology of TBI, including fluid-percussion (FP), controlled cortical impact (CCI), and weight drop (Awasthi et al., 1997; Scheff and Sullivan, 1999; Shimamura et al., 2004). In consideration of experimental design, the species of animal, location of injury and choice of anesthesia are also considered. Cats (Bedell et al., 1998), pigs (Pfenninger et al., 1989), rats (DeKosky et al., 2004), and mice (Hall et al., 2005) are now commonly used animals in studies of TBI. The injury locations include midline for whole brain concussion injury and parietal cortex for lateral percussion injury. For FP injury, the injury is produced when a metal pendulum strikes the piston of an injury device from a certain height leading to a pulse injection of saline into the closed cranium, which induces a brief increase of intracranial pressure with

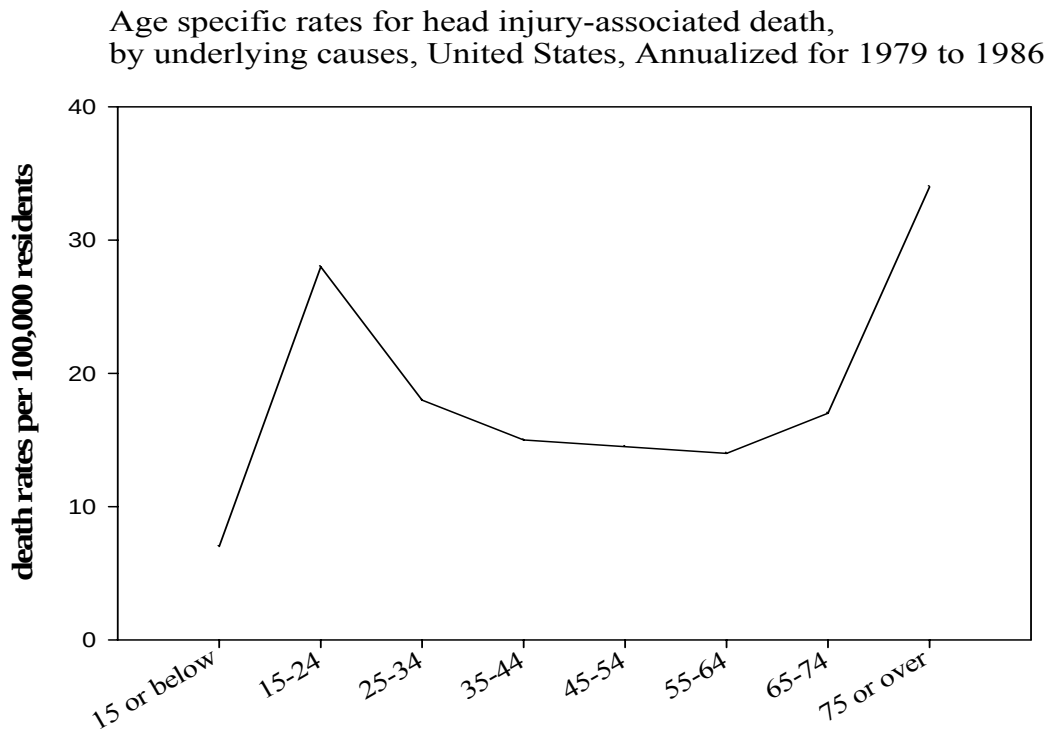


Figure 1.1. Death rate distribution of TBI in aging (Sosin et al., 1989).

associated displacement and deformation of brain tissue. The severity of injury is controlled by the height of the pendulum. The FP injury was first described by Linden and Ringer (1965) using a rabbit model of TBI (Carbonell et al., 1998). This model produces concussive injury, hippocampal cell death and cortical contusion, vascular and blood-brain barrier disruption injury, cerebral edema, tissue shearing, and intraparechymal hemorrhage. The CCI model, which allows better control of the biomechanical parameters of injury leading to reproducible injuries, was first introduced by Lighthall and colleagues (Lighthall, 1988). The injury is easier to quantify by regulation of the impact force, velocity and dwell time and to measure the tissue deformation and functional impairment. In the CCI model, a cylinder is used with a round or flat edge, and is pneumatically driven to produce injury. Like the FP injury, the CCI model also produces injury that resembles certain aspects of human TBI, but it produces a significantly more pronounced cortical contusion. Another injury model is the weight drop model, which can be used with either an open or closed skull. Injury with an open skull produces a focal lesion that progresses from hemorrhages in the white matter to a necrotic cavity. A closed skull injury produces a diffuse injury without noticeable contusions or hippocampal cell loss. In this model, a weight is released through a guide tube and subsequently strikes a footplate resting directly on the exposed brain or directly on a cemented disk on the skull. The severity of injury is controlled by the weight and distance the weight travels.

1.4 Current Understanding of Mechanism of TBI

TBI is a result of direct and indirect brain damage, resulting from primary mechanical injury and secondary injury. Primary mechanical injury can be reduced or avoided through protective measures, such as education of potential victims and use of safety equipment (helmets, airbags). In TBI, primary mechanical brain injury happens in seconds, resulting in subdural hematoma, laceration, diffuse axonal injury, diffuse vascular injury, intracerebral hemorrhage, and concussion (BIROS, 1998a; Armin et al., 2006). With current technology, it is believed that the brain damage from mechanical injury is irreversible. Secondary injury follows primary mechanical injury in minutes, hours and days, and is potentially treatable (Hall et al., 2005).

Previous studies show secondary injury results in delayed dysfunction and death of neuronal populations near and distant to the site of injury. Neuron death resulting from secondary injury is complicated, and the pathogenesis is unclear. However, It has been shown neuron death involves both cell apoptosis and necrosis (Raghupathi, 2004). There are some contributors to the cellular apoptosis and necrosis, including excess excitatory amino acids, mitochondrial dysfunction, oxidative damage, and adenosine and triphosphate adenine nucleotide depletion, inflammatory events, ion homeostasis disruption, proteolytic enzyme activation, DNA damage, and cholinergic receptors dysfunction (Simon et al., 1984; Azbill et al., 1997; Sullivan et al., 1998; Tyurin et al., 2000; Verbois et al., 2002; Mendez et al., 2004; Lu et al., 2005; Cimatti, 2006). This dissertation is focused on studies of oxidative damage and mitochondrial dysfunction in TBI.

1.4.1 Mitochondrial Dysfunction in Aging and TBI

Mitochondria, believed to have originated from evolution of bacteria having a symbiotic relationship with ancient eukaryotic cells, are the power house of eukaryotic cells (Margulis, 1981). As the power supply, mitochondria synthesize ATP and consume molecular oxygen by reducing O_2 to H_2O in oxidative phosphorylation. Structurally, mitochondria consist of an outer membrane, an inner membrane, the intermembrane space and the matrix. However, mitochondria have different shapes and population densities in different cells (Figure 1.2) (Perkins and Frey, 2000; Scheffler, 2001). The inner membrane folds into cristae to increase the inner membrane area, and is relatively rich in protein, consisting of ~ 75% protein and 25% lipids (DePierre and Ernster, 1977). One third of mitochondrial proteins, including complexes I, II, III IV and V, adenosine triphosphate (ATP) and ion translocators, are involved in the respiratory chain and ATP syntheses. The lipids of the inner membrane are predominantly phospholipids, including phosphatidylcholine, phosphatidylethanolamine and cardiolipin (Ardail et al., 1990; Camici and Corazzi, 1995; de Kroon et al., 1997). The outer membrane contains receptor proteins, signaling proteins, and channel proteins, including the Bcl-2 family of proteins, hexokinase, and voltage-dependent anion channel (VDAC) proteins (Adams and Cory, 1998; Verrier et al., 2003; McBride et al., 2006). The outer membrane is permeable to both charged and uncharged molecules with a molecular mass less than 1000 Daltons

(Verrier et al., 2003). Ketones, mitochondrial DNA and the enzymes and cofactors associated with Krebs cycle, amino acid metabolism and fatty acid oxidation and synthesis, and mitochondrial protein folding are located in the matrix. Although mitochondria have their own circular DNA, only 13 polypeptides are encoded by mitochondrial genes, including seven subunits of complex I, one subunit of complex III, three subunits of complex IV, and two subunits of the H^+-translocating ATP synthase (Scheffler, 2001). There are thousands of mitochondrial proteins encoded by nuclear DNA, which are transferred to mitochondria by transporting proteins (Neupert, 1997; Pfanner, 1998; Schatz, 1998).

Mitochondria serve not only as power house, but also as a calcium sink to control ion homeostasis through two modes of calcium influx and two of efflux as well as through calcium induced formation of the MTP. The most studied Ca^{2+} influx mechanism is the uniporter that transports Ca^{2+} through its electrochemical gradient without coupling the transport to other ions or molecules. Another Ca^{2+} influx mechanism is the 'rapid mode' or RAM (Gunter et al., 2000). Calcium efflux pathways include Na^+-dependent and Na^+-independent mechanisms (Crompton et al., 1977; Crompton et al., 1978; Murphy and Fiskum, 1988). In addition, mitochondria play a key role in cell signaling, regulating cell survival via apoptosis involving Bcl-2 family protein, cytochrome c and caspase 9 and 3, and apoptosis-inducing factor (AIF) (Liu et al., 1996; Danial et al., 2003; Saelens et al., 2004).

Glycolysis is an alternative energy production process in cells. Under aerobic conditions, glycolysis can produce ATP, NADH and pyruvate. NADH can be transported by the glycerol phosphate shuttle or the malate-aspartate shuttle to mitochondria for oxidative phosphorylation. Pyruvate is the initial substance in the Krebs cycle for NADH, FADH and GTP production. Therefore, glycolysis and mitochondrial oxidative phosphyorylarion are closely connected with each other. Hexokinase locates in mitochondrial outer membrane and catalyzes ATP-dependent phosphorylation of glucose to form glucose-6-phosphate, and it is also a key protein in mitochondrial permeability transition pore (Robey and Hay, 2006).

The mitochondrial respiratory chain is the major intracellular source of reactive

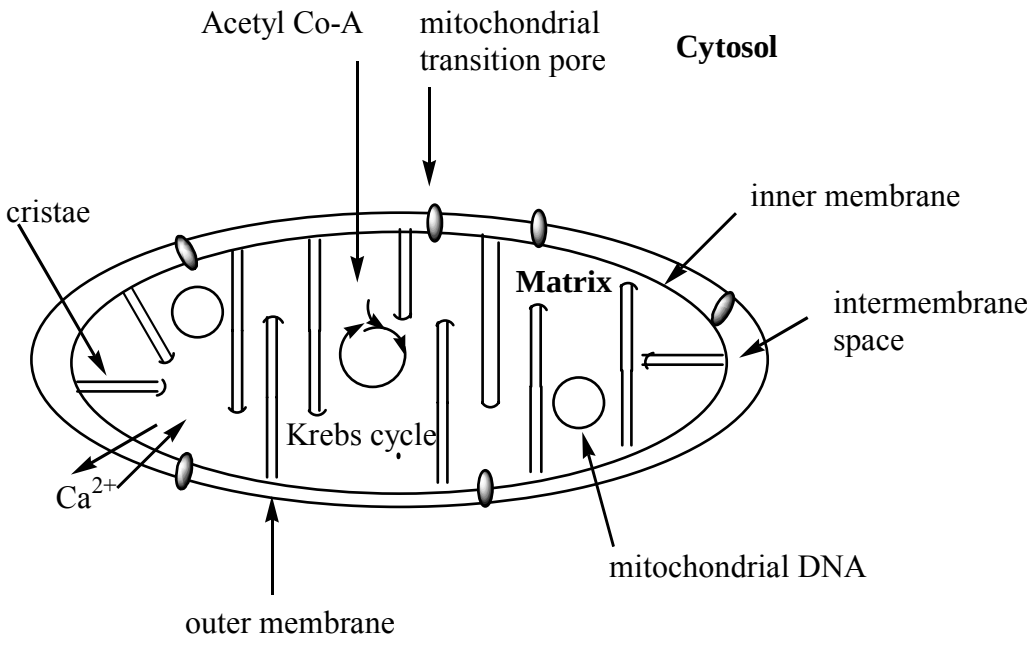


Figure 1.2. Mitochondrial structure.

oxygen species and free radicals under normal physiologic and pathologic conditions. Mitochondria play a key role in aging since they appear to be particularly susceptible to damage, as evidenced by dramatic declines in respiratory function, mitochondrial transcripts, cytochrome c oxidase activity and ATP production (Calleja et al., 1993; Schwarze et al., 1998). Respiratory chain damage, particularly complex I and IV, are widely measured to evaluate mitochondrial function (Navarro and Boveris, 2007). Using electron microscopy, structurally abnormal mitochondria are observed to accumulate during aging (Miquel et al., 1976; Fleming et al., 1985). The "mitochondrial theory of aging" postulates that damage to mitochondrial DNA (mtDNA) by free radicals leads to loss of mitochondrial function and loss of cellular energy (Cortopassi and Wong, 1999). Human mtDNA consists of 16,569 base pairs with 37 genes. Unlike nuclear DNA, mtDNA has limited protection, and is more easily damaged. In addition, mtDNA repair is less efficient than nuclear DNA. Accumulated mtDNA damage could lead to mutations and disrupt the mitochondrial protein expression. In addition to damage to mtDNA, free radicals can oxidatively damage proteins. For instance, oxidatively damaged aconitase and adenine nucleotide transporter (Yan et al., 1997; Yan and Sohal, 1998) have been shown to accumulate during aging. In addition, free radicals oxidize unsaturated fatty acids and could compromise the integrity of mitochondrial membranes and alter permeability. Changes in mitochondrial permeability could lead to structure changes, leakage of protons and membrane associated proteins, and reduce mitochondrial potential, resulting in compromised the energy production. The reduced mitochondrial function and ATP production would decrease the rate of protein synthesis and turnover, which in turn could increase protein half-life and the potential for further damage.

It has been shown that mitochondria play a key role in chronic and acute neurodegenerative disorders except aging, including Parkinson's disease (PD), AD, amyotrophic lateral sclerosis (ALS), ischemic brain injury, and stroke (Kristian and Siesjo, 1998). Studies of these diseases show damaged mitochondrial proteins and DNA, disrupted signal processing, increased oxidative stress and reduced Ca^{2+} buffer capacities, impairment of respiratory chain function and other enzymes (Xiong et al., 1997b; Fiskum et al., 1999; Lifshitz et al., 2004; Chen et al., 2006; Kaddour-Djebbar et al., 2006).

Studies of mitochondrial dysfunction in TBI have been limited. The role of mitochondria in neuron damage following TBI is unclear. Previous studies show metabolic disruption occurs following TBI (Yoshino et al., 1991; Ginsberg et al., 1997) as evidenced by reduced capacity to produce glutamine in mitochondria (Ashwal et al., 2004). The reduced capacity to regenerate glutamine from glutamate in astrocytes could lead to excess glutamate in the synaptic cleft, lead to overactivation of ion channels, and disrupt ion homeostasis of neurons. In addition, mitochondria show reduced sequestration capacity of calcium as early as 3 h post injury (Singh et al., 2006) and provoke cell death. C.P. Lee and coworkers (Xiong et al., 1997b), using a controlled cortical impact model, also demonstrated considerable impairment of brain mitochondrial respiration following injury. Previous research from Hall et al. showed mitochondrial respiration is damaged as early as 30 mins post injury (Singh et al. 2006), and other studies showed respiratory damage persisted for up to 14 days following injury (Xiong et al., 1997b). The compromised respiration could reduce the ATP production and could significantly affect the function of ATP dependent enzymes. Significant ATP shortage could lead to cell necrosis. Additionally, contact proteins between the inner membrane, outer membrane and intermembrane space form channels called mitochondrial permeability transition (MPT) pores and trigger cell death under oxidative stress or excess calcium loading. Alterations in mitochondria could trigger free radical leakage and apoptosis through cytochrome c release from the mitochondria (Sullivan et al., 2002). Lipid peroxidation and mitochondrial protein modifications by reactive oxygen species were shown to be significantly increased following severe injury (Singh et al., 2006), and indicate free radicals play a role in mitochondrial dysfunction in TBI. In addition, uncoupling proteins are shown to play a pivotal role in cell survival and to be interrupted in TBI (Mattiasson et al., 2003; Sullivan et al., 2004), and the uncoupling protein inhibitor could reduce the mitochondrial damage. Although previous studies show mitochondrial damage in TBI, there has been little study of changes in protein expression in mitochondria. are not clear. Additionally, the mitochondria are damaged during aging, and respond differently to injury in aged animals compared with young animals. The studies described here help elucidate the different response of mitochondria in aging and may help explain the exacerbated mitochondrial defecits observed in aged patients.

1.4.2 Oxidative Stress

Oxidative stress is a condition in which the balance between antioxidant defenses and reactive oxygen species (ROS) generation is disrupted, and favors ROS accumulation (Braughler and Hall, 1989). These ROS include superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), lipid oxyl or peroxyl radicals ($ROO^{\cdot}$), singlet oxygen, and peroxinitrite ($NOOO^{-}$), which behave as a unit and are now named **"**free radicals**"**(Valko et al., 2007). The original oxidative species *in vivo* is partly reduced superoxide ($O_2^{\cdot -}$) and nitric oxide (NO). Free radical species contain unpaired electrons in their atoms, and are very chemically reactive.

Mitochondria are the major source of superoxide radicals, although other enzymes such as xanthine oxidase and NADH peroxidase are also contributors (Antoine et al., 2006; Valko et al., 2007). It is estimated that 70-80% of $O_2^{\cdot -}$ from mitochondria is vectorially released to mitochondrial matrix and 20-30% to the intermembrane space (Navarro and Boveris, 2007). The two major $O_2^{\cdot -}$ production reactions have been described as auto-oxidation of intermediate semiquinones: UQH· for the ubiquinone/ubiquione redox pair ($UQH\cdot + O_2 \rightarrow UQ + H^+ + O_2^{\cdot -}$) (Boveris and Chance, 1973) and the $FMN^{\bullet}$ of the $FMNH_2$/FMN coenzyme of NADH dehydrogenase ($FMNH^{\bullet} + O_2 \rightarrow FMN + H^+ + O_2^{\cdot -}$) (Turrens and Boveris, 1980). The reactions are nonenzymatically oxidized by molecular oxygen to yield superoxide.

Iron is one of the most abundant metal ions in humans. Iron is a late transition metal and has two oxidation states (+2 and +3). Fe^{2+} is a reducing reagent and Fe^{3+} is an oxidizing agent Iron is generally incorporated into the heme complex. In most organism, heme is an essential component of cytochrome proteins, which mediate redox reactions, and of oxygen carrier proteins such as hemoglobin and myoglobin. Iron toxicity occurs when there is free iron in the cell, which generally occurs when iron levels exceed the capacity of transferrin to bind the iron. Iron is involved in ROS production of superoxide, and hydroxyl radicals by Fenton's reaction (Figure 1.3) (Braughler et al., 1986), in which Fe^{2+} is oxidized to Fe^{3+}, and H_2O_2 is reduced and decomposed to form hydroxyl radical. Fe^{3+} can be reduced by superoxide to regenerate Fe^{2+}. Free radical damage is a major theory of aging and neurodegenerative diseases. This theory of aging postulated by Denham Herman suggested that free radical damage and the accumulation of the

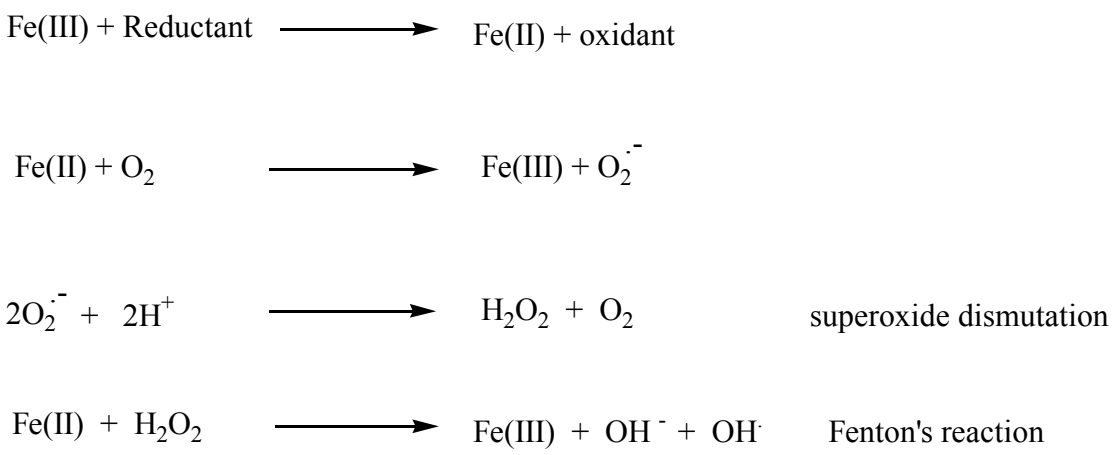
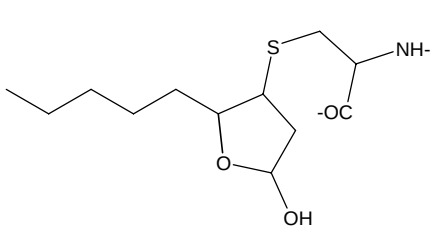


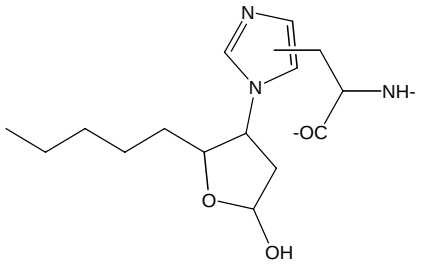
Figure 1.3. Iron is the most abundant transition metal ion in brain, and is derived from hemoglobin or transfer protein release. Increased iron exposure leads to increased oxidative damage. The most important reaction in the formation of $OH^{\bullet}$ is the Fenton's reaction, which was established in 1894.

oxidative damage is the cause of cell death, organ dysfunction and aging (Harman, 1972). It is suggested the ability of cells to detoxify ROS and replace oxidatively damaged macromolecules is apparently inefficient over time, as oxidative damage products accumulate in every aging organism examined. Previous studies show significantly increased superoxide in brain in aging, and superoxide levels in hippocampus are higher than in cortex or striatum (Antier et al., 2004). Superoxide and other free radicals with unpaired electrons can damage polyunsaturated fatty acids (arachidonic acid and decosohexaenic acid), DNA and proteins, resulting in formation of toxic aldehydic products (Figure 1.4) and oxidized DNA bases, protein thiol loss, protein cross linking, protein and DNA cross linking, and protein or DNA adducts with aldehyde products of lipid peroxidation (Braughler and Hall, 1989). As the major source of ROS, mitochondria appear to be also a key target of oxidative damage during aging (Nicholls, 2002; Lesnefsky and Hoppel, 2003). Previous studies show increased lipid peroxidation products, DNA oxidation products and abnormal proteins in aging (Sohal et al., 1995; Schwarze et al., 1998; Orr et al., 2003), and that mitochondrial DNA are more severely oxidatively damaged than nuclear DNA (Wang et al., 2003).

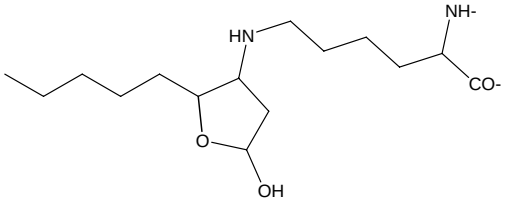
Lipid peroxidation products are stable markers of membrane lipid damage and oxidative stress. Studies of lipid oxidation can be traced back to the 1940's when it was shown that hydroperoxides are the primary products of hydrocarbon oxidation (Farmer et al., 1942). Detailed studies of products of polyunsaturated fatty acid oxidation were initiated in the 70's, and the first demonstration of free radical oxidation of membrane phospholipids was in 1980 (Porter et al., 1980a, b). Many lipid peroxidation products have been identified, including primary products such as F_2-isoprostanes and hydroperoxides of oleic acid, linoleic acid, and other highly unsaturated fatty acids like arachidonic acid. These primary products could be oxidized to secondary products, such as 4-hydroxy-2-nonenal (Schneider et al., 2001), 4-hydroxy-2,3–hexenal**,** acrolein, and malondialdehyde (MDA). Previous studies show increased lipid peroxidation following TBI, including significant elevations of thiobarbituric acid reactive substances (TBARS) and 8-isoprostaglandin $F_{2\alpha}$ in injured cortex (Hoffman et al., 1996; Hsiang et al., 1997). Recently, 4-hydroxynonenal (HNE) and acrolein, two neurotoxic byproducts of lipid peroxidation of arachidonic acid (Benedetti et al., 1980), have become of interest in a



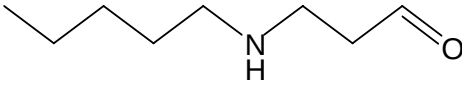
Cysteine-HNE



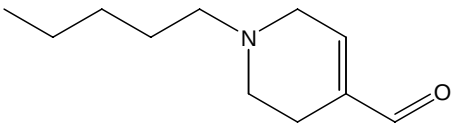
Histidine-HNE



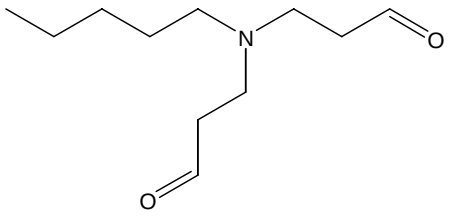
Lysine-HNE



Lysine-acrolein



Lysine-acrolein



Lysine-acrolein

Figure 1.4. HNE/acrolein modification of amino acid residues.

variety of neurological diseases (Lovell et al., 1997; Lovell et al., 2000). HNE levels were shown to be significantly increased in brain and spinal cord injury (Baldwin et al., 1998; Zhang et al., 1999; Cao et al., 2001; Hall et al., 2004; Singh et al., 2006) in young animals, although the lipid peroxidation in TBI in aging remains unclear.

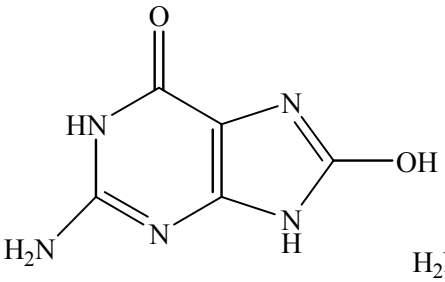
HNE and acrolein are most toxic byproducts of lipid peroxidation. It is well established that HNE and acrolein can modify proteins, DNA/RNA *in vivo* and *in vitro*, and these modifications could lead to protein dysfunction (Hiratsuka et al., 2000; Akagawa et al., 2006). Previous studies show HNE modification can lead to dysfunction of oxidative phosphorylation complexes, Na^+/K^+-ATPase, $NADP^+$-dependent isocitrate dehydrogenase, proteasome, and cathepsin B (Siems et al., 1996; Okada et al., 1999; Crabb et al., 2002). In addition, HNE can improve AP-1 binding activity (Camandola et al., 2000) and trigger apoptosis (Bruckner et al., 2003), leading to neuron death and tissue loss in injured animals. Additionally, HNE can trigger an epidermal growth factor receptor-linked signal pathway for growth inhibition by phosphorylation of adaptor protein Shc and activation of MAP kinase (Liu et al., 1999). It has also been shown that HNE can induce the heat shock protein factor and upregulate the heat shock protein expression in cell culture (Cajone, 1988), triggering cell defense. A small number of studies show lipid peroxidation increases immediately after TBI and is maintained for several days (Hall et al., 2004).

When ROS are produced close to DNA, it can result in DNA damage. It is suggested that hydroxyl radicals generated from hydrogen peroxide are extremely active with a life time of $\sim 10^{-6}$ s, and can react close to its formation (Pastor et al., 2000). DNA oxidation includes damage to base and sugar moieties, leading to DNA strand breaks, gene mutation and cell apoptosis (Chen et al., 1997; Johnson et al., 2005). Currently, about 20 DNA base oxidative products have been identified. For example, oxidized guanine in DNA by reactive oxygen species leads to formation of 7,8-dihydro-8-oxoguanine (8-OHG), and ring opening products 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapyguanine, FG). Adenine oxidative products include 8-hydroxyadenine (8-OHA) and 4,6-diamino-5-formamidopyrimidine (fapyadenine, FA). For cytosine and thymine, oxidative damage results in formation of 5-hydroxycytosine (5-OHC), 5,6-dihydroxycytosine, 5-hydroxy-6-hydrothymine and thymine glycol. The

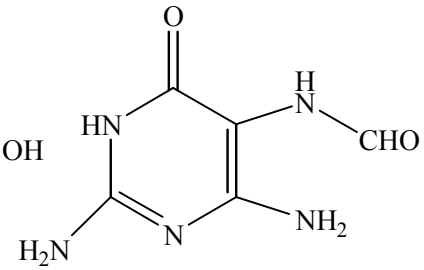
DNA base oxidative products can be used as markers to evaluate the oxidative damage in cell. The effect of oxidative stress in DNA damage has been studied extensively in aging, cancer, AD, HD, diabetes and stroke, but limited in TBI (Alam et al., 1997; Rehman et al., 1999; Nagayama et al., 2000; Wang et al., 2005). The accumulation of free radical induced damage to DNA is illustrated by an age-related increase in human serum over an age range of 15-91 years (Valko et al., 2007). Numerous studies have reported the accumulation of 8-OHG and other lesions with age, both *in vivo* and *in vitro*, in nuclear and mitochondrial DNA. Although 8-OHG levels are increased in injured brain cortex in TBI in young animals(Mendez et al., 2004), it is unclear whether DNA oxidative damage is exacerbated in TBI in aging. In order to evaluate DNA oxidative damage in TBI in aging, 8-OHG, FG, 8-OHA, FA, and 5-OHC are oxidized bases investigated in this dissertation (Figure 1.5).

1.4.2.1 Mechanism of Lipid Peroxidation

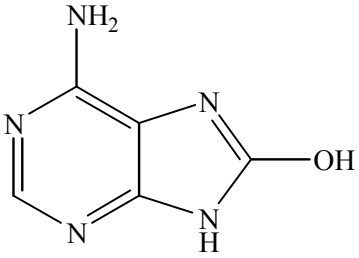
Unsaturated fatty acids, especially ω-3 such as linoleic acid, and ω-6, including arachidonic (AA, 20:4n-6), docosatetraenoic acid (DCS, 22:4n-6) and decosohexaenic acid (DCH, 22:6n-6), are major components of cell membranes and organelles. These ω-6 fatty acids are abundant in brains and highly susceptible to free radical attack. Lipid peroxidation forms a variety of products, including malondialdehyde, 4-hydroxynonenal, acrolein, and other aldehydes (Williams et al., 2005). The mechanism of lipid peroxidation *in vivo* is difficult to investigate because of the complicated cellular environment. However, studies in organic chemistry help suggest possible pathways of lipid peroxidation. Using arachidonic acid and linoleic acid as substrates, their autoxidation and enzyme catalyzed oxidations were investigated. Arachidonic acid or linoleic acid may be attacked by free radicals and lose hydrogens attached to bis- allylic carbons (Thomas and Pryor, 1980). Oxygen can then add to allyl radicals and form peroxyl radicals. The peroxyl radical may abstract hydrogen from hydrogen donor forming hydroperoxide. Hydroperoxides are precursors of products such as 4-HNE and acrolein (Porter et al., 1981). In arachidonic acid autoxidation, six major conjugated diene hydroperoxide, hydroperoxyeicosatetraenoic acids (HPETE), have been identified (Porter et al., 1979), and all have trans,cis conjugated diene stereochemistry



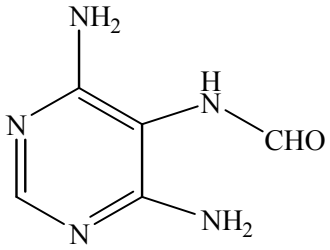
8-hydroxyguanine



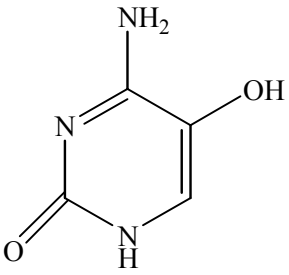
Fapyguanine



8-hydroxyadenine



Fapyadenine



5-hydroxycytosine

Figure 1.5. DNA base oxidation products investigated in this dissertation.

with hydroperoxide substitution being at carbon 5, 8, 9, 11, 12, or 15. Further oxygenation can form dihydroperoxides, which fragment to 4-hydroperoxy-nonenal by Hock rearrangement. 4-Hydroperoxy-nonenal could form 4-hydroxynonenal (Figure 1.6). The mechanism for acrolein formation from unsaturated fatty acids is not well investigated yet. It's possible the formation of acrolein follows a different mechanism.

1.4.2.2 Mechanism of DNA Oxidation

Free radicals attack of the DNA strand leads to DNA base and ribose moiety oxidation. The attack of ribose could lead to strand break and DNA fragmentation. Damage to DNA bases could result in a variety of base oxidation products. However, radicals have a high preference for interaction with the base part of the molecule rather than with the ribose (phosphate) (O'Neill and Davies, 1987). The addition of OH• to purines could form C4-OH, C5-OH, and C8-OH products. The C8-OH adducts of purines could form 8-OHG, FG, 8-OHA, and FA products (Figure 1.7). As for pyrimidine base, hydroxyl radical (OH·) could attack C5 and C6 to from cytosine glycol, which dehydrates to form 5-OHC (Figure 1.8). Among the bases, guanine is the most easily oxidized, which is supported by MO calculation, ionization potential, and aqueous-phase redox (Jovanovic and Simic, 1986; Steenken, 1989).

1.5 Antioxidant Enzymes

Exposure to free radicals from a variety of sources has led to development of a series of defense mechanisms. Defense mechanisms against free radical-induced oxidative stress include: preventive mechanisms, repair mechanisms, physical defenses, and antioxidant defenses. Non-enzymatic antioxidants incude ascorbic acid (Vitamine C), alpha-tocopherol (Vitamine E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Antioxidant enzymes include superoxide dismutase (Cu/ZnSOD and MnSOD), catalase and glutathione peroxidase (GPx), glutathione reductase (GSSG-R), and glutathione transferase. These enzymes work in concert to protect cells (Figure 1.9).

1.5.1 Superoxide Dismutase (SOD)

SOD, a metalloenzyme was first discovered by McCord and Fridovich (McCord and Fridovich, 1969). SOD catalyzes the dismutation of toxic superoxide radicals with the conversion of hydrogen ions to hydrogen peroxide, which is less toxic. It has been established that all organisms living in the presence of oxygen have at least one form of

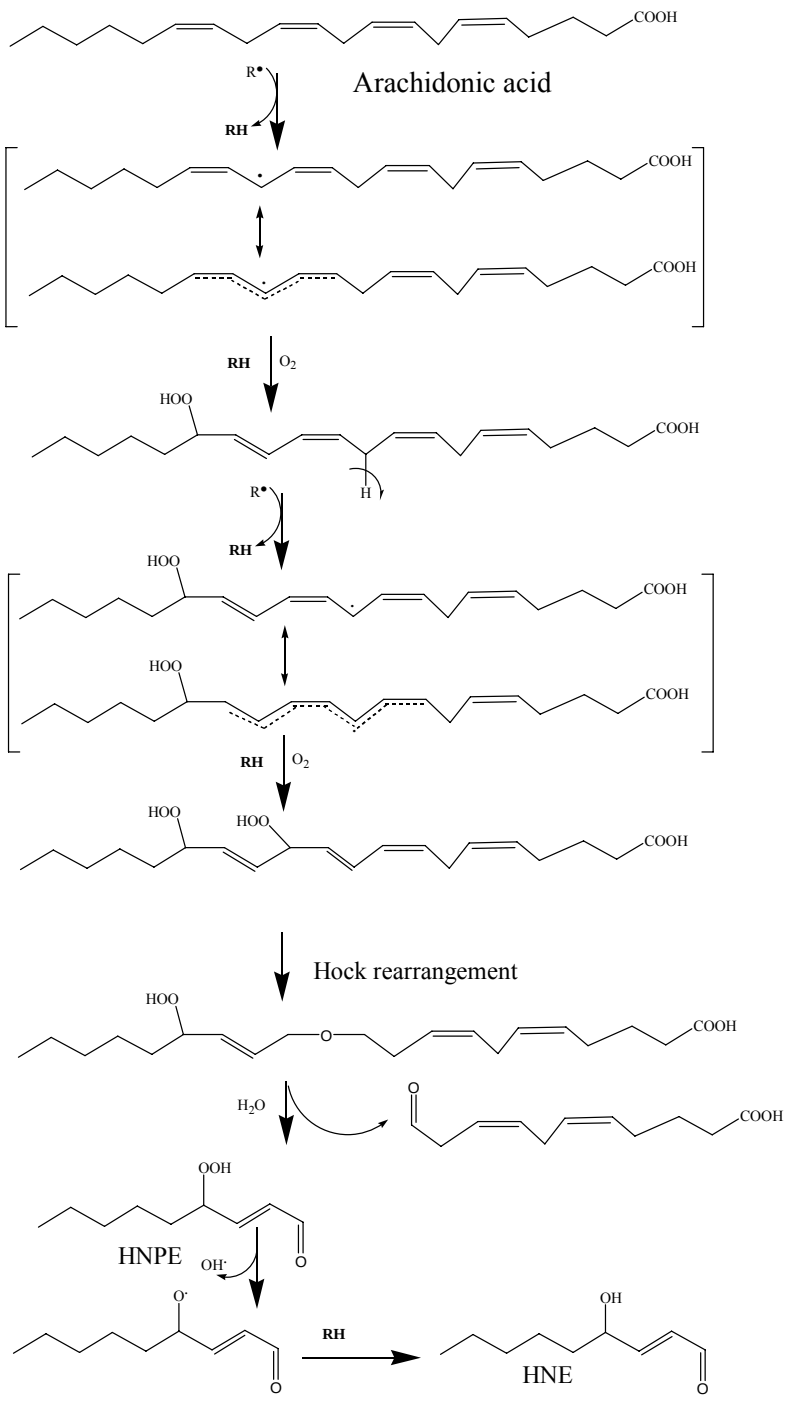


Figure 1.6. Mechanism of arachidonic acid autoxidation lipid peroxidation generates HNE. R• represents free radical.

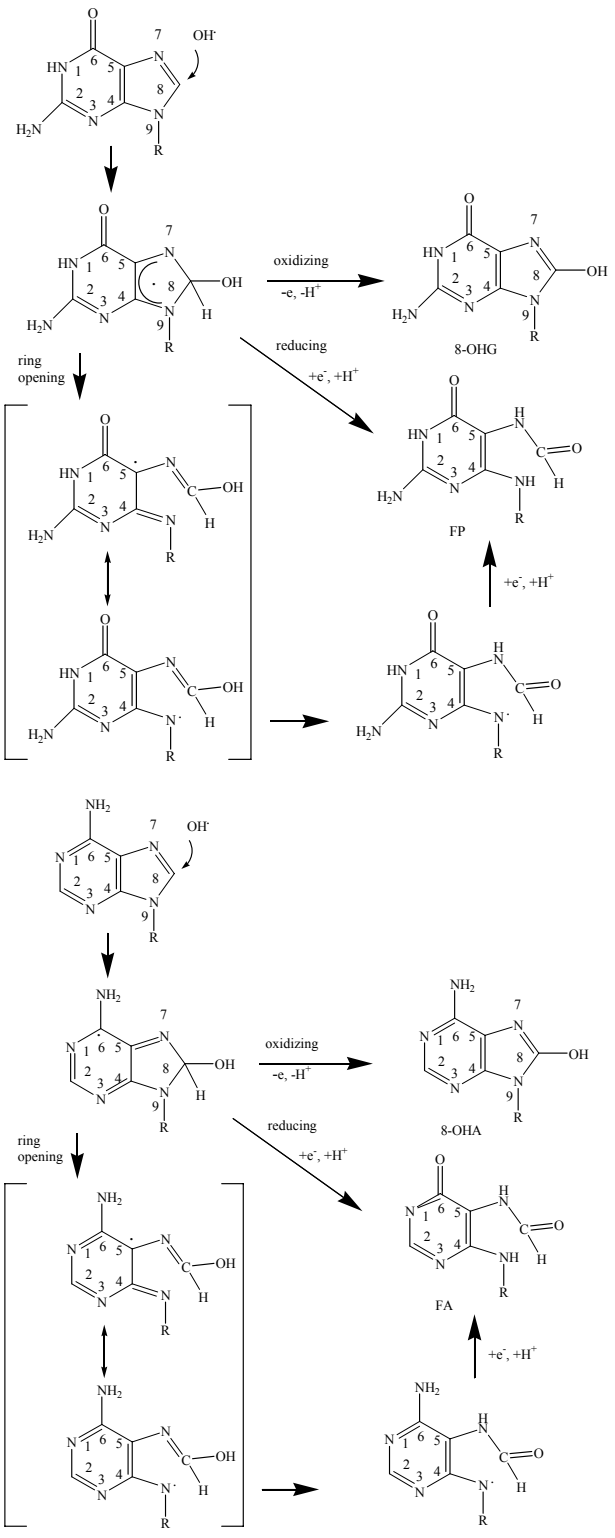


Figure 1.7. Mechanism of 8-hydroxyguanine, fapyguanine, 8-hydroxyadenine, and fapyadenine formation. R=H.

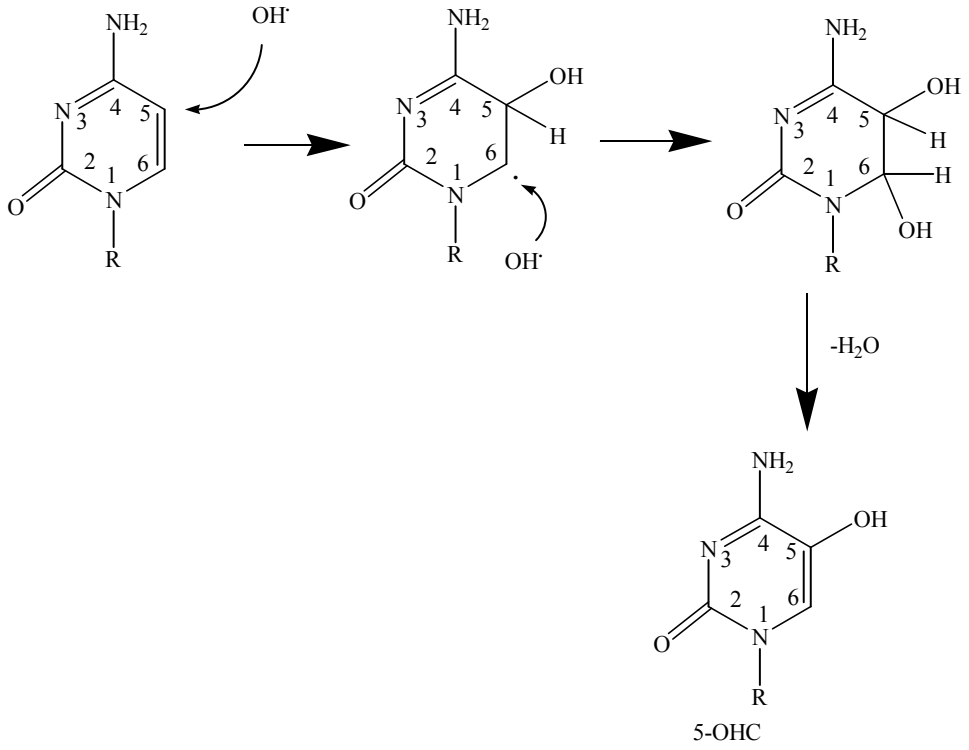


Figure 1.8. Mechanism of 8-hydroxycytosine formation.

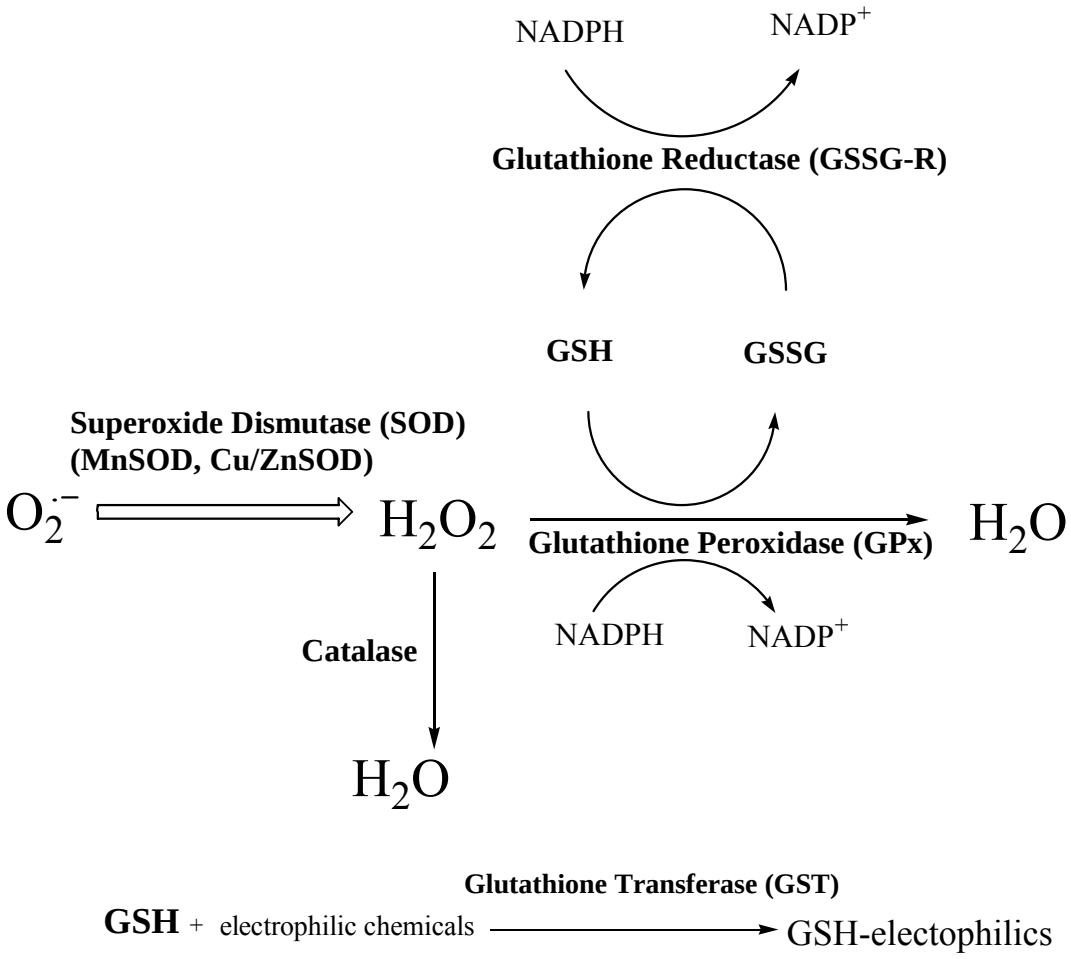


Figure 1.9. Antioxidant enzymes.

SOD, with an active site containing manganese, iron, nickel or copper-zinc (Cadelli et al., 1999; Niviere and Fontecave, 2004). Manganese (Mn) and copper-zinc (Cu/Zn) dismutases are present in human and animals. MnSOD (MW 26.6 KDa) is located in mitochondrial matrix, and Cu/ZnSOD (32 KDa) is found in cytosol, mitochondrial intermembrane space and extracelluar fluid. The gene encoding SOD human mRNA resides on chromosome 21.

SOD plays a key role in antioxidant defense. Cu/ZnSOD activity decreased in stroke, SOD protein and gene transcription levels, and the enzyme activity are investigated have been widely investigated in a variety of neurodegenerative diseases, including ALS, AD, PH, spinal and brain injury (Trotti et al., 1999; Demirkaya et al., 2001; Earnhardt et al., 2002; Antier et al., 2004; Vaziri et al., 2004). Transgenic animals with increased expression of SOD show increased life span and less damage following ischemic injury (Saito et al., 2003). SOD mimetics were reported to be effective in attenuating oxidative associated neurodegenerative disease.

1.5.2 Glutathione Peroxidase and Catalase

As mentioned above, superoxide dismutase catalyzes the dismutation of superoxide radical ($O_2^{\bullet -}$) to produce hydrogen peroxide (H_2O_2), a quite stable and invasive ROS. Moreover, through the Fenton and Haber-Weiss coupled reactions, H_2O_2 forms reactive hydroxyl radical ($OH^{\bullet}$). To efficiently remove hydrogen peroxide, two antioxidant enzymes are available: catalase and GPx, which both transform H_2O_2 into a harmless product, H_2O. These two enzymes do not work in identical conditions although they do similar jobs. First, catalase only uses H_2O_2 as a substrate, whereas GPx uses both H_2O_2 and lipid peroxides as substrates (Cohen and Hochstein, 1963). Catalase functions when H_2O_2 concentrations are significantly above physiological levels that can occur in oxidative bursts characteristic of stress responses. However, GPx functions during small variations of H_2O_2 concentrations (Drevet, 2006). Therefore, GPx activity represents the first protective response for small adjustments in H_2O_2 concentrations under normal physiological conditions. Glutathione peroxidase also needs glutathione as a substrate, in which reduced glutathione (GSH) is converted to oxidized form (GSSG).

Glutathione peroxidase is a family of proteins, divided into five classes (GPx1 to GPx5) (Chu, 1994) based on their primary sequence, substrate specificity and subcellular

localization. GPX1, the so-called cytosolic or cellular GPx, was the first GPx to be discovered (Mills, 1957), and is abundant in kidney, lung, red cells and liver, but relatively low in brain. GPx2, another cytoplasmic enzyme, exists in stomach and intestines (Chu et al., 1993). GPx3, an extracellular GPx or plasma-type GPx (pGPx), was found to be preferentially expressed in the mammalian kidney with lower levels in a wide array of tissues (Maser et al., 1994; Schwaab et al., 1998). GPx4, also called phospholipid hydroperoxide glutathione peroxidase (PHGPx), is a membrane-associated GPx expressed in the mammalian testis, brain and heart (Ursini et al., 1982). GPX5, also previously known as MEP24 (for mouse epididymal protein of 24 KDa) or epididymal GPX (epGPx), is a highly restricted GPx and is expressed only in the mammalian caput epididymidis (Ghyselinck and Dufaure, 1990). In higher animals, GPxs are selenium-containing proteins. They contain a selenocysteine residue, which form so-called catalytic triad with glutamine and tryptophan residues (Maiorino et al., 1995).

1.5.3 Glutathione Reductase

Glutathione, a triamino acid peptide (γ-glutamylcysteinylglycine), is an antioxidant molecule, and also functions in DNA and protein synthesis regulation, signal transduction, cell-cycle control, proteolysis, immune response and cytokine synthesis (Harding et al., 1996; Wu et al., 2004). The synthesis of GSH is catalyzed by γ-glutamylcysteine synthetase (GCS) and GSH synthetase. GCS activity, cysteine availability, and GSH feedback inhibition are the main factors regulating GSH synthesis. In addition, recycling of oxidized GSH produced by GPx is important in the maintenance of reduced GSH levels in the cell. Glutathione reductase is the enzyme that converts oxidized GSH (GSSG) back to reduced GSH, and is involved in the pathogenesis of many diseases. The conversion from GSSG to GSH requires NADPH, which is produced from reactions catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. GSSR-R is important not only for maintaining the required GSH level but also for reducing protein thiols to their native state (Ganea and Harding, 2006).

1.5.4 Glutathione Transferase

Glutathione transferase (GST) is a family of detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of xenobiotics (McIlwain et al., 2006). GST isoforms contain a highly conserved N-terminal domain which has a

catalytically active tyrosine, cysteine or serine residue. The catalytic residue interacts with the thiol group of GSH (G-site), and the substrate binding site (H-site) near the G-site to facilitate catalysis (Dirr et al., 1994; Armstrong, 1997). The detoxification ability of GST plays a role in cellular protection from environmental and oxidative stress. Human GSTs are divided into three main families: cytosolic, mitochondrial and membrane-bound microsomal.

1.5.5 Antioxidant Capacity in Aging and Neurodegenerative Diseases

Aging is correlated with a reduced redox state and diminished antioxidant capacities. Reducing equivalents, especially glutathione, are involved in maintaining redox balance throught the cell. Reduced antioxidant defenses could change the redox condition of cells. Many proteins contain redox-sensitive regulatory amino acid residues. The reduced levels of glutathione could lead to the inactivity of redox sensitive proteins (Kim et al., 2002; Droge, 2003). Aging studies using mice showed a 40% increase in median life span and a 17% increase of maximal life span with supplementation with Vitamine E (Navarro et al., 2005). Studies of Cu/ZnSOD show increased activity as a function of age in brain, although other studies show the Cu/ZnSOD decreased in cortex, hippocampus, and striatum in aged animals compared with young animals, which could contribute to the increase of superoxide in aging (Antier et al., 2004). Additionally, studies show no significant changes of Cu/ZnSOD and catalase levels among age groups of 35-39, 50-54, and 65-69 years (Barnett and King, 1995). Because of the ability to convert very active superoxide to less active hydrogen peroxides Cu/ZnSOD can reduce the damage to DNA, RNA, protein, and unsaturated fatty acids. However, SOD defense appears unable to keep pace with endogenous and exogenous sources of damage even though antioxidant enzyme levels are unchanged or increased, leading to progressive deterioration of structure and function with age. In neurodegenerative disease, Cu/ZnSOD mutantions lead to oxidative damage, and associated with diseases such as ALS (Trotti et al., 1999). MnSOD, a mitochondrial SOD, reduces the potential damage to mitochondrial proteins and mtDNA. MnSOD expression is down regulated in smooth muscle cells (Li et al., 2006), and human granulosa cells (Tatone et al., 2006), and could contribute to their dysfunction in aging. In brain, MnSOD activity decreases linearly upon aging (Navarro et al., 2002). It has been shown that partial MnSOD deficiency (+/-)

results in mice with increased mitochondrial damage during aging (Wallace, 2002). However, other studies indicate no significant changes in brain in aging (Antier et al., 2004).

In AD, elevated SOD, catalase, GPx and GSSG-R and decreased GST activities were reported, indicating a compensationary response in disease for most antioxidant enzymes (Lovell et al., 1995; Lovell et al., 1998). In spinal cord injury, Azbill et al. showed SOD was unchanged following injury (Azbill et al., 1997). In TBI, Dekosky et al. showed SOD activity decreased, GPx and catalase increased significantly following injury in young animals (DeKosky et al., 2004). However, the response of antioxidant enzymes has not been investigated in TBI in aging.

1.6 Measurement of Oxidative Damage

Oxidative damage is widely investigated in a variety of diseases, including cancer, AD, HD, diabetes, ALS, aging, stroke, cardiovascular disease, and TBI. There are several methods to evaluate oxidative stress *in vivo* and *in vitro*, including measurement of the reducing capacity of tissue or cell (Shohami et al., 1999); trapping the superoxide radicals using electron spin resonance trapping reagents (Awasthi et al., 1997); measuring products of oxidative stress such as lipid peroxidation (Hsiang et al., 1997) and DNA oxidation (Mendez et al., 2004) or protein modification (Chirino et al., 2006). Measuring lipid peroxidation byproducts, DNA oxidation markers, and protein carbonyls are currently the most commonly used methods.

1.6.1 Analysis of By-products of Lipid Peroxidation

The measurement of markers of lipid peroxidation can be carried out using fluorescence spectrometry, chromatography, and immunochemistry (Awasthi et al., 1997; Hsiang et al., 1997; Lovell et al., 1997; Lovell et al., 2000; Hall et al., 2004; Davis et al., 2006). Immunochemistry methods include Western blot, dot blot, slot blot and tissue section staining. Immunochemistry measures the protein-bound lipid peroxidation byproducts, which look at the protein modifications under oxidative stress (Springer et al., 1997). Chromatography and fluorescence spectrometry methods quantify the free or unstable protein-bonded lipid peroxidation byproducts *in vivo*. For example, using fluorescence method, MDA, HNE and acrolein can be measured following derivatization (Ohkawa et al., 1979; Lovell et al., 1997; Lovell et al., 2001).

1.6.2 DNA Oxidation Analysis

DNA oxidative damage is widely investigated as a major marker of oxidative damage in a variety of diseases (Loft and Poulsen, 1996; Kowalczyk et al., 2004; Mendez et al., 2004). Various chemical and biochemical methods have been proposed, including indirect measurements based on the use of antibodies (Mendez et al., 2004), single-cell gel electrophoresis (Collins et al., 1995), ^{32}P-postlabeling assays (Cadet et al., 1992), and direct analysis by HPLC (Kasai, 1997) and GC/MS (Dizdaroglu and Bergtold, 1986). The direct approach involves isolation of DNA, followed by hydrolysis for nucleosides or nucleobases of DNA sample. The indirect methods are usually highly sensitive, but their specificity is still open to debate. High performance liquid chromatography associated with electrochemical detection (HPLC-EC) and gas chromatography coupled to mass spectrometry (GC/MS) have been the most widely applied methods during the last decade with detection limit of 50 fmol of oxidized base, which corresponds to one lesion per 10^6 normal base or in a DNA sample size of 10 pg. HPLC-EC assays are aimed at monitoring the formation of oxidized bases with a low oxidation potential. The assay was initially developed for the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHG) (Floyd et al., 1986), and then extended to 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-OHA) (Berger et al., 1990), 5-hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine, 5-hydroxy-2'-deoxyuridine, and 5-hydroxy-2'-deoxycytidine (Wagner et al., 1992).

However, one limitation of HPLC-EC deals with the fact that only bases with a low oxidation potential can be measured by electrochemical detection. In contrast, GC/MS is a more versatile technique since it is less dependent on the chemical properties of the oxidized base of interest (Dizdaroglu, 1991). The mass spectrometer may be set in the selective ion monitoring (SIM) mode that detects only the ions corresponding to the major peaks of the mass spectrum of the bases, which provides a more specific and sensitive detection. One point that should be noted is the bases have to be derivatized into volatile compounds prior to their injection into the GC/MS. In the current method, staple isotopically enriched internal standards, which differ from the oxidized DNA bases in samples by three units of mass, are used. Internal standards could reduce errors from the derivatization step and also compensate for the possible lack of reproducibility of the injection. The GC/MS assay was first applied to the detection of 8-OHG in isolated DNA

that was exposed to γ-radiation in aqueous solution (Dizdaroglu, 1985). Subsequently, the method has been applied numerous times to the measurement of many types of oxidative base damage in isolated DNA (Aruoma et al., 1989; Spencer et al., 1994; Fuciarelli et al., 1995; Wang et al., 2005) and within isolated cells (Djuric et al., 1991).

1.6.3 Protein Carbonyl Modification and Nitration Analysis

Under oxidative stress, proteins are targets of reactive oxygen species and lipid peroxidation byproducts. Modification of thiol group on amino acid side chains can result in loss of protein folding and incorrect folding (de Venevelles et al., 2006). Aldehydic products of lipid peroxidation can react with amine groups to form Shiff base adducts or react with cysteine to form Michael addition products (Hashimoto et al., 2003). The peroxynitrite can modify tyrosine to inactivate proteins (Chirino et al., 2006; Tao et al., 2006). Protein carbonyl and nitration can be measured using immunochemistry, immunohistochemistry and mass spectrometry (Hall et al., 2004; Matters et al., 2006).

1.7 Proteomics

Currently, biologists try to understand the function of cell at the molecular level and to look for biological markers in cells with disease. Over the past several decades, considerative effort has been directed at the polynucleotide level. Complete sequencing of genome in a wide variety of simple organisms and great progress in the human genome project provide the chance for the development of research at the protein level. The expected proteins in human may be as many as millions if splice variants and post translation modifications are considered (Wilkins et al., 1996; Binz et al., 1999; Wilkins et al., 1999). Based on genome and protein databases such as SwissProt and NCBI thousands of proteins are predicted. The complete set of proteins that result from a genome is called the proteome (Wasinger et al., 1995), which is dynamic and highly dependent on the type of cell and its state. The development of Mass Spectrometry tools, such as the Matrix Assisted Laser Desorption Ionization (MALDI) and electrospray ionization, provides effective tools to investigate protein levels in cell. Proteomics is a method to display and detect all cellular proteins, and study multiple gene/protein functions and networks (Schilling et al., 1999).

1.7.1 Two Dimensional Gel Proteomics

In current proteomic studies, protein separation before identification is critical. The separation of a complicated protein mixture from cells was firstly achieved more than 30 years ago using two dimensional polyacrylamide gel electrophoresis (2D PAGE) (O'Farrell, 1975). In this method, proteins are separated by their isoelectric point in one dimension and molecular weight in the second dimension. In the first dimension, proteins are loaded onto immobilized pH gradient strips, and separated under high voltage from hundreds to thousands volts. The secondary dimension is similar to running a 1D gel. The proteins in the 2D page are visualized by Coomassie blue staining, silver staining, or by Sypro ruby staining and imaged by fluorescence scanning. Protein spots of interest are cut out, and proteolytically digested to release peptides. Peptides from in-gel digestion are loaded onto matrix for MALDI MS and the peptide can be sequenced by mass spectrum fingerprint and then identified based on comparision to a database. It is predicted that up to 5000 proteins can be displayed by this method, and post-translational modifications and single-site mutations can be detected with proteins differing in concentration by ratio of 10^{-4} to 10^{-5} detected.

Two dimensional PAGE proteomics has allowed many proteins in different organisms or animal and human tissues to be mapped, but this method is labor intensive and difficult to match protein spots on various gels. In addition, less abundant proteins, proteins with low or high molecular weight as well as proteins with acidic or basic side chains are difficult to detect. Additionally, membrane protein analysis is also difficult using this method (Godovac-Zimmermann and Brown, 2001).

1.7.2 Isotope-coded Affinity Tag Proteomics

To overcome problems associated with 2D PAGE, Isotope-coded Affinity Tag (ICAT) was developed by Gygi et al., and uses heavy and light reagents to label control and treatment samples separately (Gygi et al., 1999). This gel free method allows more automatic protein analysis. In this method, cysteine residues in amino acids are labeled by a tag, which contains a biotin moiety for subsequent isolation of the labeled peptides. Rather than displaying the cellular proteins by 2D PAGE, the cellular protein mixtures are subjected to tryptic digestion after labeling, and the Cys-containing peptides isolated by chromatography on an avidin column. Purified Cys-containing labeledpeptides are

separated by 2D microcapillary liquid chromatography and ionized by electrospray ionization, and identified by 2D LC-MS/MS. The ICAT proteomics method requires much less sample for analysis compared with 2D PAGE, which is useful for studies of subcellular organelle or where limited material is available. The heavy and light labeling reagents differ in mass since they have different isotopes, and this mass difference help MS determine if the peptide is from control or treatment sample. So far, ICAT reagents have been labeled with deuterium or ^{13}C, and the mass difference between heavy and light can be 8 or 9 Da (Lovell et al., 2005). The peptides are sequenced by mass spectrometry and used for database searches to identify proteins based on their peptides.

1.8 The Effort to Find Efficient Treatment for TBI

Currently, research on TBI is directed at understanding the mechanisms of neuron injury and the search to find an effective therapy for clinical application. Based on the understanding of the injury, some treatment methods such as hypothermia (Davies, 2005) and many kinds of drugs have been proposed. Current drugs under investigation include: inhibitors or regulators targeting specific proteins such as lithium (Wada et al., 2005), magnesium (Enomoto et al., 2005; Ghabriel et al., 2006), N-(4-fluorophenylsulfonyl)-L-valyl-L-Lleucinal (Inoue et al., 2003), cyclosporine A (Rabchevsky et al., 2001), progesterone (Fiskum G, 2006, p235), dexanabinol, and dexamethasone; antioxidants such as metalloporphyrin (Tauskela et al., 2006), bromocriptine (Kline et al., 2004) and N-acetylcysteine (Yi et al., 2006), and protective drugs with unknown mechanisms such as methylprednisolone (Rabchevsky et al., 2002).

Creatine has been shown to increase lean body mass, muscular strength, and sprint power, and is one potentially protective compound of interest in TBI (Matthews et al., 1999; Sullivan et al., 2000). Creatine is an endogenous substance synthesized mostly in kidney and liver. Creatine biosynthesis has two sequential steps catalyzed by L-arginine:glycineamidinotransferase and S-adenosylmethionine:guanidinoacetate N-methyltransferase (Braissant et al., 2005). Cells with high energy demand can take up creatine from the blood stream with the involvement of a specific creatine transporter (CRT). Imported creatine can be converted to phosphocreatine by creatine kinase with consumption of ATP provided through oxidative phosphorylation and glycolysis at contact sites of mitochondria or in cytosol (Jacobus, 1985) (Figure 1.10).

Phosphocreatine is an energy pool and can be available in cytosol. ATP levels in cytosol can be maintained at a stable level with the hydrolysis of phosphocreatine to regenerate ATP to satisfy the demand of different biological process, such as ATPase and other ATP-regulated process, which contribute to the protective function of creatine. It was shown that creatine can regulate the mitochondrial transition pore by stimulating creatine kinase located in the intermembrane space of mitochondria (O'Gorman et al., 1997). Creatine supplemented animals maintained better calcium buffering capacity compared to animals without suppleentation following brain injury (Sullivan et al., 2000). Studies also show that creatine can function as an antioxidant *in vitro*, showing a significant ability to remove superoxide and peroxynitrite (Lawler et al., 2002).

Oral supplementation of creatine (Cr) has recently been shown to be protective in a variety of neurodegenerative diseases, including HD, PD, ALS, and TBI (Klivenyi et al., 1999; Matthews et al., 1999; Ferrante et al., 2000; Sullivan et al., 2000). In animal models of ischemia, creatine supplementation resulted in neuroprotection and a remarkable reduction in ischemic brain infarction. Caspase-3 activation and cytochrome c release were significantly reduced, and ATP levels were maintained (Zhu et al., 2004). Despite its well documented neuroprotective properties and its possible mechanism, creatine's protective role in TBI is not well understood.

1.9 Experiment Design

Because secondary injury is the only possible curable damage so far in TBI we decide to investigate the factors in secondary injury to find a clue for clinical therapy. As mentioned ealier in the desertation that oxidative damage and mitochondrial dysfunction play key roles in TBI. In order to understand the effect of age on TBI, this dissertation was to investigate the oxidative damage in TBI in aging, and to elucidate the exacerbated injury in TBI in aging. In our study, young (3 months), middle aged (12 months) and aged (22 months) animals were investigated using a controlled cortical impact injury. To evaluate mitochondrial dysfunction, mitochondria were isolated following TBI and subjected to proteomics. To test potential therapeutics to prevent against secondary injury, oxidative damage in creatine supplemented animals was measured.

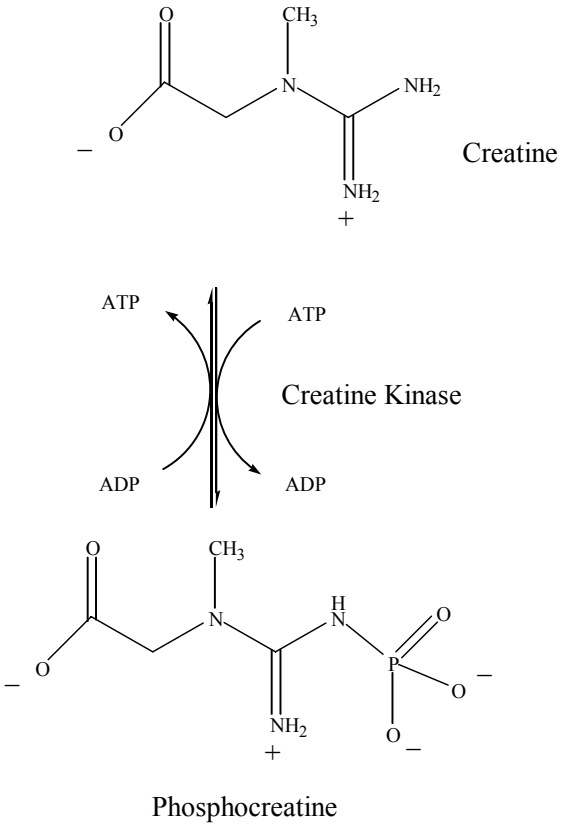


Figure 1.10. Creatine phosphorylation.

1.9.1 Oxidative Stress in TBI in Aging

As mentioned earlier, aging is an important factor in brain injury, and aged patients are more likely to sustain more severe injury than young adult patients. The reason for this phenomenon is not well understood. Previous studies show oxidative stress could be a factor for many pathological processes, but has not been investigated in head trauma in aging. In the current project, Fisher-344 rats were subjected to CCI, and HNE and acrolein, toxic markers of lipid peroxidation, evaluated using immunochemistry (dot-blot) at 1 and 7 days post injury. Further studies were performed to determine antioxidant defense capacities of animals in head trauma in aging. The antioxidant enzyme activities, including MnSOD, Cu/ZnSOD, GPx, GSSG-R, and GST, were investigated.

DNA oxidation may contribute to gene mutation or protein expression changes, and is widely used as a marker for oxidative damage. To determine if the oxidative stress in TBI in aging impacts DNA, 8-OHG, FG, 8-OHA, FA, and 5-OHC were investigated in injured rat brains using GC/MS-SIM. Stable isotope labeled oxidized DNA bases labeled with ^{15}N and ^{13}C were used as internal standards for quantification.

1.9.2 Protection by Creatine in TBI

Creatine has been shown to be neuroprotective in neurodegenerative disorders. In order to investigate the effect of creatine on TBI, Sprague-Dawley rats with 1% and 2% creatine supplementation were subjected to CCI, sacrificed at 24 h post injury, and HNE and acrolein levels in cortex and hippocampus measured using dot-blot immunochemistry. Additionally, enzyme activities, including MnSOD, Cu/ZnSOD, GPx, GSSG-R, and GST, were investigated.

1.9.3 Mitochondrial Proteomics

Previous studies showed oxidative damage, ion homeostasis disruption, and ATP depletion following TBI. Mitochondria play a key role in these pathologies and regulate the fate of neurons in TBI. Mitochondrial dysfunction is also the reason of aging. Although studies showed that mitochondrial swelling, mitochondrial membrane

permeability changes, reduced amount of isolated mitochondria, respiration chain damage in TBI, the individual mitochondrial protein expression level is not evaluated. In this dissertation, young Fisher-344 rats were subjected to CCI and the mitochondria were isolated at 1 and 24 h post injury. Mitochondrial protein expression was quantified using isotope-coded affinity tag (ICAT) labeling and 2D-LC/MS/MS. In the further, we will use middle aged and aged animals to evaluate mitochondrial dysfunction in TBI in aging.

Chapter Two

Materials and methods

2.1 Materials

Stable isotope labeled oxidized bases were obtained from Cambridge Isotope laboratories (Andover, MA, USA), and included 8-[8-^{13}C,7,9-$^{15}N_2$] hydroxyguanine, [formyl-^{13}C, diamino-$^{15}N_2$] fapyguanine, 8-[8-^{13}C,6,9-diamino-$^{15}N_2$] hydroxyadenine, [formyl-^{13}C, iamino-$^{15}N_2$] fapyadenine, and 5-[2-^{13}C,1,3-$^{15}N_2$] hydroxycytosine. Other chemicals are purchased from Sigma (St Louis, MO, USA) if not specified.

2.2 Animal Surgical Procedures

The present studies were conducted in adult male Fisher 344 rats or Sprague-Dawley rats. All injury procedures were performed under isofluorane anesthesia and were approved by the Animal Care and Use Committee of the University of Kentucky. Every effort was made to minimize both the possible suffering and number of animals used. All animals were housed 2/cage on a 12 hour light/dark cycle with free access to water and food. 51 Young (3 mo), 30 Middle-aged (12 mo) and 27 Aged (22 mo) Fisher-344 rats (205-500 g/animal) were subjected to a unilateral cortical impact on the surface of the brain utilizing an electronic controlled pneumatic impact device (ECPI) previously described (Baldwin and Scheff, 1996) and performed by Dr. Stephen Scheffs laboratory. Briefly, each animal was anesthetized with 2% isofluorane and placed in a Kopf stereotaxic frame with the incisor bar set at -5. Following a midline incision and retraction of the skin, a 6 mm diameter craniotomy was made approximately midway between bregma and lambda with a Michele hand trephine (Miltex, NY). The skull disk was removed without disturbing the dura. The exposed brain was injured using the ECPI. The impactor rod had a beveled tip with a 5 mm diameter that was used to compress the cortex to a depth of 1.3 mm at 3.5m/sec. Following injury, Surgicel (Johnson & Johnson, TX) was placed over the injury site, the skull disk replaced, and sealed with a thin layer of dental acrylic. The incision was closed with surgical staples and animals maintained at 35 °C--37 °C with a heating pad. Eighteen young (3 month), 6 middle aged (12 month), and 6 aged Fisher-344 rats were subjected to sham surgery following the same procedure except brains were not impacted. For the creatine study, Sprague-Dawley rats were used.

The animals were fed a 1% (N = 8) or 2% (N = 8) creatine diet 2 weeks before surgery. Control animals (N = 8) were fed a regular diet. Animals on the creatine or regular diet were subjected to ECPI following the procedure described above.

2.3 Tissue Collection

Animals were anesthetized using 2% isofluorane and subjected to decapitation and the brains removed. For studies of HNE and acrolein in TBI in aging, twenty-one young, twenty-four middle aged, and twenty-one aged Fisher-344 rats were used. Ten young, ten middle-aged, and eight aged animals were allowed to survive for 24 h. Eleven young, fourteen middle-aged, and thirteen aged animals were euthanized 7 days post injury. To study the effects of TBI and aging on DNA oxidation, young (N = 6), middle-aged (N = 6), and aged (N = 6) Fisher-344 rats were subjected to injury, an additional 6 middle-aged, 6 aged animals were subjected to sham surgery. Injured and sham animals were euthanized 24 h post injury. For mitochondrial proteomics studies, 12 injured young Fisher-344 rats were sacrificed 1 hr post injury, 12 injured and 12 sham young animals sacrificed 24 hr post injury. All animals used in these studies were dissected on ice following decapitation. Ipsilateral (IP) and contralateral (CON) cortex and hippocampus were obtained and then frozen immediately in liquid nitrogen. In addition, four naive rats were anesthetized using isofluorane and brains were extracted as described for injured animals. An additional four naive animals were anesthetized using isofluorane and brains flash-frozen in skull using liquid nitrogen following a midline incision. The animals were stored at -80°C, and cortex and hippocampus were extracted the next day.

For creatine studies, 24 young (3 month) Sprague-Dawley rats were provided a creatine supplementation or regular diet for 2 weeks and were sacrificed at one day after injury. IP cortex and hippocampus and CON cortex and hippocampus were dissected as described above. All the tissue samples were stored at -80°C until used for analysis.

2.4 Method for Estimating Tissue Sparing

In order to examine tissue sparing/loss in TBI in aging, 6 young, 6 middle-aged and 5 aged animals were subjected to injury, overdosed with sodium pentobarbital and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS, and decapitated. Whole brains were removed, postfixed in

4% paraformaldehyde containing 10% sucrose at 4°C overnight and subsequently cryoprotected in a 20% sucrose/4% paraformaldehyde solution for 24 hrs. Coronal 50 μm sections were cut with a cryostat throughout the rostral-caudal extent of the injured cortex extending from the septal area to the posterior aspect of the hippocampus. Sections were stained with cresyl violet and subjected to image analysis (NIH Image v. 1.62). Quantitative assessment of spared tissue employed the Cavalieri method as previously described (Michel and Cruz-Orive, 1988; Sullivan et al., 1999). The amount of tissue sparing was expressed as percentage of the total cortical volume of injured hemisphere compared to the contralateral uninjured hemisphere. In this way each animal is used as its own control. These methods obviate a need to adjust values due to possible differential shrinkage resulting from fixation and tissue processing. A total of twelve evenly spaced sections were used for each animal. All sections were assessed blind with respect to age group.

2.5 Tissue Processing for Enzyme Activity and Oxidative Stress Assays

Tissue samples (cortex, peumbra, and hippocampus) for enzyme and lipid peroxidation assays were homogenized using a chilled Teflon Dounce homogenizer on ice in 1 mL HEPES buffer (pH 7.4), containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, pepstatin A (1 μM), leupeptin (1.2 μM), aprotinin (0.1 μM) and PMSF (0.23 mM). Samples were centrifuged at 100,000 × g for one hour at 4 °C for 1 hour and the supernatant were kept for analyses.

2.6 Protein Assay

Total protein concentrations were determined using the Pierce BCA method (Sigma, St. Louis, MO). In detail, a UV-Vis spectrometer (Labsystem Multiscan MCC/340) was programmed using protein standards with concentrations of 0, 1, 5, 10, 15, and 20 μg/μl. For the protein assay, standards were loaded in duplicate sequentially in the "A" row of 96-well plate, and protein samples were loaded in duplicate in other wells of the same plate. After adding 100 μL BCA reagent (bicinchoninic acid, containing 1% Cu^{2+}), the volume in each well was 200 μL, and the plate was incubated at 37 °C for 20 min. The Cu^{2+} was reduced to Cu^{+}, which coordinates with bicinchoninate to form a complex with purple color that absorbs at 562 nm. The plate was read at wavelength of

562 nm using a multiwell plate reader and the data were converted to protein concentration in the well based on calibration curve from known protein standards.

2.7 Method of Nuclear DNA Isolation

The method of Mecocci et al. (Mecocci et al., 1993) with modification (Wang et al., 2005) was used to isolate nuclear DNA. Briefly, brain samples were homogenized on ice using a Teflon-coated Dounce homogenizer in MSB-Ca^{2+} buffer (pH 7.5), containing 0.21 M mannitol, 0.07 M sucrose, 0.05 M Tris-HCl, 3 mM $CaCl_2$. Na_2EDTA was added to the homogenate with a final concentration of 0.01 M. The homogenate was centrifuged at 600 × g and 4 °C for 5 min, and nuclear pellets collected, rinsed in MSB-Ca^{2+} and repelleted. The pellets were suspended in 700 µl digestion buffer (0.5% sodium dodecyl sulfate, 0.05 M Tris-HCl, 0.1 M Na_2EDTA) with proteinase K (0.5 mg/ml) in a 55 °C water-bath overnight. After digestion, 30 µl 5 M NaCl solution was added, and solution extracted 3 times with phenol (containing 5.5 mM 8-hydroxyquinoline) and chloroform (containing 4% isoamyl) to remove proteins from the digestion mixture. 8-Hydroxyquinoline was used to avoid artificial oxidation as previously described (Wang et al., 2005). Following extraction, the upper aqueous layer was collected and 20 µl NaCl (5 M) and two volumes of cold absolute ethanol added, and the DNA precipitated. Following centrifugation, the DNA was collected, washed three times using 60% ethanol, and dried. The isolated DNA was dissolved in autoclaved water, and purity and concentration measured at 260 nm and 280 nm using a Genesys 10 UV/Vis spectrometer (Rochester, NY, USA).

2.8 Isolation of Mitochondria

A modification of the procedure by Mecocci et al (1993) was used to isolate mitochondria (Mecocci et al., 1993). The cortices or hippocampi from three animals were used to make a pooled sample. Brain samples were homogenized on ice using a Teflon-coated Dounce homogenizer in MSB-Ca^{2+} buffer. The homogenate was centrifuged at 600 × g for 5 min at 4 °C (Beckman Avanti J25 centrifuge), and the supernatant collected. The supernatant was then centrifuged at 20,000 × g for 20 min, and a mitochondrial enriched fraction obtained. The raw mitochondria were suspended

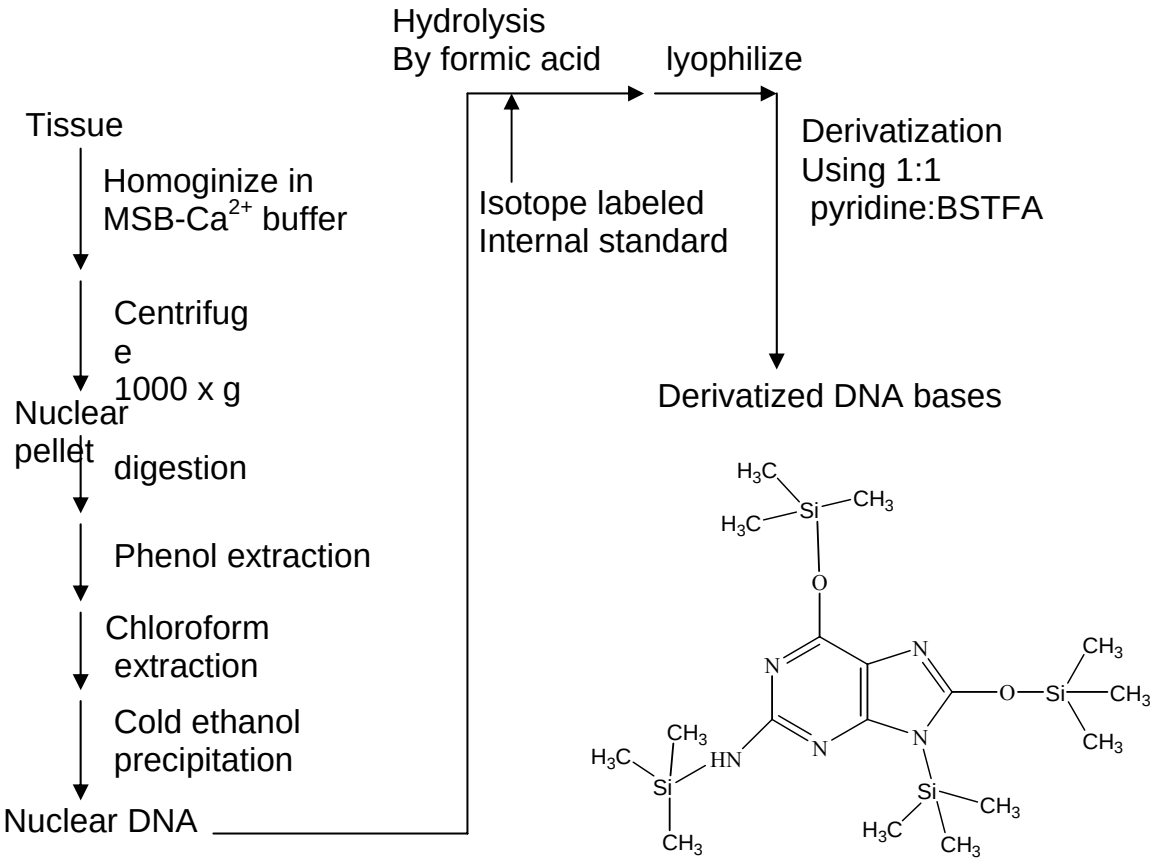


Figure 2.1. Scheme of nuclear DNA isolation and representative molecular structure of derivatized DNA bases.

in 2 mL MSB-Ca^{2+} buffer, loaded on the top of Percoll/ MSB-Ca^{2+} gradient (1:1) in a 10 mL centrifuge tube, and centrifuged at 50,000 × g for 1 h (Beckman optima L-90K ultracentrifuge). Several bands were observed after centrifugation and the light brown band (ρ = 1.035) was collected. This fraction was centrifuged at 10,000 × g at 4 °C to obtain enriched mitochondria. The mitochondrial pellets were washed twice using MSB-Ca^{2+} buffer. The pellets were homogenized in micro Douncer homogenizers on ice using distilled water, and the protein concentration measured using the Pierce BCA method. Four samples (75 μg) for each group were pooled, and aliquoted to 100 μg samples for proteomics.

2.9 4-Hydroxynonenal and Acrolein Assay

Dot-blot analyses of HNE and acrolein modified proteins were carried out using a Schleicher & Schuell Dot-blot apparatus, and the method described by Saiki et al. with modification (Saiki et al., 1986). Briefly, 20 μg of 100,000 × g supernatant protein were loaded in triplicate onto nitrocellulose membrane in a 40-50 μL volume. Vacuum was applied until the solution was evacuated from the wells. After air drying, blots were incubated overnight in 5% dry milk in 0.05% Tween-20/tris buffered saline (TTBS, 0.1 M Tris-HCl, 0.155M NaCl, pH = 7.5) to block nonspecific binding of the primary antibody. Blots were probed with a 1:2000 dilution of anti-HNE (Alpha Diagnostic, San Antonio, TX) or anti-acrolein (United States Biological, Swampscott, MA) polyclonal antibodies for 1 h at room temperature, washed in TTBS 3 × 10 minutes and then incubated with a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. Detection of bound antibodies was achieved by enhanced chemiluminescence (Amersham ECL™ western blotting detection reagents, Amersham Pharmacia Biotech, Piscataway, NJ) and exposure of membrane to Hyperfilm (Film developer, Kodak, 2000). Blots were scanned and measured using Scion Image (NIH). The average density of the triplicate dots was calculated for HNE or acrolein content in an individual sample.

To validate linearity of response of the dot-blots, aliquots of 100,000 × g protein from a representative rat were incubated for 4 h at 37 °C with increasing concentration of HNE (10, 25, 50 100 μM) or acrolein (1.0, 5.0, 10, 25 μM). The acrolein and HNE spiked sample were subjected to dot-blot analyses as described above.

2.10 Enzyme Acyivity Assays

The enzyme activity could reflect the antioxidant capacity of cell, and shows the antioxidant response of cells following injury. In this dissertation, MnSOD, Cu/ZnSOD, GPx, GSSG-R, and GST activities were quantified using UV/Vis spectrometry.

2.10.1 Superoxide Dismutase

The method for assay of total SOD (Cu/Zn- and MnSOD) activity was that of Misra and Fridovich (Misra and Fridovich, 1972) as modified by Mizuno (Mizuno, 1984). Briefly, 70-µl aliquots of the 100,000 × g supernatant were placed in UV-grade cuvettes and 1.46 mL 68mM NaH_2PO_4 containing 1.35 mM EDTA (pH 7.8), 100 µL 4 mM xanthine, 170 µL 3.53 mM epinephrine (pH 11.5) added. Following incubation at 30 ºC for 5 minutes, 10 µL xanthine oxidase was added to the solution and the absorbance followed at 320 nm (Genesys 10 UV, Rochester, NY, USA) for 3 minutes. The blank for the assay contained 70 µL supernatant boiled in water for 5 minutes. For MnSOD, the same protocol was followed with the addition of 200 µL 20 mM KCN. Enzyme activities were expressed as units per microgram protein, where 1 unit reduces the absorbance change by 50%. Cu/Zn activity was obtained by subtracting MnSOD activity from total activity. Activities are expressed as mean ± SD percent of control.

2.10.2 Glutathione Transferase

GST activity was assayed using the method of Ricci et al, which measures the rate of conjugation of 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) with glutathione catalyzed by glutathione transferase (Ricci et al., 1994). The NBD-Cl complex is a stable, yellow compound that strongly absorbs at 419 nm with a molar absorption coefficient of 14.5 $mM^{-1}cm^{-1}$. For the assay, 1.6 mL of 0.1 M sodium acetate buffer (pH 5.0) was mixed with 200 µL 0.2 mM NBD-Cl and 100 µl 0.5 mM reduced glutathione. The reaction was initiated by addition of 100 µL aliquots of the 100,000 × g tissue supernatant. The absorbance was followed for 3 minutes. Enzyme activity was expressed in units/µg total protein, with 1 unit = 1 nmol NBD-Cl complex formed per minute. Results are expressed as mean ± SD percentage of control activity.

2.10.3 Glutathione Peroxidase

For GPx activity, the method of Paglia and Valentine (Paglia and Valentine,

1967) as modified by Mizuno (Mizuno, 1984) was followed, which measures the rate of oxidation of reduced glutathione to oxidized glutathione by H_2O_2 as catalyzed by GSH-Px present in the tissue supernatant. For the assay, 100 µL aliquots of the 100,000 × g supernatant were added to 1.68 mL 68 mM KH_2PO_4 buffer (pH 7.0) containing 1mM EDTA, 100 µL 2 mM NADPH, 10 µL 66 U/mL GSSG reductase, 10 µL 200 mM sodium azide. The assay was initiated with 100 µL 15 mM H_2O_2. The decrease in absorbance was followed for 2 minutes at 340 nm. Quantification was based on a molar absorption coefficient of 6,270 M^{-1} cm^{-1} for NADPH (Braughler et al., 1986). The blank in the enzyme assay consisted of 100 µL supernatant heated at 100 ºC for 5 minutes, and its value was subtracted from the sample value to correct for nonenzymatic oxidation of glutathione. Activity of the enzyme was expressed in units/µg total protein, with 1 unit = 1 nmol NADPH oxidized per minute, and final data for the activity in ipsilateral tissue was expressed as mean ± SD percent of control activity.

2.10.4 Glutathione Reductase Assay

The method of assay for GSSG-R is from Mizuno (Mizuno, 1984) and is based on the reduction of oxidized glutathione by GSSG-R and exogenous NADPH. The assay measures a loss in absorbance at 340 nm as NADPH is converted to $NADP^+$. One-hundred-microliter aliquots of the 100,000 × g supernatant were added to 1.58 mL 68 mM KH_2PO_4 buffer containing 1 mM EDTA with 100 µL 2 mM NADPH. The absorbance was followed at 340 nm for 3 minutes following initiation of the reaction initiation by addition of 220 µL 13.6 mM oxidized glutathione. Enzyme activities were calculated as described for GPx and expressed as mean ± SD percent of control activity.

2.11 DNA Hydrolysis and Derivatization

For GC/MS-SIM, 20 µg DNA was aliquoted into glass vials to which internal standards (1 µg for each) were added, and the samples freeze-dried overnight under vacuum. Acid hydrolysis was performed by heating the DNA and standards in 250 µl 90% formic acid in evacuated and sealed conical glass tubes for 30 min at 140 °C. After lyophilization, 200 µl derivatization reagent (pyridine:BSTFA 1:1 (vol/vol)) added, the tube evacuated, and derivatization carried out at room temperature for 2 h. The

derivatized samples (Figure 2.1) were freeze-dried, and 20 µl BSTFA added immediately before GC/MS injection.

2.12 GC/MS-SIM

Derivatized DNA samples/standards were analyzed on a Hewlett-Packard 5972A mass selective detector interfaced with a Hewlett-Packard 6890 gas chromatograph and equipped with a computer workstation. The injection port and the GC/MS interface were kept at 250 °C. Separation was performed on a fused-silica capillary column (30 m × 0.25mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.25 µm, HP-5MS). Ultra pure helium was used as carrier gas with a constant flow rate of 1.1 ml/min. Derivatized samples (2 µl) were injected directly into the GC injection port using the splitless mode. The initial temperature was held for 2 min after sample injection, and the oven temperature was programmed through the following four ramps: ramp 1, column temperature was increased from 100 °C to 178 °C at a rate of 3 °C /min; ramp 2, temperature was increased from 178 °C to 181 °C at rate 0.3 °C /min; ramp 3, column temperature was raised at a rate of 3 °C /min from 181 °C to 208 °C; ramp 4, rate was 10 °C /min from 208 °C to 280 °C. The final temperature was maintained for 2 min. The total run time was 56.2 min for each sample and the temperature of the mass spectrometer ion source was maintained at 180 °C. Mass spectra were recorded from 9 to 56.2 min. The five bases of interest were well separated at following retention times: 5-hydroxycytosine
(22.0 min), fapyadenine (28.0 min), 8-hydroxyadenine (32.5 min), fapyguanine (39.8 min) and 8-hydroxyguanine (46.0 min) (Figure 2.2).

2.13 Isotope-coded Affinity Tag (ICAT) Labeling Mitochondrial Protein for MS Analysis

In the current study, 100 µg mitochondrial proteins from sham animals and injured animals were labeled with light ($C_{10}H_{17}N_3O_3$) and heavy ($^{13}C_9CH_{17}N_3O_3$) ICAT reagents (ICAT kit, Applied Biosystems, Foster City, CA), respectively as following manufacturer's instructions (Figure 2.3). Briefly, mitochondrial protein from sham animals and the same amount of protein from injured animals were aliquoted, denatured by adding 80 µL denaturing buffer and 2 µL reducing reagent, and heated in a boiling water bath for 10 min. For labeling, proteins from injured animals were mixed with

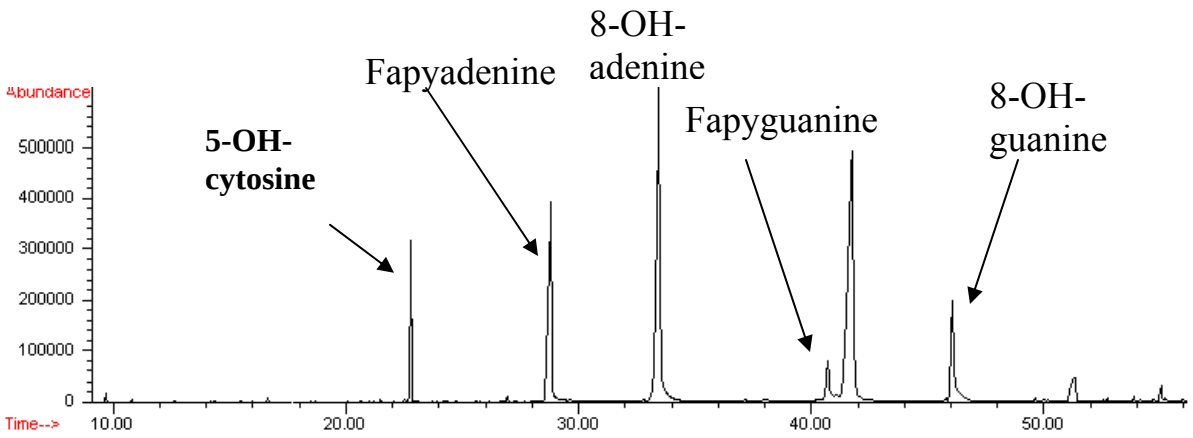


Figure 2.2.Representative chromatogram of DNA oxidized adducts.

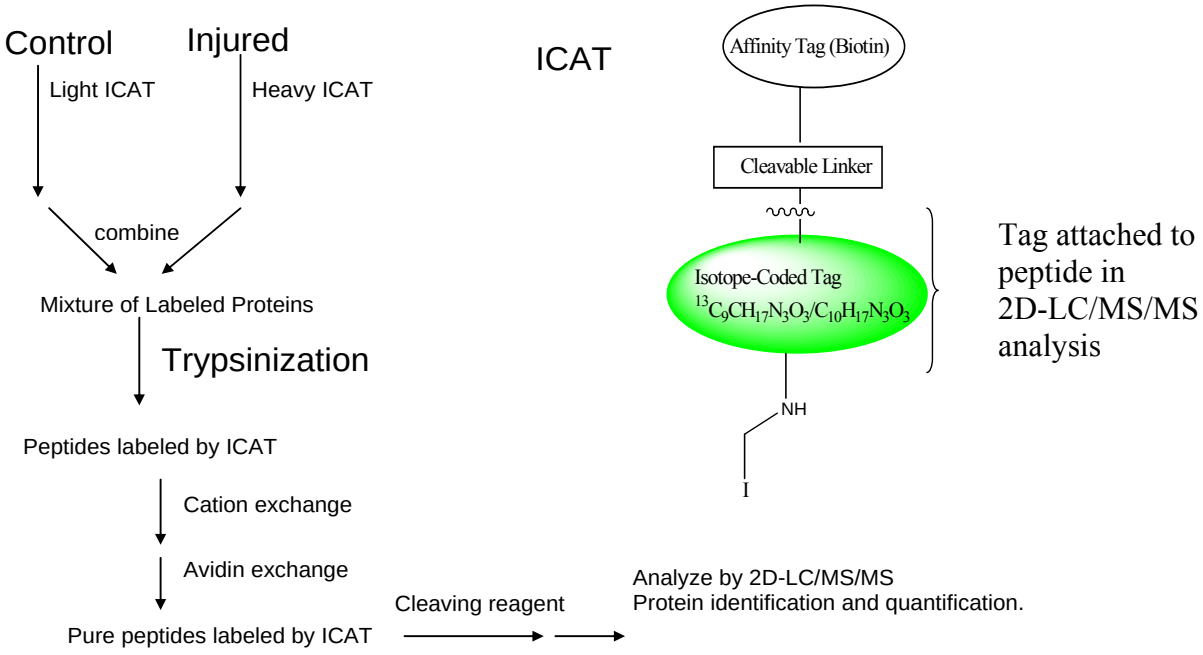


Figure 2.3. Scheme of ICAT labeling.

heavy ICAT reagent, and proteins from control mixed with light ICAT reagent. The labeling was performed in water bath at 37 °C for 2 hours. During labeling, proteins containing cysteine residues react with iodoacetamine group of the ICAT reagent. Proteins from control and injured animals were then combined and digested 16 h at 37 °C by using sequencing grade modified trypsin (Promega, Madison, WI). Excess ICAT reagents, Tris(2-carboxyethyl)phosphine (TCEP) and SDS in sample mixtures were removed using cation exchange. Resulting peptides were added to avidin column that isolated ICAT labeled peptides. The isolated labeled peptides were lyophilized, resuspended in trifluoroacetic acid (TFA) and incubated in a water bath 2 hours at 37 °C to cleave the biotin portion of the ICAT tags. Peptides were dried in a speed vacuum centrifuge and reconstituted in 10 μL 5% ACN/95% aqueous formatic acid (0.1%) and extracted again using C_{18} zip-tips prior to 2-D-LC/MS/MS.

2.14 Analysis of ICAT-labeled Peptide Samples Using 2D-HPLC and Mass Spectrometry

In this study, ICAT lebeled samples were analyzed using 2D-LC/MS/MS (ThermoFinnigan Deca quadruple ion trap mass spectrometer), and peptides were separated using 2-D LC (Peng and Gygi, 2001; Wolters et al., 2001). 2D LC-MS/MS was carried out using a column composed of strong cation exchange (SCX) and reverse phase C_{18} resins and performed by Dr. Lynn in his lab. The peptides were separated based on charge using SCX, and further separated on C_{18} residue based on hydrophobicity. An ammonium acetate gradient from 0 to 250 mM was used for peptide separation in SCX. Thirteen individual chromatographic profiles were obtained for each sample, indicating 13 fractions separated by SCX. Each fraction from SCX separation was subjected to further separation using an acetonitrile gradient from 5% ACN/95% aqueous formic acid (0.1%) to 70% ACN/aqueous 30% formic acid (0.1%) over 80 min (Figure 2.4). Peptides in the column effluent were directly subjected to electrospray ionization, and introduced into a Finnigan LCQ Deca ion trap mass spectrometer for mass analysis. Mass spectra of peptides were saved for later data base searches.

2.15 Protein Identification

In order to identify proteins, acquired tandem mass spectra of peptides were subjected to protein database searches using TurboSEQUEST (ThermoFinnigan). First

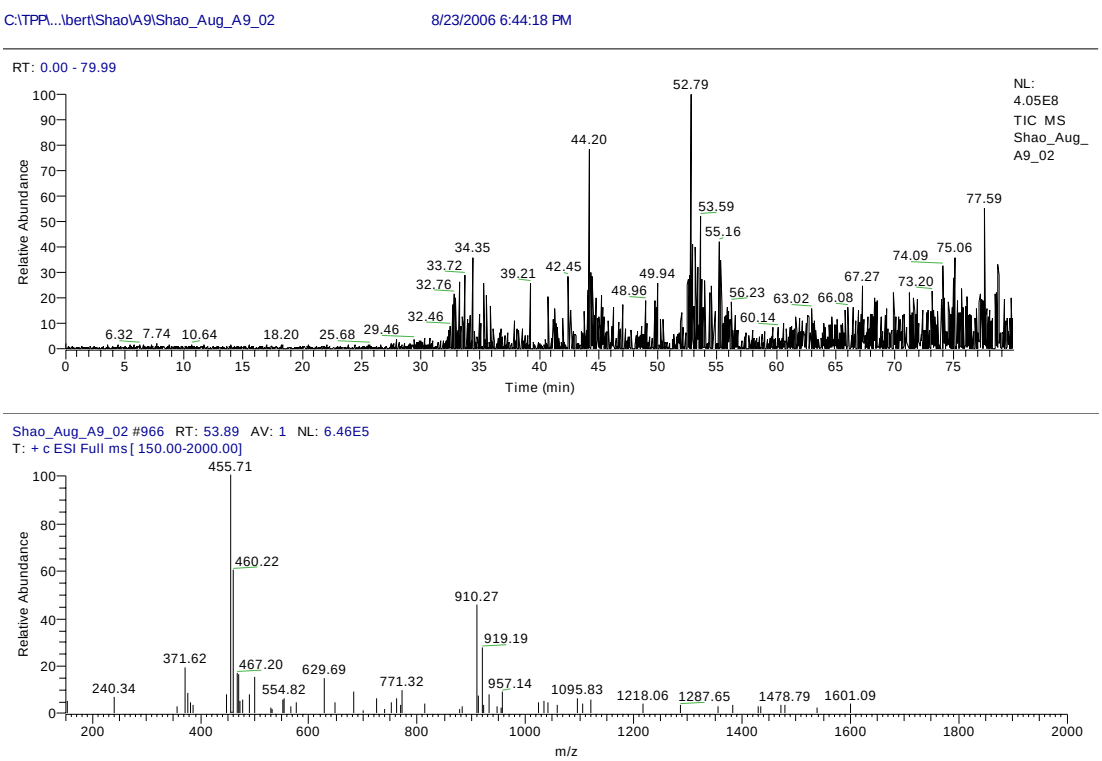


Figure 2.4. Representative chromatogram and MS spectrum of ICAT labeled peptides.

of all, modifications were made to the protein database and the search parameters of TurboSEQUEST. Briefly, a sub-database of the Uniprot SwissProt non-redundant rat/mouse protein database was set up using the Fasta Database Utilities of Bioworks. This sub-database was specified to have only tryptic, cysteine-containing peptides. In addition, search parameters of TurboSEQUEST were edited to expect static modifications of cysteine residues that corresponded to 227.1273 u which is the mass of the light ICAT label or 236.16 u, the mass of expected heavy ICAT modifications. TurboSEQUEST results were filtered by cross-correlation score (Xcorr) criteria, Xcorr > 1.8 for +1 peptides, >2.3 for +2 peptides, and >3.0 for +3 peptides. Proteins identified in database search were scored and the protein with best score was considered the hit in the data base search. Relative ratios of heavy/light (injured/sham) peptides were obtained using the XPRESS program of Bioworks 3.1 (Figure 2.5). ICAT ratios for all tryptic peptides identified from a protein were averaged to obtain the relative ratio of that protein.

2.16 Western Blot

To verify the purity of mitochondria isolated using our extraction protocol, mitochondrial proteins, cytosolic protein samples and nuclear specimens were subjected to SDS-PAGE on a 4-20% gradient gel (Bio-Rad) and transferred to nitrocellulose membrane for Western blot analysis. The blots were incubated 1 h in 5% dry milk in 0.05% Tween-20/Tris buffered saline (TTBS) to block nonspecific binding of the primary antibodies. Blots were incubated with a 1:3000 dilution of anti-VDAC (Calbiochem, 1:3000), anti-Lamin B (1:1000) or anti-MAP-2 (1: 1000) primary antibody for 1 h at room temperature, washed in TTBS 3 × 10 min and then incubated with a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit for 1 h. Detection of bound antibodies was achieved by enhanced chemiluminescence (Amerisham Pharmacia Biotech) and exposure of the membrane to Hyperfilm (Amerisham) (Figure 2.6).

To verify proteomics data, equal amounts of mitochondrial protein (20 μg) from 3 independent samples from IP or CON cortex at each time point were subjected to Western blot and probed using anti-VDAC (Calbiochem, 1:3000) or anti-hexokinase 1 (Chemicon, 1:3000) antibodies. Staining intensity was quantified using Scion Image

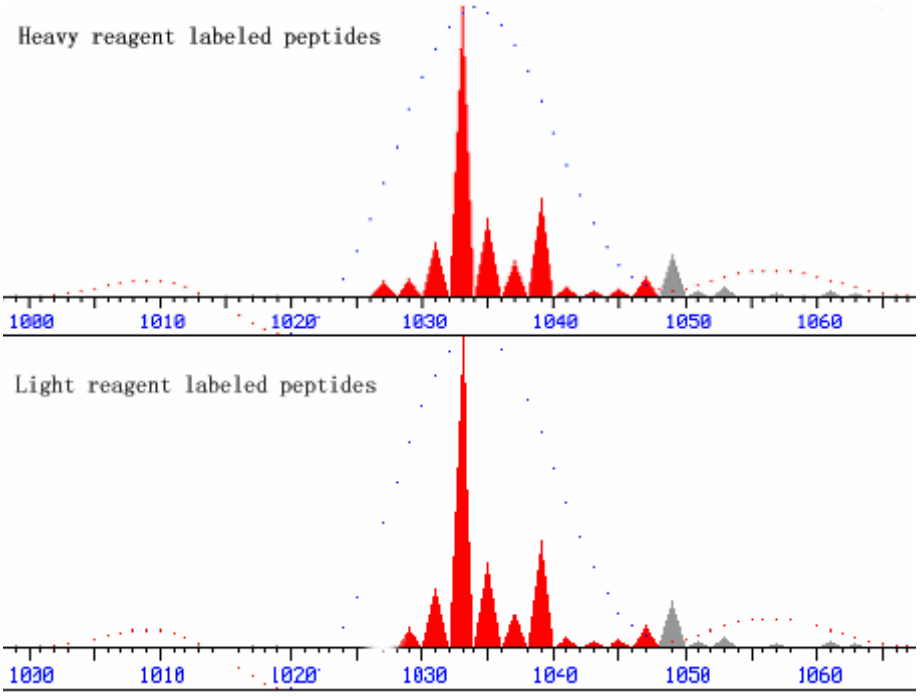


Figure 2.5. Representative chromatogram showing the heavy and light ICAT labeled peptide

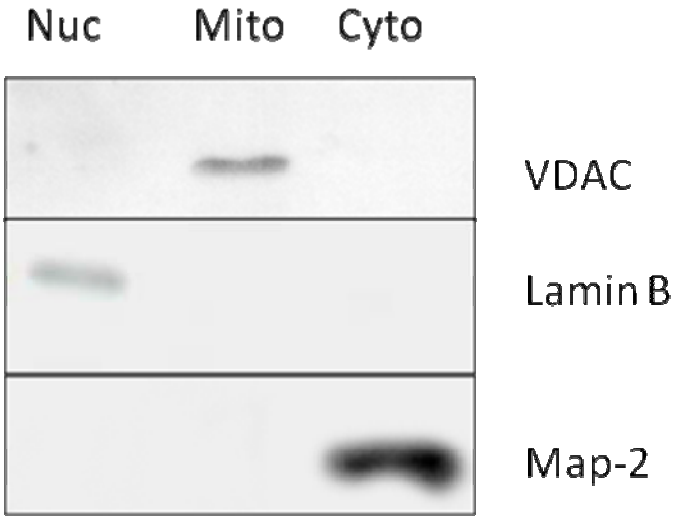


Figure 2.6. Representative Western blots of mitochondrial, cytosolic, and nuclear protein specimens to evaluate purity of fractions. VDAC is a mitochondrial protein marker, Lamin B is a nuclear envelope protein, and MAP-2 is cytosolic protein. Results of the analyses show no cross contamination of fractions.

(NIH). The average band densities from three samples of IP or CON cortex at a time point indicates the protein content in the tissue at that time point.

2.17 Statistical Analyses

A two-tailed Student *t* test was used for time course differences in lipid peroxidation and enzyme activities. A two-tailed Student Paired *t* test was used for IP and CON differences in lipid peroxidation, DNA oxidation, and antioxidant enzyme activities. Age dependent differences in enzyme activities, lipid peroxidation, DNA oxidation, and percent tissue sparing of the injured cortex were compared using one-way ANOVA and the student-Newman-Keuls *post hoc* test for age dependent differences using the commercially available ABSTAT software. Significance was established as $p < 0.05$. One-way ANOVA and student-Newman-Keuls post hoc test was also used for enzyme and lipid peroxidation measures in the creatine study. For ICAT proteomics data, one-sample *t* tests were used to calculate the *p* values for the rejection of the null hypothesis that the mean ratio of the peak areas is 1.0 (signifying no difference in the protein levels between injured animals and sham). Correction for multiple comparisons was done according to the Bonferroni procedure.

Chapter Three
Experimental Results

3.1. Oxidative Damage and Antioxidant Enzyme Activities in TBI in Aging

Oxidative damage is shown to play a key role in neurodegeneration following TBI. In order to quantify oxidative damage in aging, markers of oxidative damage, 4-HNE, acrolein, 8-OHG, FG, 8-OHA, FA, and 5-OHC, were quantified in cortex and hippocampus following TBI. To evaluate antioxidant defense capacity, MnSOD, Cu/ZnSOD, GPx, GSSG-R, and GST activities were measured.

3.1.1 Assessment of Tissue Sparing in TBI in Aging

At 7 days post injury, 6 young, 6 middle-aged and 5 aged rats were assessed for cortical tissue sparing immediately below the site of impact (Figure 3.1). Analysis of variance showed a statistically significant difference among group means [$F(2,14) = 17.36$, $p < 0.001$]. Post-hoc analysis showed significant differences ($p < 0.05$) between young and middle aged, and young and aged groups. Young animals showed $89.4 \pm 4.4\%$ (SD) tissue sparing with middle aged and aged animals showing $77.1 \pm 3.9\%$ (SD) and $77.0 \pm 3.8\%$ (SD), respectively ($p < 0.05$) (Figure 3.2). There were no significant differences in tissue sparing between middle aged and aged animals.

3.1.2 HNE and Acrolein Dot Blot Calibration

To validate antibody response for the dot blot assays, representative protein samples were incubated with HNE or acrolein and subjected to dot blot analysis. Figure 3.3 shows a statistically significant linear response with increasing HNE levels ($r = 0.95$, $p < 0.0001$). Figure 4.4 shows similar results for acrolein ($r = 0.84$, $p = 0.0006$).

3.1.3 Comparison of HNE and Acrolein in Sham, and Naive Animals with and without Flash-freezing Prior to Cervical Dislocation and Tissue Extraction

In order to determine if artifactual ROS are produced during post mortem tissue extraction, HNE and acrolein levels of naive animals with and without *insito* flash-freezing before tissue extraction were evaluated. Our results show no significant differences in HNE or acrolein levels between the animals subjected to different tissue extraction methods (Figure. 3.5). Comparison of HNE and acrolein levels in CON cortex and hippocampus of injured animals to levels in CON and IP tissues of sham,

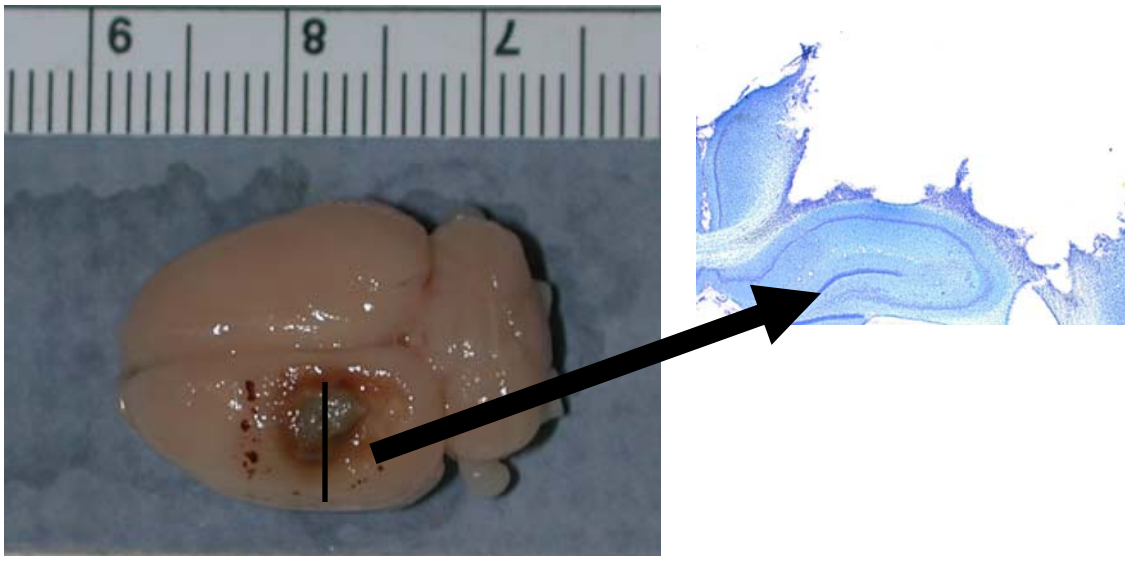


Figure 3.1. Representative rat brain and coronal section at 7 days post injury.

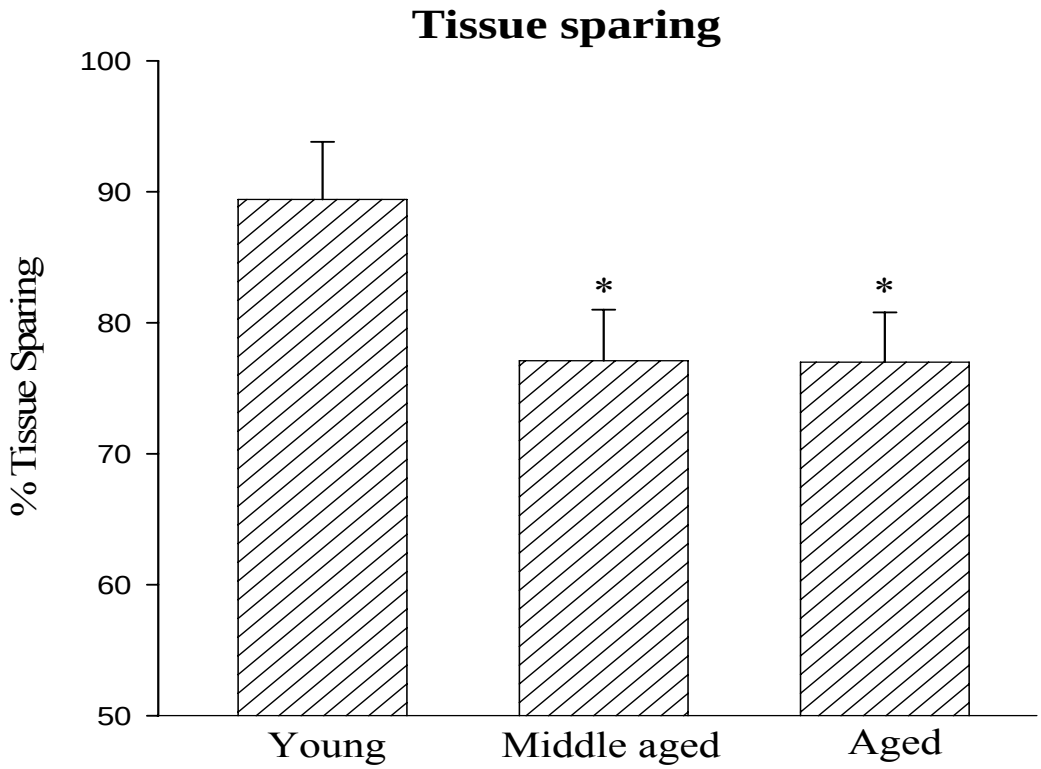


Figure 3.2. Percentage of tissue sparing in Fisher-344 rats subjected to 1.5 mm injury. ANOVA showed a significant difference between the three age groups. Bars represent the mean ± SD. Student-Newman-Keuls, *$p < 0.05$.

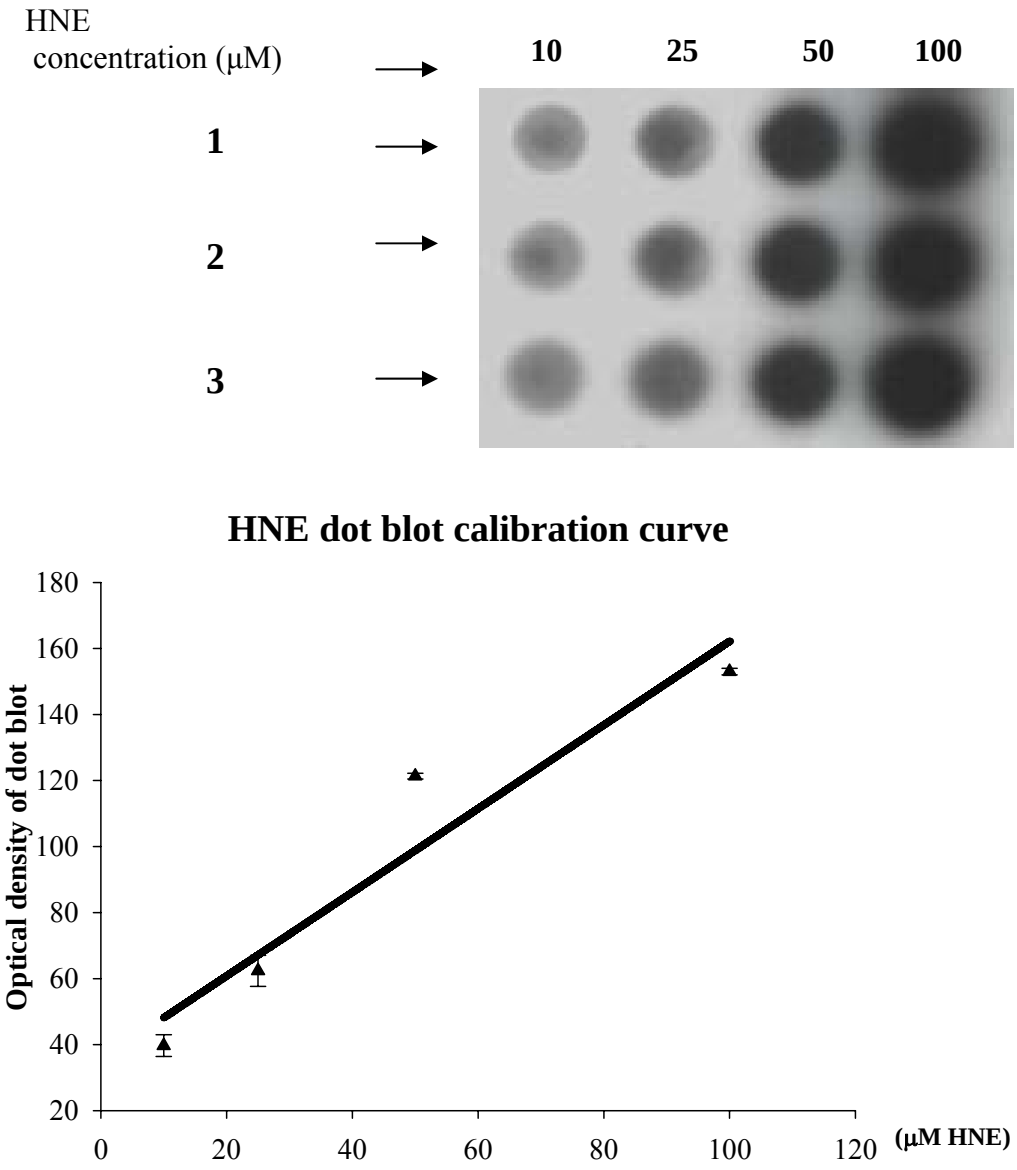


Figure 3.3. Correlation of HNE immunohistochemistry staining of protein samples incubated with increasing concentrations of HNE. Each sample was loaded in triplicate. Calibration curve of HNE concentration vs immunohistochemical staining, r = 0.9532 (bottom). Representative dot-blot with increasing HNE concentrations (top).

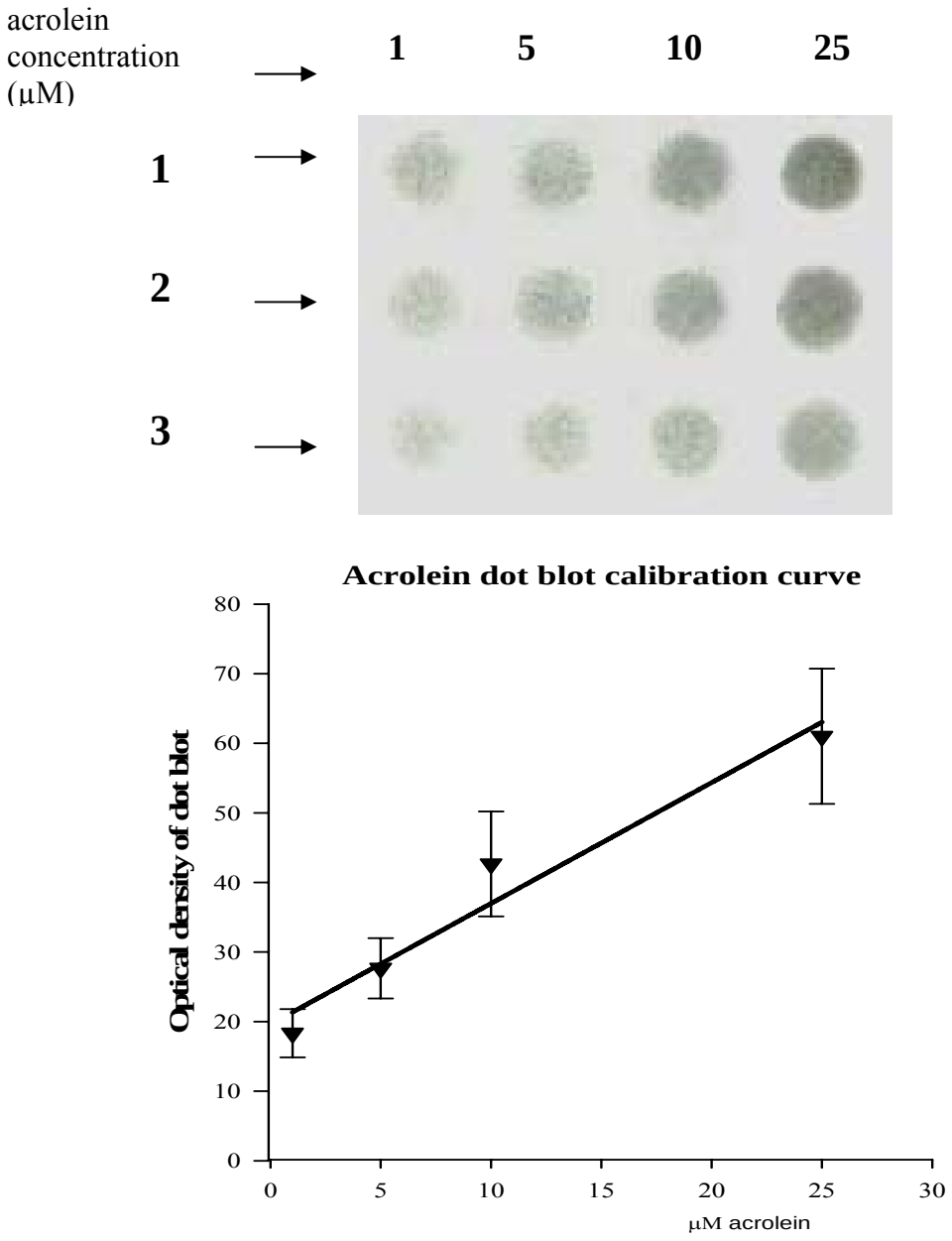


Figure 3.4. Correlation of acrolein immunohistochemical staining of protein samples incubated with increasing concentrations of acrolein. Each sample was loaded in triplicate. Calibration curve of acrolein concentration vs immunohistochemical staining, r = 0.8414 (bottom). Representative dot-blot with increasing acrolein concentrations (top).

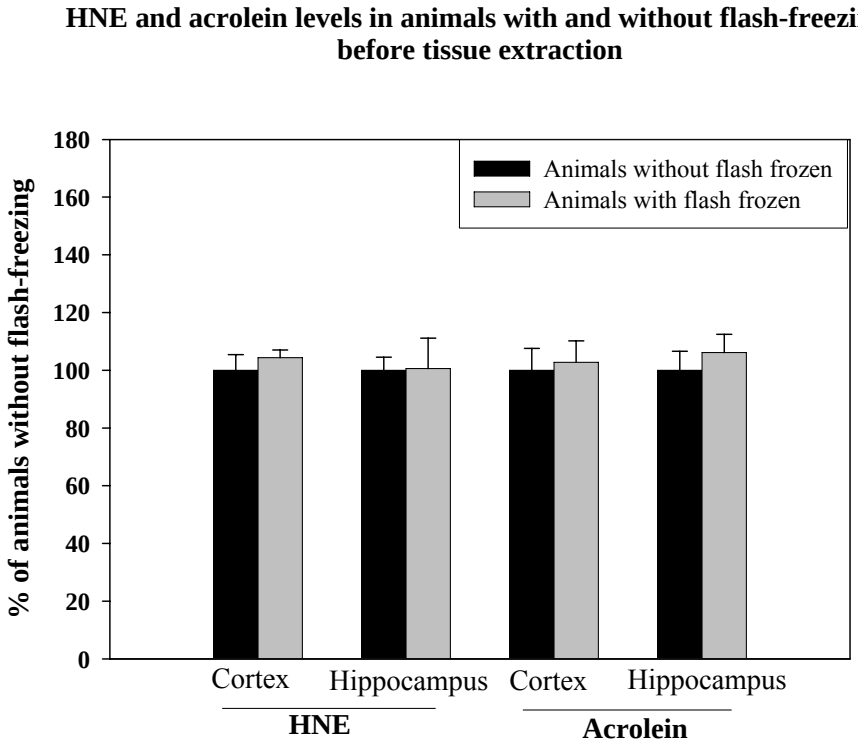


Figure. 3.5. 4-Hydroxynonenal and acrolein levels in cortex and hippocampus of naïve animals with and without flash-freezing before brain extraction. For flash-frozen animals, the brains were frozen using liquid nitrogen following isofluorane anesthetization and midline cranial incision. The frozen animals were stored at -80 °C, and the brain removed the next day. Bars represent mean ± SD of animals without flash-freezing before cervical dislocation.

animals showed no significant differences (Figure. 3.6).

3.1.4 HNE and Acrolein Relative Content in Hippocampus Following TBI in Aging

To evaluate lipid peroxidation in IP and CON hippocampus 1 and 7 days post injury, we measured levels of HNE and acrolein. Because the CCI injury model produces a gradient of secondary injury with no significant changes of antioxidant response in distal areas (DeKosky et al., 2004), we used CON tissue as internal controls to limit animal variability, and focused on oxidative response in IP tissues. The data in IP tissues are expressed as percentage of CON tissues and show HNE levels in IP hippocampus are significantly ($p < 0.01$) increased 24 h post injury in young (230.1 ± 53.4%), middle aged (253.2 ± 45.1%) and aged animals (288.6 ± 46.3%) (Figure 3.7). Statistical analyses showed an age dependent difference [$F(2,27) = 3.2334$, $p < 0.05$], with aged animals showing significantly higher HNE levels than young animals ($p < 0.05$) 24 h post injury. Another lipid peroxidation marker, acrolein, showed a significant increase ($p < 0.05$) in aged IP hippocampus compared with CON hippocampus. ANOVA showed a significant age dependent increase 24 h post injury [$F(2,27) = 3.5055$, $p < 0.05$], although the differences were more modest compared with HNE levels in aging (Figure. 3.7). At 7 days post injury, HNE levels remained elevated in an age dependent manner [$F(2,19) = 9.598$, $p < 0.01$]. HNE levels in IP hippocampus of aged animals were significantly increased compared with aged CON hippocampus (Figure 3.8). For young (104.3 ± 10.3%) and middle aged (138.8 ± 55.6%) animals, the changes in HNE levels in IP hippocampus were not statistically significant compared to CON tissues. No significant changes were observed for acrolein in hippocampus 7 days post injury for any age group (Figure 3.8).

3.1.5 HNE and Acrolein Relative Content in Cortex Following TBI in Aging

In cortex, mechanical injury triggers cell necrosis and apoptosis with concomitant production of ROS and toxic byproducts of lipid peroxidation including HNE and acrolein. In the current study, HNE and acrolein levels in IP and CON cortices were measured, and expressed as percentage of CON cortex. Statistical

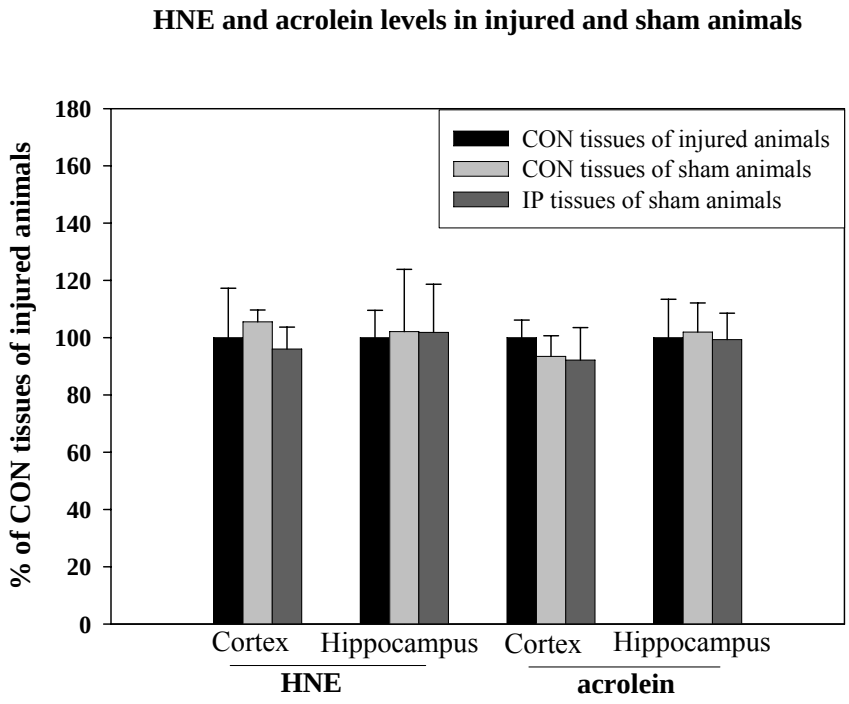


Figure. 3.6. 4-Hydroxynonenal and acrolein levels in CON tissues of injured animals and CON and IP tissues of sham animals. Bars represent mean ± SD of CON tissues of injured animals.

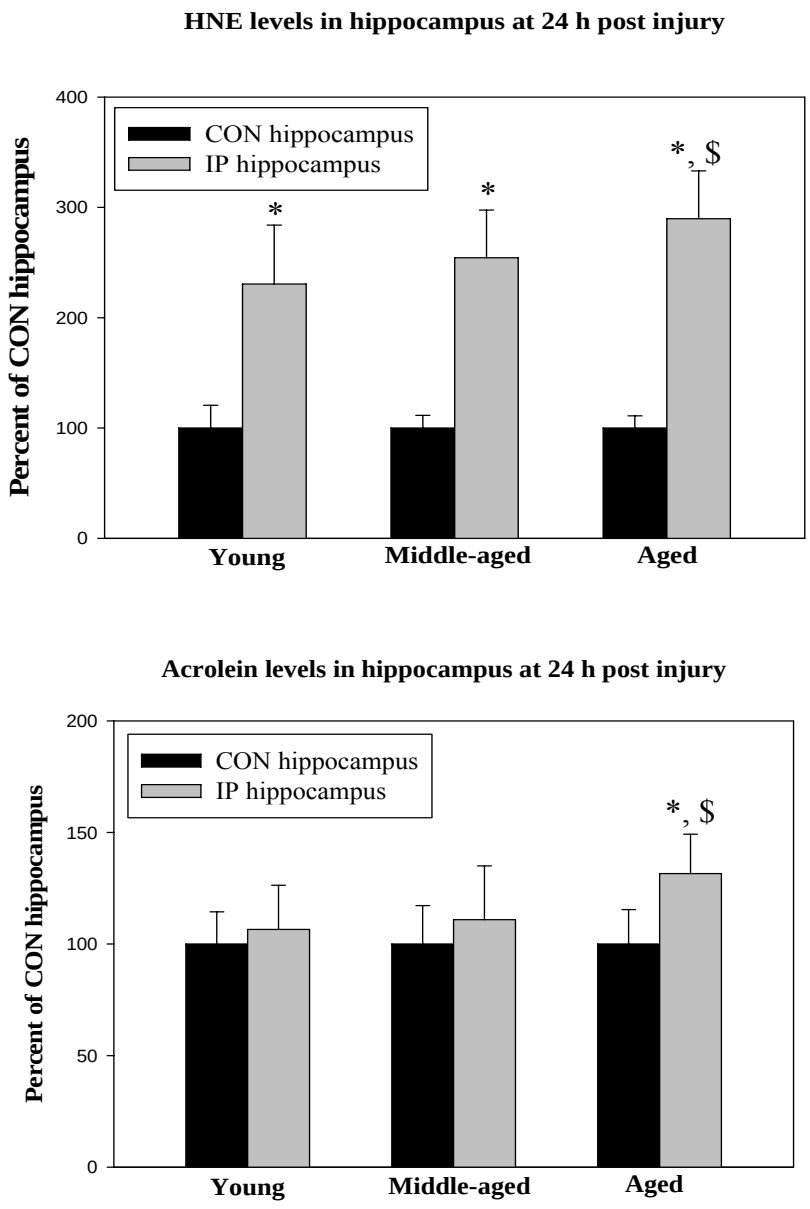


Figure 3.7. HNE and acrolein levels in hippocampus at 24 h post injury. Bars represent the mean ± SD. *$p < 0.01$ compared with CON hippocampus of the corresponding age, $^{\$}p < 0.05$ compared with young animals IP hippocampus.

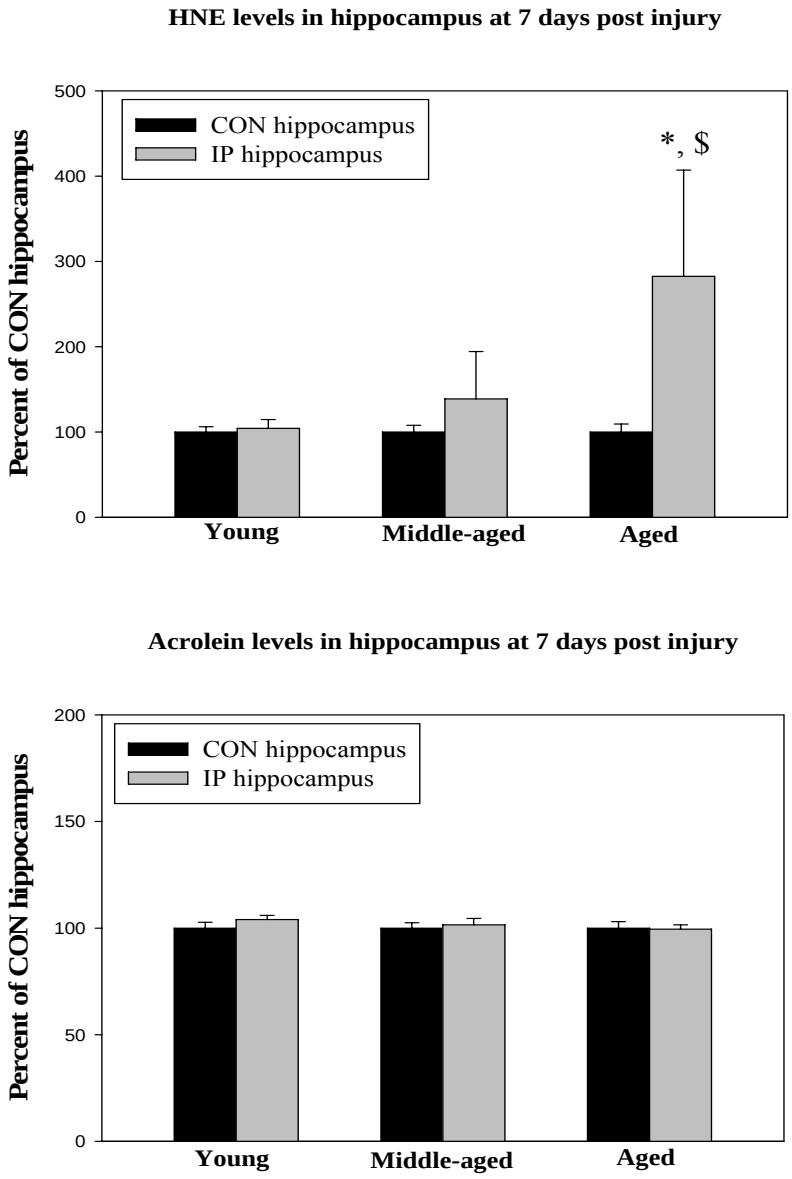


Figure 3.8. HNE and acrolein levels in hippocampus at 7 day post injury. Bars represent the mean ± SD. *$p < 0.01$ compared with CON hippocampus of the corresponding age, $^{\$}p < 0.05$ compared with young animals IP hippocampus.

analysis of levels of HNE modified proteins in IP cortex in young, middle aged, and aged animals 24 h post injury showed HNE levels in IP cortices were significantly ($p < 0.01$) increased compared to CON tissues for each age. No age dependent changes were observed among young, middle-aged, and aged animals (Table 3.1). Acrolein levels in IP cortex did not change significantly in any age group compared with their CON cortices at 24 h post injury. At 7 days post injury, HNE levels increased significantly ($p < 0.01$) for middle aged animals and showed a trend toward an elevation ($p < 0.1$) for aged animals in cortex (Table 3.1). Post *hoc* testing following ANOVA showed HNE levels were significantly higher in middle aged animals compared with young animals [$F(2,17) = 87.037$, $p < 0.001$]. Our data also showed significantly increased acrolein in middle aged and aged animals compared with young animals 7 days post injury (Table 3.1).

3.1.6 DNA Oxidative Damage in TBI in Aging

Using the method of Mecocci et al. (1993) as modified in our laboratory (Wang et al., 2005), we isolated nuclei with high purity. Using stable isotope labeled internal standards, multiple oxidized DNA base adducts were quantified, providing a comprehensive measure of DNA oxidation. Our data show an age dependent increase of 8-hydroxyguanine levels in IP cortex [$F(11,66) = 6.76$, $p < 0.0001$] and hippocampus [$F(11,67) = 5.93$, $p < 0.0001$] of injured animals (Figure 3.9 and 3.10). 8-OHG levels in IP cortex of aged injured animals were significantly ($p < 0.05$) increased compared to young and middle aged animals. In addition, levels of 8-OHG in aged and middle aged IP hippocampus were significantly ($p < 0.05$) higher than young animals. Our data also show 8-hydroxyguanine levels in aged IP cortex of injured animals were significantly ($p < 0.05$) increased compared to CON and IP cortices of aged animals subjected to sham surgery (Figure 3.9). In hippocampus, we observed significantly ($p < 0.05$) higher levels of 8-hydroxyguanine in middle aged and aged IP hippocampus compared to age matched CON hippocampus. 8-hydroxyguanine levels in IP hippocampus of sham middle aged and aged animals were also significantly increased compared to IP hippocampus of young animals (Figure 3.10). FP, 8-OHA, FA and 5-OHC levels (Tables 3.2 and 3.3) were not significantly different in IP hippocampus or cortex among the three age groups, or between injured and sham animals.

Table 3.1. HNE and acrolein levels and antioxidant enzyme activities in cortex in aging.

		24 h post injury		7 days post injury	
		CON	IP	CON	IP
HNE	Young	100 ± 15.4	166.5 ± 17.8†	100 ± 8.0	110.7 ± 16.0
	Middle aged	100 ± 18.8	166.5 ± 29.9†	100 ± 7.8	371.7 ± 28.7*,†
	Aged	100 ± 12.0	160.8 ± 22.7†	100 ± 5.8	148.2 ± 52.9$^{\$}$
acrolein	Young	100 ± 13.0	103.1 ± 19.5	100 ± 3.5	104.7 ± 10.6
	Middle aged	100 ± 10.1	104.4 ± 19.3	100 ± 1.0	179.3 ± 38.9*†
	Aged	100 ± 6.9	115.2 ± 30.0	100 ± 0.5	169.2 ± 72.8$^{*,\#}$
MnSOD	Young	100 ± 41.0	98.7 ± 28.2	100 ± 8.3	73.2 ± 12.3†
	Middle aged	100 ± 30.0	63.5 ± 27.2$^{*,\#}$	100 ± 21.2	61.1 ± 16.5†
	Aged	100 ± 34.6	60.7 ± 20.8*,†	100 ± 26.5	48.4 ± 20.3†
Cu/ZnSOD	Young	100 ± 21.7	71.9 ± 21.1†	100 ± 8.4	77.7 ± 12.7†
	Middle aged	100 ± 22.8	97.7 ± 37.0	100 ± 9.8	63.8 ± 23.7†
	Aged	100 ± 16.2	92.4 ± 22.6	100 ± 30.7	54.1 ± 11.6†
GPx	Young	100 ± 14.9	116.4 ± 35.3	100 ± 19.9	149.2 ± 38.8$^{\#}$
	Middle aged	100 ± 19.8	106.2 ± 30.3	100 ± 46.4	180.7 ± 106.3
	Aged	100 ± 29.2	94.8 ± 40.2	100 ± 19.5	101.8 ± 31.0
GSSG-R	Young	100 ± 12.8	99.8 ± 20.0	100 ± 7.6	93.2 ± 22.5
	Middle aged	100 ± 11.0	81.5 ± 14.1*,†	100 ± 27.3	66.0 ± 33.8
	Aged	100 ± 20.9	82.0 ± 16.6$^{*,\#}$	100 ± 25.2	102.2 ± 29.4
GST	Young	100 ± 15.21	81.5 ± 17.7†	100 ± 4.9	65.3 ± 23.3†
	Middle aged	100 ± 11.6	69.6 ± 20.8†	100 ± 27.3	43.3 ± 18.6†
	Aged	100 ± 20.2	70.5 ± 20.7†	100 ± 25.2	87.7 ± 28.9$^{\$,\#}$

All values are mean ± SD. The value is percentage of that of CON tissue.* $p < 0.05$ compared with activities in young IP tissues at the same time point. $^{\$}$ $p < 0.05$ compared with activities in middle aged IP tissues at the same time point. $^{\#}p < 0.05$, $^{\dagger}p < 0.01$ compared with activities in CON hippocampus of the corresponding age.

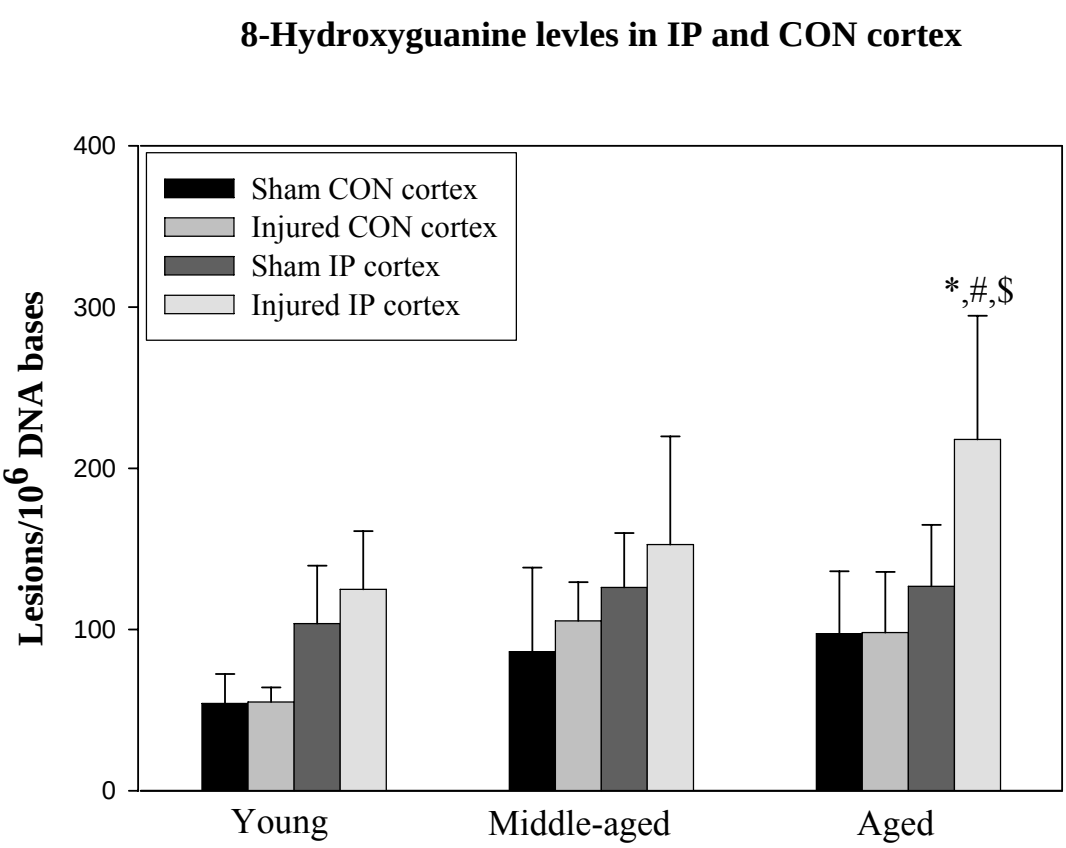


Figure 3.9. 8-hydroxyguanine levels in cortex in TBI in aging. $^{*}p < 0.05$ compared with young and middle aged IP cortex of injured animals; $^{\#}p < 0.05$ compared with CON cortex of injured aged animals; $^{\$}p < 0.05$ compared with aged CON cortex of injured animals. Results are expressed as mean $\pm$ SD.

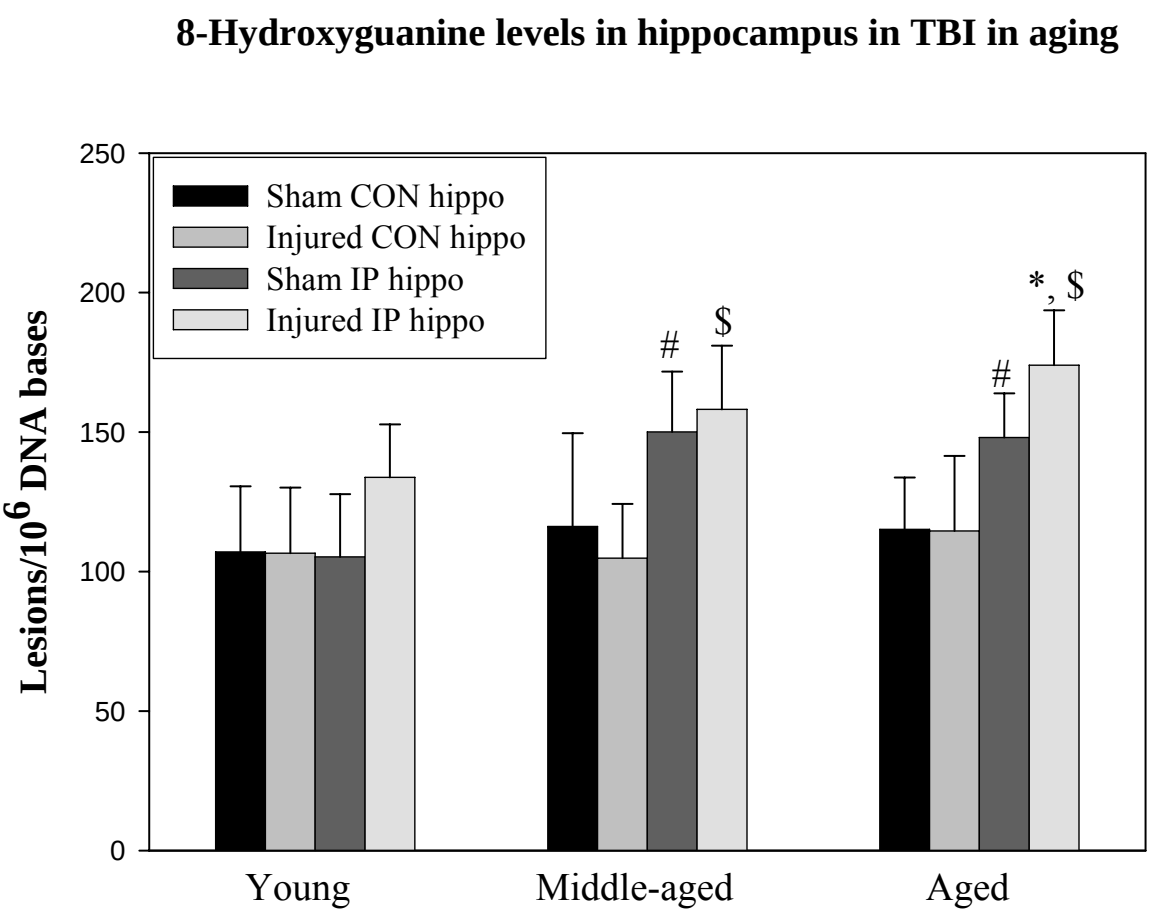


Figure 3.10. 8-hydroxyguanine levels in hippocampus in TBI in aging. $^{*}p < 0.05$ compared with young IP hippo of injured animals, $^{\#}p < 0.05$ compared with young IP hippo of sham animals, $^{\$}p < 0.05$ compared with age matched CON hippo of injured animals. Results are expressed as mean $\pm$ SD.

Table 3.2. DNA oxidation levels in cortex.

			FG	8-OHA	FA	5-OHC
Young animals	CON cortex	sham	105.4 ± 60.0	7.4 ± 3.3	188.2 ± 111.7	17.2 ± 7.3
		injured	120.5 ± 63.2	6.7 ± 3.0	168.0 ± 65.5	13.5 ± 4.3
	IP cortex	sham	154.0 ± 61.2	13.0 ± 4.0	209.0 ± 81.9	23.9 ± 9.7
		injured	160.7 ± 79.8	11.7 ± 4.6	279.0 ± 134.8	23.5 ± 10.4
Middle-aged animals	CON cortex	sham	160.2 ± 39.9	9.5 ± 3.2	232.1 ± 103.4	16.5 ± 3.4
		injured	138.3 ± 75.6	11.5 ± 3.6	167.9 ± 84.6	18.7 ± 9.6
	IP cortex	sham	206.2 ± 82.4	15.0 ± 2.4	213.7 ± 102.6	23.1 ± 5.3
		injured	228.4 ± 91.4	15.4 ± 5.8	299.6 ± 90.2	29.8 ± 9.5
Aged animals	CON cortex	sham	202.8 ± 34.9	11.4 ± 4.0	272.4 ± 78.3	14.9 ± 3.9
		injured	201.0 ± 86.3	11.2 ± 2.6	267.4 ± 91.2	19.3 ± 5.5
	IP cortex	sham	207.8 ± 83.6	16.6 ± 1.5	328.8 ± 141.4	28.9 ± 4.4
		injured	262.3 ± 114.7	16.6 ± 7.1	289.0 ± 89.3	27.8 ± 6.7

Results are expressed as mean ± SD.

Table 3.3. DNA oxidation in hippocampus.

			FG	8-OHA	FA	5-OHC
Young animals	CON hippo	sham	135.0 ± 39.5	7.9 ± 5.0	207.7 ± 104.8	19.1 ± 9.8
		injured	133.5 ± 49.3	10.1 ± 4.6	314.7 ± 216.7	17.3 ± 9.1
	IP hippo	sham	143.2 ± 49.4	10.5 ± 7.6	326.4 ± 52.4	14.3 ± 5.3
		injured	159.7 ± 25.0	12.5 ± 5.0	333.2 ± 114.5	20.3 ± 3.7
Middle-aged animals	CON hippo	sham	162.0 ± 64.0	11.1 ± 5.3	309.4 ± 66.1	22.5 ± 5.2
		injured	161.2 ± 29.2	10.2 ± 3.7	310.8 ± 128.4	21.0 ± 5.6
	IP hippo	sham	197.6 ± 51.3	10.2 ± 3.8	346.5 ± 133.1	24.1 ± 5.1
		injured	242.3 ± 50.8	10.1 ± 3.6	313.0 ± 78.6	24.2 ± 8.0
Aged animals	CON hippo	sham	209.2 ± 60.5	10.1 ± 9.6	338.2 ± 152.3	19.6 ± 6.4
		injured	255.8 ± 50.4	10.6 ± 5.7	341.7 ± 75.0	24.9 ± 7.1
	IP hippo	sham	227.8 ± 64.2	13.3 ± 9.7	347.3± 78.7	23.4 ± 6.3
		injured	308.2 ± 92.6	8.8 ± 2.5	425.2 ± 151.6	30.3 ± 5.8

Results are expressed as mean ± SD.

3.1.7 Antioxidant Enzyme Activities in IP Hippocampus and Cortex in Aging

Measurement of markers of lipid peroxidation showed increased oxidative damage in aging following TBI. To determine if alterations in antioxidant enzyme capacity occurred as a function of age, Cu/Zn and MnSOD, GPx, GSSG-R and GST activities were determined in IP and CON hippocampus and cortex 24 h and 7 days post injury. The data from IP tissues were expressed as a percentage of CON tissues. At 24 h post injury, the hippocampus showed a trend toward decreased MnSOD activity ($p < 0.1$) for aged animals compared to aged CON hippocampus (Table 3.4). ANOVA showed a significant age dependent decrease of MnSOD [$F(2,27) = 4.54$, $p < 0.05$] in IP hippocampus, with a more pronounced loss of MnSOD activity ($p < 0.05$) in aged animals compared with middle aged animals. At 7 days post injury, our data showed MnSOD activities in aged animals decreased significantly ($p < 0.01$) compared with aged CON hippocampus, but not for young and middle-aged animals (Table 3.4). ANOVA [$F(2,20) = 3.5004$, $p < 0.05$] showed an age dependent decrease of MnSOD activity in IP hippocampus, with aged animals showing significantly lower activities compared with young animals. In IP cortex, our data showed MnSOD activity decreased significantly ($p < 0.01$) in middle aged and aged animals compared with their age matched CON cortices and also IP cortices of young animals 24 h post injury [$F(2,25) = 5.9072$, $p < 0.01$] (Table 3.1). At 7 days post injury, young, middle aged and aged animals all showed significant ($p < 0.01$) depletion of MnSOD compared to their corresponding CON cortices, although there were no significant age dependent changes at this time point (Table 3.1).

Analysis of Cu/ZnSOD activities showed no significant changes in IP hippocampus compared to CON tissues at 24 h post injury (Tables 3.1, 3.4). In cortex, Cu/ZnSOD activity deceased significantly in IP cortex compared with CON cortex for young animals at 24 h post injury. At 7 days post injury, Cu/ZnSOD activity decreased significantly in IP cortex compared to CON cortex for each age animals (Table 3.4). However, there were no age dependent changes observed for this enzyme activity at any time point.

Levels of GPx, which reduces oxidative stress by converting H_2O_2 and a variety of organic peroxides to stable alcohols and water, showed a trend of increase in young IP hippocampus 24 h post injury (Table 3.4). Only slight changes were observed in response to injury in middle aged and aged IP hippocampus (Table 3.4).

Table 3.4. Antioxidant enzyme activities in hippocampus in aging.

		24 h post injury		7 days post injury	
		CON	IP	CON	IP
MnSOD	Young	100 ± 21.1	97.7± 19.8	100 ± 19.1	95.8 ± 24.6
	Middle aged	100 ± 24.9	111.8 ± 23.5	100 ± 22.8	66.7 ± 38.9
	Aged	100 ± 26.4	83.9 ± 17.9$^{\$}$	100 ± 22.7	71.2 ± 8.9*†
Cu/ZnSOD	Young	100 ± 18.5	104.3 ± 22.5	100 ± 19.2	95.8 ± 10.1
	Middle aged	100 ± 21.7	115.8 ± 50.5	100 ± 33.4	129.5± 71.6
	Aged	100 ± 17.0	113.4 ± 24.7	100 ± 25.2	105.1 ± 12.1
GPx	Young	100 ± 31.6	169.3 ± 68.9	100 ± 32.0	143.9 ± 88.0
	Middle aged	100 ± 41.0	111.0 ± 39.7*	100 ± 19.2	113.4 ± 13.1
	Aged	100 ± 26.4	99.6 ± 20.0*	100 ± 13.2	107.2 ± 14.0
GSSG-R	Young	100 ± 9.3	111.2 ± 13.6	100 ± 9.4	98.1 ± 24.2
	Middle aged	100 ± 7.6	107.4 ± 8.8	100 ± 8.8	89.3 ± 6.1†
	Aged	100 ± 9.9	108.4 ± 18.0	100 ± 7.6	87.5 ± 8.7†
GST	Young	100 ± 7.3	95.9 ± 8.1	100 ± 12.0	94.3 ± 16.6
	Middle aged	100 ± 10.4	92.8 ± 12.6	100 ± 14.4	84.8 ± 14.6
	Aged	100 ± 17.7	94.1 ± 13.5	100 ± 8.1	87.7 ± 5.7†

All values are mean ± SD. The value is percentage of that of CON tissue. * $p < 0.05$ compared with activities in young IP tissues at the same time point. $^{\$}$ $p < 0.05$ compared with activities in middle aged IP tissues at the same time point. $^{\dagger}p < 0.01$ compared with activities in CON hippocampus of the corresponding age.

Statistical analysis showed GPx activity was significantly lower ($p < 0.05$) in middle aged and aged animals compared to young animals 24 h post injury in IP hippocampus. In IP cortex, GPx activity did not change significantly in any age group 24 h post injury, although levels increased significantly ($p < 0.05$) in young animals 7 days post injury (Table 3.1).

Glutathione transferase activities showed a significant decrease ($p < 0.01$) in IP cortices in young, middle aged and aged animals 24 h post injury compared with their CON cortices. At 7 days post injury, GST activities in young and middle aged IP cortices were also decreased significantly ($p < 0.01$) compared to CON cortices. ANOVA showed GST activity was significantly lower in middle aged animals compared with aged animals at this time point (Table 3.1). In IP hippocampus, GST activities decreased significantly ($p < 0.01$) in aged animals compared with their corresponding CON hippocampi at 7 days post injury. However, no age dependent changes were observed at 24 h or 7 days post injury (Table 3.4).

Another antioxidant enzyme, GSSG-R, which functions to reduce oxidized GSH to reduced GSH for participation in the GSH reaction, was significantly depleted in IP cortex ($p < 0.01$) of middle aged and aged animals compared to their CON cortices 24 h post injury. ANOVA showed GSSG-R activities were significantly ($p < 0.05$) lower in middle aged and aged animals compared with young animals at this time point (Table 3.1). In IP hippocampus, GSSG-R activity did not change significantly compared to CON hippocampus for any age group 24 h post injury. At 7 days post injury, GSSG-R activity decreased significantly ($p < 0.01$) in middle aged and aged animals compared with their corresponding CON hippocampus. Our data showed no age dependent differences at either time point in IP hippocampus (Table 3.4).

3.2 Protection of Creatine in TBI

Considerable research and effort has been directed toward finding efficient therapeutics that may reduce secondary injury, including oxidative damage. Although no breakthrough was emerged to date, some drug candidates have recently arisen. Creatine has been shown to be neuroprotective in other neurodegenerative diseases, although there has been limited study in TBI. In the current study, we evaluated creatine on oxidative damage in TBI.

3.2.1 HNE and Acrolein Relative Content in Hippocampus of Young Animals with Creatine Supplementation

The hippocampus is subject to mild mechanical injury in TBI, and experiences delayed secondary injury, including oxidative damage, which could trigger or accelerate neuron death in this tissue and result in memory deficits. Our previous study showed extensive oxidative stress in IP cortex and hippocampus 24 h post injury in animals following TBI. Creatine has been shown to ameliorate oxidative damage in TBI. To determine the effects of creatine on lipid peroxidation and antioxidant capacities, we measured oxidative damage using markers of lipid peroxidation at 24 h post injury in animals provided a creatine supplemented diet. Our data show HNE levels in IP hippocampus of animals treated with creatine decreases significantly ($p < 0.05$) compared to animals provided a control diet [$F(2,23) = 4.3897$, $p < 0.05$] (Figure. 3.11). Another lipid peroxidation marker, acrolein, showed the same trend in the three groups although it was not statistically significant (Figure. 3.11). No significant changes were observed for HNE or acrolein in IP hippocampi from animals treated with 1% and 2% creatine. For HNE and acrolein levels in CON hippocampus, there were no significant differences between creatine treated and control animals

3.2.2 HNE and Acrolein Relative Content in Cortex of Young Animals provided Creatine Supplementation

In cortex, mechanical injury triggers cell necrosis and apoptosis, with production of ROS and toxic byproducts of lipid peroxidation, including HNE and acrolein. Analysis of levels of HNE modified proteins in IP cortex showed that HNE protein adducts decreased significantly ($p < 0.05$) in IP cortex in animals provided 1% creatine supplemented diet compared to animals on a control diet [$F(2,23) = 4.2013$, $p < 0.05$] (Figure. 3.12). Similarly, acrolein decreased as a trend in animals provided creatine diets. In CON cortex there were no significant changes in HNE or acrolein levels between control and creatine treated animals.

3.2.3 Antioxidant Enzyme Activities in Hippocampus and Cortex of Young Animals with Creatine Supplementation

Measurement of markers of lipid peroxidation showed increased oxidative stress in TBI. Creatine has shown protective effects in a variety of neurodegenerative disease,

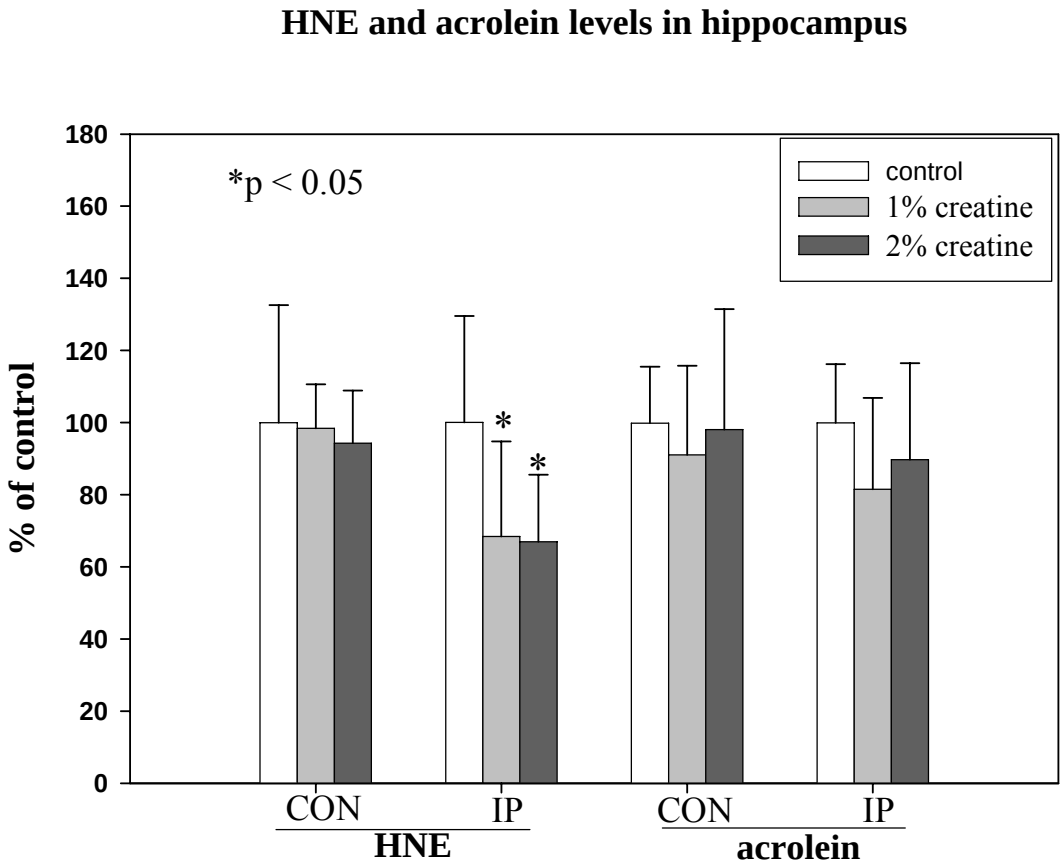


Figure 3.11. 4-Hydroxynonenal and acrolein levels in hippocampus following TBI. Bars represent mean ± SD of control. $p^* < 0.05$ compared with control animals with regular diet.

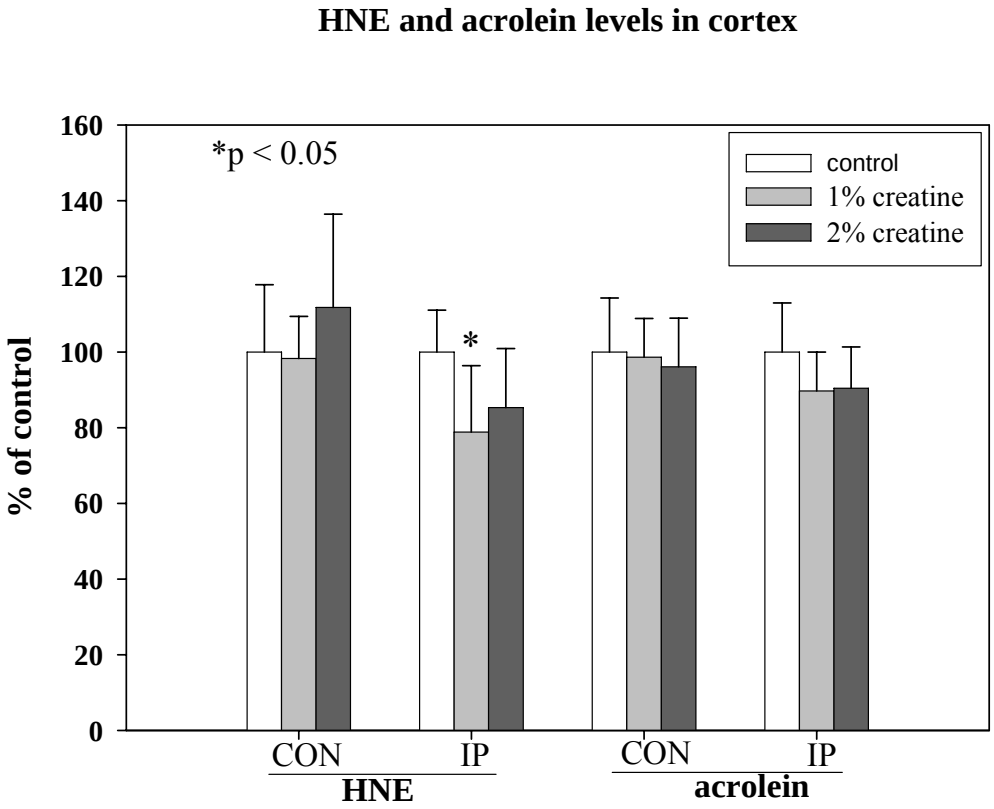


Figure 3.12. 4-Hydroxynonenal and acrolein levels in hippocampus following TBI. Bars represent mean ± SD of control.

*p < 0.05 compared with control animals with regular diet.

including TBI. Our data showed reduced lipid peroxidation in animals on a creatine diet 24 h post injury. To determine if protective effects of creatine supplementation in TBI are due to enhanced antioxidant capacities, activities of Cu/Zn and Mn SODs, GPx, GSSG-R and GST were determined in IP and CON hippocampus and cortex 24 h post injury. Our data show increased MnSOD activity [$F(2,23) = 4.0551$, $p < 0.05$] in IP hippocampus of animals with both 1% and 2% creatine diets compared to controls (Table 3.5). In CON hippocampus, MnSOD activity increased in 1% and 2% creatine treated animals, but did not reach statistical significance. In IP and CON cortex, our data showed creatine supplementation did not significantly improve MnSOD activity (Table 3.6).

Our data showed Cu/ZnSOD activities increased significantly ($p < 0.05$) in IP hippocampus of 1% and 2% creatine treated animals relative to controls [$F(2,23) = 3.9727$, $p < 0.05$] (Table 3.5). In IP cortex, Cu/ZnSOD activity also increased significantly in creatine treated animals compared to controls (Table 4.6). No significant differences in Cu/ZnSOD were observed in CON tissues between creatine treated and control animals (Tables 3.5 and 3.6).

Levels of GPx, which reduces oxidative stress by converting H_2O_2 and a variety of organic peroxides to corresponding stable alcohols and water, showed
significant decreases in IP hippocampus of animals treated with 1% creatine but not 2% (Table 3.5). In IP cortex, GPx also decreased in creatine treated animals compared to controls (Table 3.6).

Glutathione transferase activities showed a significant increase ($p <0.05$) in IP hippocampus in 2% creatine treated animals compared with controls [$F(2,23) = 7.5320$, $p < 0.01$] (Table 3.5). In both IP and CON cortex and CON hippocampus, there were no significant changes in GST activity between control and creatine treated animals (Tables 3.5 and 3.6).

Another antioxidant enzyme, GSSG-R which functions to reduce oxidized GSH to reduced GSH as the substrate for the GPx reaction, was significantly ($p < 0.05$) increased in IP cortex of 1% and 2% creatine treated animals relative to controls (Table 3.6). There were no significant changes in IP or CON hippocampus or CON cortex (Tables 3.5 and 3.6).

Table 3.5. Antioxidant enzyme activities in hippocampus.

IP hippo	MnSOD	Cu/ZnSOD	GPx	GSSG-R	GST
control	100.0±25.9	100.0±17.9	100.0±17.3	100.0±11.3	100.0±5.9
1% creatine	126.8±28.1*	122.8±18.5*	66.3±11.3*	103.2±12.3	106.5±16.2
2% creatine	127.1±19.7*	121.7±18.2*	86.1±24.3	95.7±11.2	121.0±8.3*
CON hippo					
control	100.0±34.0	100.0±14.0	100.0±16.4	100.0±10.7	100.0±15.2
1% creatine	118.6±19.1	115.3±17.0	98.7±18.8	105.4±22.9	95.7±13.7
2% creatine	130.6±18.5	109.8±9.0	108.7±31.0	97.2±11.0	105.6±15.3

$p^* < 0.05$ compared with control.

Data were expressed as percent of control, mean ± SD

Table 3.6. Antioxidant enzyme activities in cortex.

IP cortex	MnSOD	Cu/ZnSOD	GPx	GSSG-R	GST
control	100.0±11.0	100.0±15.3	100.0±7.3	100.0±10.3	100.0±9.0
1% creatine	101.3±27.9	117.2±12.1*	71.8±8.3*	120.2±21.3*	103.6±23.9
2% creatine	112.6±29.1	126.1±17.0*	71.8±15.1*	122.1±17.9*	102.4±22.5
CON cortex					
control	100.0±12.9	100.0±16.4	100.0±22.4	100.0±18.4	100.0±18.1
1% creatine	103.2±18.5	104.7±11.3	108.1±13.1	100.6±16.1	96.6±14.5
2% creatine	110.3±14.5	96.7±7.7	118.8±57.1	94.7±14.6	103.6±13.3

$p^* < 0.05$ compared with control.
Data were expressed as percent of control, mean ± SD

3.3 Mitochondrial Proteomics

Mitochondria function as a signaling center, calcium sink and major ROS source in the cell, and previous study showed compromised respiration chain and calcium sequestion capacity following TBI. The current study shows increased oxidative damage and decreased antioxidant enzyme activities, especially MnSOD, suggesting there could be significant changes in mitochondria following TBI. In order to uncover the changes in mitochondria, mitochondrial protein levels were evaluated.

Isotope-coded affinity tag (ICAT) proteomics is a very efficient method to compare protein levels in two different samples. Although most proteomic studies use 2D gel electrophoresis, the amount of protein necessary for analysis limits its usefulness when only small amounts of protein are available as observed for mitochondria. To analyze differences in mitochondrial protein levels the use of ICAT proteomics is the most appropriate choice because it only requires 100 μg proteins from each sample for analysis. In the current study, using ICAT labeling 2D LC-MS/MS, mitochondrial proteins isolated from cortex and hippocampus at 1 h or 24 h post injury were quantified to examine individual protein changes.

3.3.1 Evaluation of ICAT Labeling for 2D LC-MS/MS

In this study, ICAT labeling and 2D LC-MS/MS was first evaluated using two aliquots of mitochondrial sample from the same pool for heavy and light ICAT reagent labeling and MS quantification. Our analyses identified 60 unique proteins of which 63.3% identified with multiple unique peptides. Most of these proteins (73.3%) were mitochondrial based on SwissProt database, which suggest mitochondria were enriched by Percoll gradient centrifugation. The mean of heavy to light ratios of all the mitochondrial proteins identified from the experiment was 1.06 ± 0.24, showing that the ICAT method is reliable to quantify protein expression changes (Table 3.7). However, it indicates variations in the methods.

3.3.2 Proteomic Analysis of Differentially Expressed Mitochondrial Proteins in Cortices 1 h and 24 h Post Injury

In this study, enriched mitochondria were isolated from IP or CON cortex of sham and injured animals 1 and 24 h post injury, and protein expression evaluated using ICAT labeling and 2D LC-MS/MS. Tissues were pooled and mitochondria isolated

Table 3.7. Protein identified from one experiment using same sample for heavy and light labeling.

Protein Name	SwissProt	Xpress	StDev	NumPep
Sodium/potassium-transporting ATPase alpha-3 chain	(P06687)	0.78		1
Aspartate aminotransferase	(P05202)	0.93	0.26	5
Acyl coenzyme A thioester hydrolase 2	(Q9R0X4)	0.79		1
Acyl-CoA dehydrogenase family member 9	(Q8JZN5)	0.71	0.66	2
ADP,ATP carrier protein, heart/skeletal muscle isoform T1	(P48962)	1.20	0.18	3
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	(P11507)	1.02		1
Sodium/potassium-transporting ATPase beta-1 chain	(P14094)	0.75		1
ATP synthase alpha chain	(Q03265)	0.88	0.22	2
ATP synthase gamma chain	(Q91VR2)	0.91	0.16	10
ATP synthase D chain	(P31399)	1.39	0.54	4
60 kDa heat shock protein	(P19226)	0.82	0.00	2
Cytochrome c oxidase polypeptide Vb	(P19536)	1.14		1
Cytochrome c oxidase polypeptide VIb	(P56391)	1.74	0.85	4
Calreticulin precursor	(P14211)	0.80		1
Cytochrome c1, heme protein	(Q9D0M3)	0.99	0.16	8
Glutamate dehydrogenase	(P26443)	0.77	0.20	8
Dihydroperidine reductase	(P11348)	0.87	0.01	2
4-aminobutyrate aminotransferase	(P61922)	0.95	0.26	7
Guanine nucleotide-binding protein	(P54311)	1.06		1
Glutaminase, kidney isoform	(P13264)	0.97	0.37	8
Glycerol-3-phosphate dehydrogenase	(P35571)	1.04		1
Hemoglobin beta chain, major-form	(P02091)	1.01		1
Hexokinase, type I	(P17710)	0.94	0.21	8
Isocitrate dehydrogenase [NAD] subunit gamma	(P70404)	1.04	0.24	4
cAMP-dependent protein kinase type II-alpha chain	(P12367)	1.04		1
Creatine kinase, B chain	(Q04447)	1.16		1
Creatine kinase, ubiquitous mitochondrial	(P30275)	1.15	0.18	2
Hematopoietic stem/progenitor cells protein MDS029 homolog	(Q91WS0)	1.40	0.42	3
Malate dehydrogenase	(P08249)	1.20	0.40	20
NADH-ubiquinone oxidoreductase B18 subunit	(Q9CR61)	0.86	0.28	3
NADH-ubiquinone oxidoreductase PDSW subunit	(Q9DCS9)	1.14	0.39	3
NADH-ubiquinone oxidoreductase 15 kDa	(Q99LY9)	1.21	0.16	2

subunit				
NADH-ubiquinone oxidoreductase 75 kDa subunit	(Q91VD9)	0.96	0.16	4
NADH-ubiquinone oxidoreductase 24 kDa subunit	(Q9D6J6)	1.25	-	2
NADH-ubiquinone oxidoreductase 19 kDa subunit	(Q9DCJ5)	1.01	0.24	5
2-Oxoglutarate dehydrogenase E1 component	(Q60597)	0.96	0.25	5
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase	(P08461)	2.08		1
Pyruvate dehydrogenase E1 component alpha subunit	(P35486)	1.54	0.23	4
Pyruvate dehydrogenase E1 component beta subunit	(Q9D051)	0.87	0.17	3
Mitochondrial precursor proteins import receptor	(Q9CZW5)	1.14		1
Dynamin-like 120 kDa protein	(P58281)	1.10		1
Proliferation-associated protein 2G4	(P50580)	1.72		1
Voltage-dependent anion-selective channel protein 1	(Q60932)	1.25	0.33	2
Voltage-dependent anion-selective channel protein 3	(Q9R1Z0)	1.02	0.21	10
Peptidyl-prolyl cis-trans isomerase A	(P10111)	1.35		1
Pyruvate carboxylase	(Q05920)	0.78	0.04	2
Retinoblastoma-like protein 1	(Q64701)	0.78		1
60S ribosomal protein L18a	(P62717)	1.20	0.00	2
40S ribosomal protein S11	(P62281)	1.30		1
Sulfated glycoprotein 1 precursor	(Q61207)	1.16		1
Succinyl-CoA ligase [ADP-forming] beta-chain	(Q9Z2I9)	0.97	0.25	3
Suppressor of cytokine signaling 4	(Q9JLY0)	1.19		1
Succinate semialdehyde dehydrogenase	(P51650)	0.61		1
Synaptophysin	(Q62277)	0.65		1
Acetyl-CoA acetyltransferase	(P17764)	1.60	0.21	2
Thiosulfate sulfurtransferase	(P52196)	1.57	0.49	2
Ubiquinol-cytochrome-c reductase complex core protein 2	(Q9DB77)	1.20	0.26	8
Ubiquinol-cytochrome C reductase complex 11 kDa protein	(P99028)	1.34	0.21	3
Hypothetical protein C030036P15Rik	(Q8BQM4)	1.30	0.16	3

as described labeling and 2D LC-MS/MS. Tissues were pooled and mitochondria isolated as described in methods and materials, and pooled samples were run triplicate to look at proteins with prominent changes (Figure 3.13) and reduce variations exist in the current method (Yi et al., 2002; Zhang et al., 2005). The results were combined with proteins identified in more than two independent experiments for each pool sample, and the proteins identified in each pool were combined. A total of 195 proteins were identified in this study using quantatitive proteomics, and 37 proteins were identified at each time point in both IP and CON tissues (Table 3.8). Fourteen proteins were identified with significant changes in at least either IP or CON tissues, or either time point post injury (Tables 3.9, 3.10). Major protein classes include those related to oxidative phosphorylation, the electron transport chain, and carrier proteins.

Several proteins involved in the respiratory chain were identified in our study. In complex III, cytochrome c1 was significantly increased (36%, p = 0.0006) 1 h post injury in CON cortex, but reduced (27%, p = 0.000) 24 h post injury. Ubiquinol-cytochrome-c reductase complex core protein 2 was reduced (31%) as a trend (p = 0.0366) 24 h post injury in IP cortex, and slightly but statistically significantly (13%, p = 0.0014) decreased in CON cortex at this time point. At 1 h post injury, this complex III core protein increased significantly (29%, p = 0.000) in CON cortex. Our data show that cytochrome c oxidase polypeptide Vb (cox5b), a component of complex IV, decreased significantly in both IP (32%, p = 0.000) and CON (26%, p = 0.000) cortex 24 h post injury. At 1 h post injury, cox5b did not change significantly in either IP or CON cortex. Cytochrome c oxidase polypeptide VIb (cox6b1), another complex IV subunit, decreased significantly 24 h post injury in IP (24%, p = 0.000) cortex.

Analyses of proteins in the TCA cycle show that 2-oxoglutarate dehydrogenase E1 component decreased significantly (25%, p = 0.000) in IP cortex 24 h post injury, and increased (41%, p = 0.0244) as a trend in CON cortex at 1 h post injury. Dihydrolipoyl (dihydrolipoamide) dehydrogenase, a component of the glycine cleavage system as well as of the alpha-ketoacid dehydrogenase complexes, decreased (19%) significantly (p = 0.000) in IP cortex 24 h post injury, and increased (16%, p = 0.0052) as a trend in CON cortex 1 h post injury.

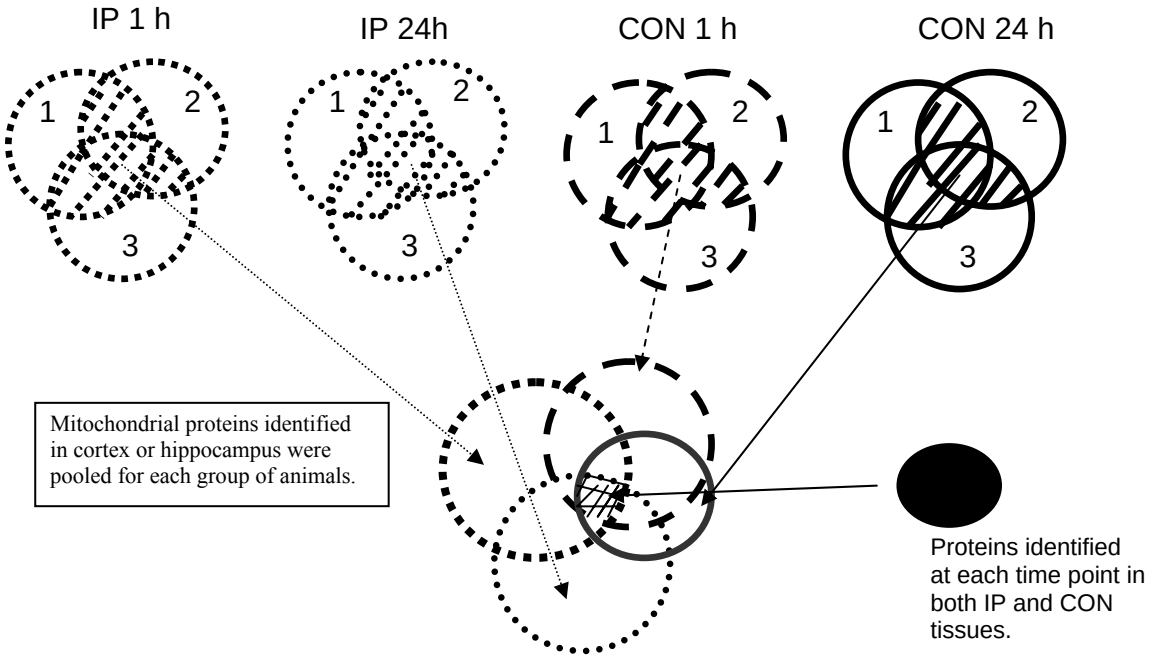


Figure 3.13. Protein organization.

Table 3.8. Proteins identified in both IP and CON cortex at 1 and 24 hour post injury.

Common name	SwissProt	SwissProt annotated location	biological process
Aspartate aminotransferase	P00507	Mitochondrial matrix	amino acid metabolism
ADP,ATP carrier protein	P48962	Mitochondrial inner membrane	carrier
ATP synthase alpha chain	Q03265	Mitochondrial inner membrane	OXPHOS
ATP synthase gamma chain	Q91VR2	Mitochondrial	OXPHOS
ATP synthase D chain	P31399	Mitochondrial	OXPHOS
60 kDa heat shock protein	P63038	Mitochondrial matrix	protein import/assembly
2',3'-cyclic-nucleotide 3'-phosphodiesterase	P16330	membrane	cell defense
Cytochrome c oxidase polypeptide Vb,	p12075	Mitochondrial inner membrane	OXPHOS
Cytochrome c1, heme protein	Q9D0M3	Mitochondrial intermembrane space	OXPHOS
Glutamate dehydrogenase	P26443	Mitochondrial matrix	TCA cycle
Dihydrolipoyl dehydrogenase	O08749	Mitochondrial matrix	amino acid metabolism
Glyceraldehyde-3-phosphate dehydrogenase	Q64467	Cytoplasmic	glycolysis
4-aminobutyrate aminotransferase	P61922	Mitochondrial matrix	amino acid metabolism
Glutaminase	P13264	Mitochondrial	amino acid metabolism
Hexokinase, type I	P05708	outer mitochondrial membrane	glycolysis
Isocitrate dehydrogenase [NAD] subunit gamma	P70404	Mitochondrial	TCA cycle
Creatine kinase, B chain	Q04447	Cytoplasmic	energy transduction
Creatine kinase, ubiquitous mitochondrial	P30275	Mitochondrial inner membrane	energy transduction
Malate dehydrogenase	P04636	Mitochondrial matrix	TCA cycle
NADH-ubiquinone oxidoreductase 51 kDa	Q91YT0	Matrix side of mitochondrial inner membrane	OXPHOS
NADH-ubiquinone oxidoreductase 13 kDa	P52504	Mitochondrial inner membrane	OXPHOS
Alpha-ketoglutarate dehydrogenase	Q60597	Mitochondrial matrix	TCA cycle
Ubiquinol-cytochrome-c reductase complex	Q9DB77	Mitochondrial inner membrane	OXPHOS

core protein 2			
Ubiquinol-cytochrome C reductase complex 11 kDa protein	P99028	Mitochondrial inner membrane	OXPHOS
Sodium/potassium-transporting ATPase alpha-1 chain precursor	Q8VDN2	Integral membrane protein	transport
Sodium/potassium-transporting ATPase alpha-3 chain	Q6PIC6	Integral membrane protein	transport
Calcium-binding mitochondrial carrier protein Aralar1	Q8BH59	Mitochondrial inner membrane	transport
Cytochrome c oxidase polypeptide VIb	P56391	Mitochondrial	OXPHOS
Mitochondrial glutamate carrier 1	Q9D6M3	Mitochondrial inner membrane	transport
Uncharacterized hematopoietic stem/progenitor call protein MDs029 homolog	Q91ws0	unknown	unknown
NADH-ubiquinone oxidoreductase 19 kDa subunit	Q9DCJ5	Mitochondrial	OXPHOS
Voltage-dependent anion-selective channel protein 1 (VDAC-1)	Q60932	outer membrane of mitochondria	channels
Voltage-dependent anion-selective channel protein 3 (VDAC-3)	Q9R1Z0	Outer mitochondrial membrane	channels
Syntaxin binding protein 1 (Unc-18 homolog)	O08599	Cytoplasmic, peripheral membrane protein	signaling
Synaptophysin	Q62277	Integral membrane protein	signaling
Tubulin beta-3	Q9ERD7	cytoskeleton	structure
Tubulin beta-4 chain	Q9D6F9	cytoskeleton	structure

Note: each protein was identified in at least two experiments at each time point in CON and IP cortices.

Table 3. 9. Cortical mitochondrial proteins.

	1h IP		24h IP	
protein	ratio	p	ratio	p
Cytochrome c1, heme protein	1.08 ± 0.20	0.4778	0.73 ± 0.08	0.000
Ubiquinol-cytochrome-c reductase complex core protein 2	0.92 ± 0.1	0.238	0.69 ± 0.21	0.0366
Cytochrome c oxidase polypeptide Vb	1.03 ± 0.31	0.865	0.68 ± 0.11	0.000
Cytochrome c oxidase polypeptide VIb	0.87 ± 0.18	0.3174	0.76 ± 0.05	0.000
Dihydrolipoyl dehydrogenase	1.05 ± 0.09	0.3844	0.81 ± 0.08	0.000
Alpha-ketoglutarate dehydrogenase	1.08 ± 0.23	0.617	0.75 ± 0.07	0.000
Hexokinase, type I	1.00 ± 0.03	0.9362	0.82 ± 0.06	0.000
Glyceraldehyde-3-phosphate dehydrogenase	1.24 ± 0.37	0.3574	2.02 ± 0.67	0.0316
Creatine kinase, ubiquitous mitochondrial	1.07 ± 0.25	0.617	3.62 ± 3.87	0.242
ANT1	1.42 ± 0.04	0.000	2.18 ± 1.95	0.3954
Aralar1	1.11 ± 0.43	0.7338	0.71 ± 0.21	0.0524
Mitochondrial glutamate carrier 1	1.12 ± 0.47	0.660	0.72 ± 0.23	0.097
VDAC1	1.31 ± 0.41	0.190	0.80 ± 0.11	0.001
VDAC3	1.04 ± 0.30	0.8494	0.80 ± 0.03	0.000

Data were expressed as mean ± SD.

Table 3. 10. Cortical mitochondrial proteins.

	1h CON		24h CON	
protein	ratio	p	ratio	p
Cytochrome c1, heme protein	1.36 ± 0.18	0.0006	0.92 ± 0.22	0.5352
Ubiquinol-cytochrome-c reductase complex core protein 2	1.29 ± 0.01	0.000	0.87 ± 0.06	0.0014
Cytochrome c oxidase polypeptide Vb	1.23 ± 0.20	0.1586	0.74 ± 0.10	0.000
Cytochrome c oxidase polypeptide VIb	1.02 ± 0.22	0.8494	0.80 ± 0.43	0.5156
Dihydrolipoyl dehydrogenase	1.16 ± 0.10	0.0052	1.03 ± 0.29	0.8572
Alpha-ketoglutarate dehydrogenase	1.41 ± 0.26	0.0244	0.94 ± 0.39	0.7794
Hexokinase, type I	1.43 ± 0.64	0.2984	0.91 ± 0.17	0.3788
Glyceraldehyde-3-phosphate dehydrogenase	0.90 ± 0.05	0.0028	0.91 ± 0.03	0.000
Creatine kinase, ubiquitous mitochondrial	1.24 ± 0.03	0.000	0.91 ± 0.17	0.2584
ANT1	1.29 ± 0.35	0.246	0.93 ± 0.07	0.1528
Aralar1	1.26 ± 0.09	0.000	1.10 ± 0.42	0.6818
Mitochondrial glutamate carrier 1	1.62 ± 0.08	0.000	0.94 ± 0.08	0.3174
VDAC1	1.05 ± 0.15	0.5286	0.96 ± 0.16	0.6242
VDAC3	1.30 ± 0.25	0.0854	1.09 ± 0.12	0.2006

Data were expressed as mean ± SD.

Our analysis also identified several proteins involved in glycolysis, NADH transport and mitochondrial transition pore (MTP) formation. Glyceraldehyde-3-phosphate dehydrogenase functions in glycolysis and apoptosis, and was increased in IP cortex 24 h post injury as a trend, and decreased significantly in CON cortex at 1 (10%, p = 0.0028) and 24 h (9%, p = 0.000) post injury respectively. Hexokinase 1, a component of glycolysis and MTP formation, decreased significantly (18%, p = 0.000) in IP cortex 24 h post injury, but did not change significantly in CON cortex 1 and 24 h post injury, or in IP cortex at 1 h post injury. Creatine kinase is involved in energy transport and MTP formation, and was increased significantly (24%, p = 0.000) 1 h post injury in CON cortex. Carrier proteins, aralar1 and cintrin were increased significantly (26%, p = 0.000 and 62%, p = 0.000, respectively) 1 h post injury in CON cortex, and decreased as a trend (29%, p = 0.0524 and 28%, p = 0.097, respectively) 24 h post injury in IP cortex. ADP/ATP carrier protein increased (42%) significantly (p = 0.000) 1 h post injury in IP cortex. VDAC proteins (VDAC1 and VDAC3), channel proteins for metabolite exchange across outer membrane and members of the MTP, decreased significantly (p = 0.001 for VDAC1 and p = 0.000 for VDAC3) in IP cortex 24 h post injury (Table 3.9).

3.3.3 Proteomic Analysis of Differentially Expressed Mitochondrial Proteins in Hippocampi 1 h and 24 h Post Injury

In this study, enriched mitochondria were isolated from IP and CON hippocampus of sham and injured animals 1 and 24 h post injury and were processed as described above. Mitochondrial proteins from four independent samples were pooled again, and samples from each pool were run in triplicate to examine proteins with prominent changes. A total of 29 proteins were identified at each time point in both IP and CON tissues (Table 3.11). Twenty proteins were identified with significant changes in either IP or CON tissues, at either time point post injury (Tables 3.12, 3.13). Major classes include those related to oxidative phosphorylation and transport.

Several proteins involved in the respiratory chain were identified in this study. NADH-ubiquinone oxidoreductase B18 subunit, a component of complex I, was significantly decreased (30%, p = 0.0025) in IP hippocampus but significantly increased 26% (p = 0.0012) in CON hippocampus 24 h post injury. Cytochrome c1, a component of complex III, was increased (26%) as a trend (p = 0.0078) in CON hippocampus 24 h post

Table 3. 11. Proteins identified in both IP and CON hippocampus at 1 and 24 hour post injury.

Common name	SwissProt	SwissProt annotated location	Biological process
Calcium\calmodulin-dependent protein kinase type II alpha chain	p11275	postsynaptic lipid rafts	signaling
Synaptophysin	p07825	integral membrane protein	signaling
Sodium\potassium-transporting ATPase alpha-3 chain	P06687	membrane protein	transport
Tubulin beta-5 chain	P69897	cytoskeleton	structure
Tubulin alpha-2 chain	Q6P9V9	cytoskeleton	structure
2',3'-cyclic-nucleotide 3'-phosphodiesterase	P13233	membrane	cell defense
Mitochondrial glutamate carrier 1	Q9D6M3	mitochondrial inner membrane	transport
Aspartate aminotransferase	P00507	mitochondrial matrix	amino acid metabolism
Glutamate dehydrogenase 1	P10860	mitochondrial matrix	TCA cycle
Voltage-dependent anion-selective channel protein 3	Q9R1Z0	mitochondrial outer membrane	channels
60 kDa heat shock protein, mitochondrial precursor	P63039	mitochondrial matrix	protein import and assembly
ATP synthase gamma chain	P35435	mitochondrial	OXPHOS
Malate dehydrogenase	P04636	mitochondrial matrix	TCA cycle
Voltage-dependent anion-selective channel protein 1	Q9Z2L0	mitochondrial outer membrane	channels
Creatine kinase, ubiquitous mitochondrial precursor	P30275	mitochondrial inner membrane	energy transduction
Ubiquinol-cytochrome c reductase complex 11 kDa protein	P99028	mitochondrial inner membrane	OXPHOS
Citrate synthase	Q9CZU6	mitochondrial matrix	TCA cycle
Isocitrate dehydrogenase [NAD] subunit alpha	Q9D6R2	mitochondrion	TCA cycle
Dihydrolipoyl dehydrogenase	Q6P6R2	mitochondrial matrix	amino acid metabolism
Aconitate hydratase	Q9ER34	mitochondrion	TCA cycle
ATP synthase subunit alpha	P15999	mitochondrial inner membrane	OXPHOS
Hexokinase-1	P05708	outer mitochondrial membrane	glycolysis
Ubiquinol-cytochrome-c reductase complex core	P32551	mitochondrial inner membrane; matrix side	OXPHOS

protein 2			
ADP\ATP translocase 1	P48962	mitochondrial inner membrane	transport
Cytochrome c1, heme protein	Q9D0M3	mitochondrial intermembrane space	OXPHOS
Mitochondrial precursor proteins import receptor	Q9CZW5	mitochondrial outer membrane	receptor
NADH-ubiquinone oxidoreductase B18 subunit	Q9CR61	mitochondrial inner membrane	OXPHOS
Excitatory amino acid transporter 2	P43006	Membrane	transport
Calcium-binding mitochondrial carrier protein aralar1	Q8BH59	mitochondrial inner membrane	transport

Table 3. 12. Hippocampal mitochondrial proteins

	1h IP		24h IP	
protein	ratio	p	ratio	p
NADH-ubiquinone oxidoreductase B18 subunit	0.90 ± 0.35	0.6892	0.70 ± 0.17	0.0025
Ubiquinol-cytochrome-c reductase core protein 2	0.92 ± 0.12	0.3174	1.33 ± 0.01	0.000
Cytochrome c1, heme protein	1.06 ± 0.12	0.4066	1.04 ± 0.15	0.6672
Ubiquinol-cytochrome c reductase 11 kDa protein	1.01 ± 0.0.1	0.8886	1.28 ± 0.01	0.000
ATP synthase gamma chain	0.85 ± 0.08	0.0016	0.94 ± 0.24	0.6672
ATP synthase subunit alpha	1.07 ± 0.06	0.0818	0.87 ± 0.25	0.3734
Aspartate aminotransferase	1.03 ± 0.07	0.5092	1.37 ± 0.36	0.0718
Glutamate dehydrogenase 1	1.09 ± 0.53	0.3222	1.20 ± 0.04	0.000
Malate dehydrogenase	0.90 ± 0.05	0.0002	1.22 ± 0.36	0.2938
Citrate synthase	1.02 ± 0.15	0.8886	1.39 ± 0.10	0.000
Isocitrate dehydrogenase subunit α	0.97 ± 0.13	0.7434	1.14 ± 0.45	0.5824
Dihydrolipoyl dehydrogenase	1.05 ± 0.14	0.6384	1.36 ± 0.19	0.0088
Hexokinase-1	0.76 ± 0.05	0.000	0.99 ± 0.33	0.9442
ADP\ATP translocase ANT1	0.84 ± 0.06	0.000	1.24 ± 0.15	0.0258
Mitochondrial precursor proteins import receptor (TOM70)	0.98 ± 0.11	0.8026	1.13 ± 0.16	0.2380
Aralar1	0.86 ± 0.07	0.0008	1.14 ± 0.20	0.9680
VDAC1	0.90 ± 0.04	0.000	1.06 ± 0.02	0.000
VDAC3	0.80 ± 0.11	0.0124	1.01 ± 0.12	0.9522
60 kDa heat shock protein	1.03 ± 0.1	0.603	1.27 ± 0.18	0.0384

Data were expressed as mean ± SD.

Table 3. 13. Hippocampal mitochondrial proteins

protein	1h CON		24h CON	
	ratio	p	ratio	p
NADH-ubiquinone oxidoreductase B18 subunit	0.97 ± 0.16	0.7872	1.26 ± 0.14	0.0012
Ubiquinol-cytochrome-c reductase core protein 2	1.19 ± 0.28	0.3422	1.34 ± 0.69	0.4902
Cytochrome c1, heme protein	1.02 ± 0.11	0.7872	1.26 ± 0.17	0.0078
Ubiquinol-cytochrome c reductase 11 kDa protein	1.07 ± 0.20	0.5156	1.54 ± 0.83	0.2584
ATP synthase gamma chain	0.96 ± 0.05	0.1646	1.28 ± 0.12	0.000
ATP synthase subunit alpha	0.87 ± 0.02	0.000	1.23 ± 0.28	0.147
Aspartate aminotransferase	0.99 ± 0.06	0.8572	1.64 ± 0.30	0.0002
Glutamate dehydrogenase 1	0.95 ± 0.07	0.215	1.12 ± 0.14	0.2802
Malate dehydrogenase	0.95 ± 0.06	0.1556	1.48 ± 0.25	0.0008
Citrate synthase	1.12 ± 0.18	0.3576	1.60 ± 0.45	0.0602
Isocitrate dehydrogenase subunit α	1.14 ± 0.05	0.000	1.37 ± 0.15	0.000
Dihydrolipoyl dehydrogenase	1.08 ± 0.29	0.6528	1.81 ± 1.28	0.2758
Hexokinase-1	0.97 ± 0.01	0.000	1.27 ± 0.22	0.034
ADP\ATP translocase ANT1	1.04 ± 0.11	0.5418	1.65 ± 0.33	0.0006
Mitochondrial precursor proteins import receptor (TOM70)	1.28 ± 0.06	0.000	1.17 ± 0.30	0.3222
Aralar1	0.85 ± 0.14	0.0026	1.09 ± 0.06	0.025
VDAC1	0.99 ± 0.24	0.9602	1.29 ± 0.44	0.246
VDAC3	1.21 ± 0.15	0.0512	1.51 ± 0.08	0.000
60 kDa heat shock protein	0.93 ± 0.04	0.0022	1.40 ± 0.16	0.000

Data were expressed as mean ± SD.

injury. Ubiquinol-cytochrome-c reductase complex core protein 2 was significantly ($p = 0.000$) increased 33% in IP hippocampus 24 h post injury. Levels of ubiquinol-cytochrome C reductase complex 11 kDa protein were increased (28%) significantly ($p = 0.000$) in IP hippocampus at 24 h post injury. ATP synthase gamma chain, a component of complex V, significantly decreased ($p = 0.0016$) in IP hippocampus at 1 h post injury. It increased ($p = 0.000$) in CON hippocampus at 24 h post injury. ATP synthase subunit alpha decreased (13%) significantly ($p = 0.000$) in CON hippocampus 1 h post injury.

Analysis of TCA cycle proteins show that glutamate dehydrogenase increased (20%) significantly ($p = 0.000$) 24 h post injury in IP hippocampus, and isocitrate dehydrogenase [NAD] subunit alpha increased significantly in CON hippocampus at 1 h (14%, $p = 0.000$) and 24 h (37%, $p = 0.000$) post injury. Citrate synthase significantly increased 39% ($p = 0.000$) 24 h post injury in IP hippocampus. Aconitate hydratase significantly increased 45% ($p = 0.002$) in CON hippocampus 24 h post injury. Malate dehydrogenase significantly increased 48% ($p = 0.0008$) 24 h post injury in CON hippocampus and deceased 10% ($p = 0.0002$) in IP hippocampus 1 h post injury. Dihydrolipoyl (dihydrolipoamide) dehydrogenase, a component of the glycine cleavage system as well as of the alpha-ketoacid dehydrogenase complexes, increased 36% as a trend ($p = 0.0088$) in IP hippocampus 24 h post injury. Aspartate aminotransferase, another enzyme thought to be expressed in liver and heart, was identified in the mitochondrial sample, and increased significantly ($p = 0.0002$) in CON (64%) hippocampus 24 h post injury. This enzyme is also involved in metabolism by catalyzing oxaloacete related reaction.

Our study also identified proteins involved in glycolysis, protein transport and folding, and the mitochondrial transition pore (MTP). Hexokinase 1, a member of the glycolytic pathway and MTP formation, decreased (24%) significantly ($p = 0.000$) in IP hippocampus 1 h post injury, and increased (27%) as a trend ($p = 0.034$) in CON hippocampus 24 h post injury. A carrier protein, ANT1, increased 24% ($p = 0.0258$) in IP hippocampus and 65% ($p = 0.0006$) in CON hippocampus 24 h post injury, and decreased significantly ($p = 0.000$) in IP hippocampus 1 h post injury. Aralar 1 decreased significantly at 1 h post injury in IP($p = 0.0008$) and CON ($p = 0.0026$) hippocampus. Mitochondrial precursor protein importer receptor TOM70 increased significantly ($p =$

0.000) 1 h post injury in CON hippocampus. VDAC1 and VDAC3 are involved in outer membrane permeability. Our data showed that VDAC1 significantly decreased 10% ($p = 0.000$) in IP hippocampus 1 h post injury but increased (6%) significantly in IP hippocampus 24 h post injury. VDAC3 decreased (20%) as a trend ($p = 0.0124$) 1 h post injury in IP hippocampus, but increased significantly ($p = 0.000$) in CON hippocampus 24 h (51%) post injury. Heat shock protein, HSP60, increased in IP (27%) as a trend ($p = 0.0384$) and significantly increased ($p = 0.000$) in CON (40%) hippocampus 24 h post injury.

3.3.4 Validation of VDAC and Hexokinase 1 with Western Blot Analyses

Proteomic analysis identified proteins with expression changes following TBI. In order to validate proteomic identification, VDAC and hexokinase 1 protein expressions were quantified by Western blot analysis. Our data showed that VDAC expression significantly ($p < 0.05$) decreased in IP cortex 24 h post injury, with 20% decrease compared to sham animals (Figure 3.14). HK1 expression also significantly ($p < 0.05$) decreased in IP cortex compared to sham (Figure 3.15). The ratio of protein expression from Western blot is comparable to those observed from proteomics using ICAT labeling and 2D LC/MS/MS. As the three independent samples were used in western blot, our data indicate the proteomics method using pooled samples is effective to determine proteins with prominent changes following TBI.

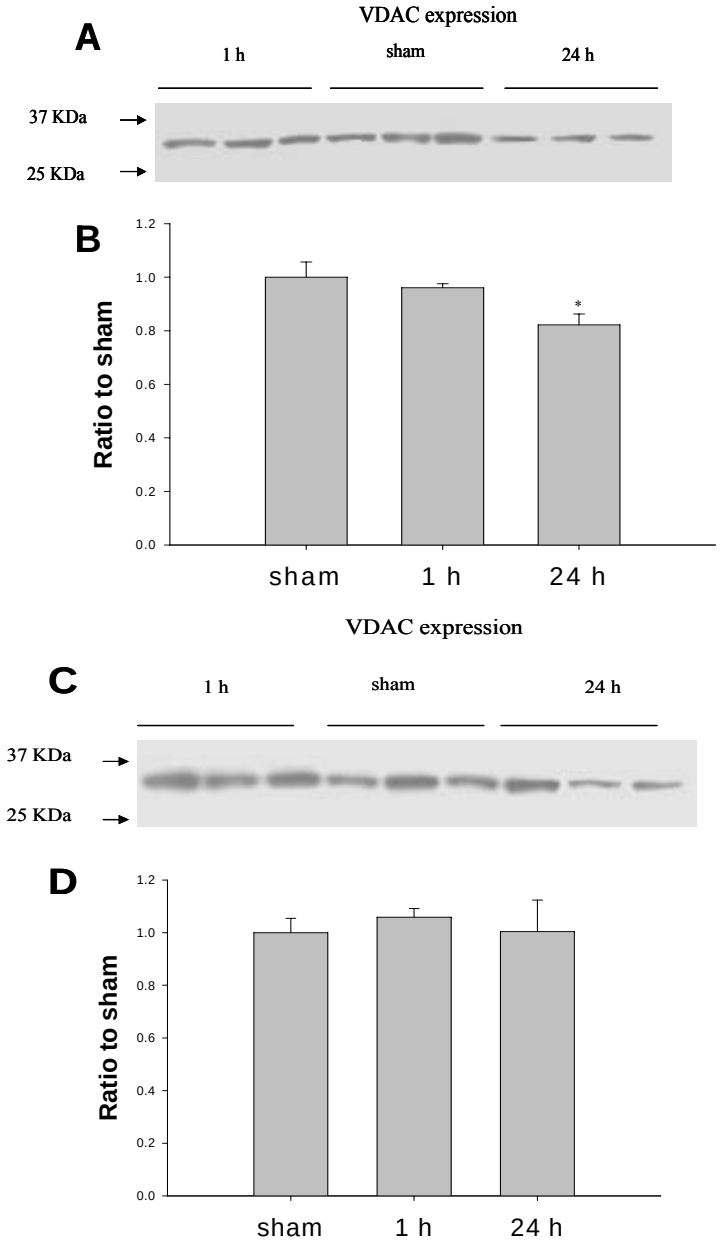


Figure 3.14. Western blot analysis of VDAC protein levels in IP cortex (panel A) and CON cortex (panel C). There is a significant ($p < 0.05$) time dependent decrease in VDAC in IP cortex (panel B), but no significant change in CON cortex (panel D).

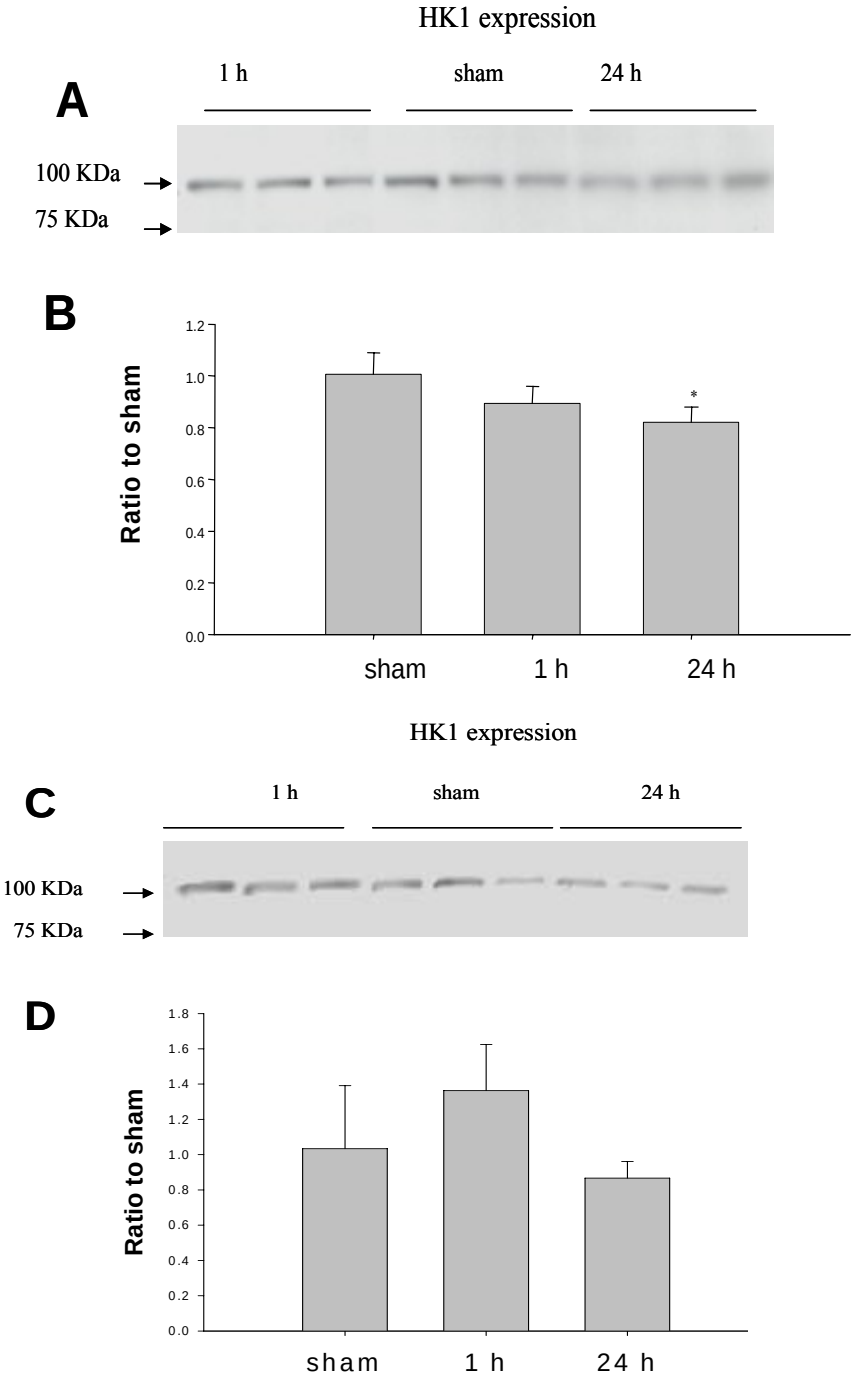


Figure 3.15. Western blot analysis of hexokinase in IP cortex (panel A) and CON cortex (panel C). There is a statistically significant ($p < 0.05$) time dependent decrease in HK1 levels in IP cortex compared to sham cortex (panel B). There were no significant differences in HK1 in CON cortex (panel D).

Chapter Four

Discussion

4.1 Reduced Tissue Sparing in TBI in Aging

Aging is associated with increased mortality rates and neurological deficits following TBI, but the mechanism is not well understood (Teasdale et al., 1979; Hamm et al., 1991; Hamm et al., 1992; Klein et al., 1996; Maughan et al., 2000; Hoane et al., 2004). Previous studies show traumatic brain injury results in delayed dysfunction and death of neuron populations near and distant to the site of injury through secondary processes (Cooper, 1985). Neuron death can lead to tissue shrinkage or loss. In an aging study, Hoane et al. (Hoane et al., 2004) showed that middle aged animals suffered more tissue loss than young animals after TBI, although aged animals were not investigated in their study. The current data described in this dissertation show middle aged and aged animals both experience significant tissue loss compared to young animals with no significant difference between middle aged and aged animals 7 days post injury. More brain tissue loss indicates more severe damage to the brain, and could lead to more problems following injury. So the current result may explain why aged animals have increased mortality rate than young adult animals, but does not provide insight into the differences between middle-aged and aged animals. To further investigate effects of age in TBI, further studies are required to evaluate the changes of brain in aging in TBI.

4.2 Oxidative Stress in Head Trauma in Aging

Oxidative damage is suggested to be an important factor for neurological deficits (Camandola et al., 2000; Anderson et al., 2005; Hall et al., 2005) following TBI, and is also hypothesized to play a role in diminished recovery from TBI in aged subjects. The oxidative damage is initiated by increased ROS in cell. There are several sources for ROS in cell, and mitochondria are believed to be major source following TBI. ROS can cause oxidative damage to biological macromolecules, including peroxidation of membrane polyunsaturated fatty acids (PUFA) (McCall et al., 1987; Beckman et al., 1993; Hall et al., 2004) and DNA (Johnson et al., 2005; Wang et al., 2005). PUFA peroxidation ultimately leads to loss of both structural and functional integrity of the cell with generation of toxic aldehydic by-products, such as HNE and acrolein (Uchida and Stadtman, 1994), which may further contribute to neuronal cell death by protein and

DNA modification (Graham et al., 1978). DNA oxidation and modification by aldehydes may lead to gene mutation and affect transcription (Barzilai and Yamamoto, 2004; Shimamura et al., 2004).

In the current study, in order to look at changes in cortex and hippocampus in TBI in aging except for tissue loss, the markers of oxidative damage, HNE, acrolein, and oxidized DNA bases, were investigated at 24 h or 7 days post injury. The oxidative damage 24 h post injury could show changes in neurons before cell death, and the damage at 7 days post injury could show the neuron damage even it survived at this time point. So the study at this two time point provided an early and late response of neuronal cell in cortex and hippocampus to TBI.

4.2.1 Lipid Peroxidation in TBI in Aging

There have been few studies of markers of oxidative stress or antioxidant capacities following TBI in aged animals (Wagner et al., 2004). The present study demonstrates age dependent increased lipid peroxidation 1 and 7 days following TBI. To minimize biological variation among animals, CON tissues of injured animals were used as control to investigate the changes in IP tissues. The data described in this dissertation show there were no significant differences in HNE or acrolein levels between CON tissues of injured animals and CON and IP tissues of sham animals. Previous studies also show distal tissues in CCI model showed no significant response in oxidative damage (DeKosky et al., 2004). In comparison of levels of lipid peroxidation in tissues isolated before and after in skull flash-freezing showed no artifactual oxidation occurring during the time between cervical dislocation and tissue extraction.

In cortical TBI, the hippocampus suffers significant neuronal loss immediately after injury that increases for up to seven days post injury, which may contribute to memory deficit. Using immunohistochemistry Wang et al. (Wang et al., 2003) showed HNE levels increased following TBI in IP hippocampus in young animals. Using dot blot immunochemistry, we showed HNE levels increased significantly in IP hippocampus 24 h post injury in each age group, suggesting pronounced lipid peroxidation and protein modification at this time point. The intensive oxidative damage could lead to delayed neuron death in IP hippocampus. Acrolein, another neurotoxic marker of lipid peroxidation, showed a similar trend as HNE with aged animals showing higher levels in

IP hippocampus compared to CON hippocampus. At 7 days post injury, levels of HNE and acrolein in IP hippocampus were reduced to CON hippocampal levels in young animals. This phenomenon may indicated young animals are able to reduce oxidative damage by its own defense. Although middle aged animals also showed reduced levels of HNE and acrolein compared to 24 h post injury, the levels were elevated compared to contralateral hippocampus. Levels of HNE remained elevated in aged animals at 7 days post injury. The increased oxidative damage following TBI could lead to mitochondrial dysfunction (Lifshitz et al., 2003) and diminished neuron recovery following TBI. The age dependent oxidative damage in hippocampus may help explain increased functional deficit following TBI in aged subjects.

In cortex, primary mechanical brain injury causes subdural hematoma, laceration, diffuse vascular injury, and cell death in the impact zone that involves extensively complicated processes (BIROS, 1998b; Raghupathi, 2004). ROS generation in cortex of young animals following TBI increases with time in early stages (Hall et al., 1993; Smith et al., 1994; Nishio et al., 1997). In addition, documented studies show lipid peroxidation products significantly increase immediately following injury, and remain elevated for several days in young animals (Hall et al., 2004). HNE levels increased in IP cortex in each age group at 24 h post injury, but did not show age dependent differences. At 7 days post injury, HNE levels decreased in IP cortex in young animals, but kept increasing in middle aged animals. Aged animals did not show more oxidative damage 7 days post injury compared to 24 h post injury. Unlike oxidative damage in hippocampus, there is no clear age dependent change in cortical lipid peroxidation, especially at 24 h post injury. Hall et al. (1993) reported that a major source of ROS is microvasculature, and saline perfusion of the injured mice eliminated ROS levels measured in their study (Hall et al., 1993). Blood is enriched by iron from hemoglobin. The free iron catalyzes the formation of ROS by Fenton's reaction and could lead to increase of lipid peroxidation. The cortex samples harvested following TBI were bloody in the current study, so it is possible that blood in the cortex tissues led to significant lipid peroxidation and masked the difference in lipid peroxidation in aging in our study.

4.2.2 DNA Oxidation in TBI in Aging

As discussed above, oxidative stress plays a pivotal role in TBI in aging. Mitochondria are major sources of superoxide radicals especially following mitochondrial dysfunction (Xiong et al., 1997b; Sullivan et al., 2002; Lifshitz et al., 2003). Superoxide can be converted to hydrogen peroxide by superoxide dismutase (SOD) (Fridovich, 1997). Hydrogen peroxide is further transformed to water through glutathione peroxidase. However, it can potentially interact with transition metals to form hydroxyl radicals that are significantly increased following TBI (Smith et al., 1994). Hydroxyl radicals can attack nucleic acids, and lead to oxidative damage (Rehman et al., 1999). More than 20 oxidized DNA bases have been identified (Candeias and Steenken, 2000; Evans et al., 2004), of these 8-hydroxyguanine is the most studied because it is the predominant marker of DNA damage (Pratviel and Meunier, 2006). Fapyguanine, 8-hydroxyadenine, fapyadenine, and 5-hydroxycytosine are other products of guanine, adenine and cytosine oxidative damage. In order to further evaluate oxidative damage in TBI in aging, we quantified five DNA base oxidation products to quantify the different oxidative response in aging. For this study, GC/MS-SIM was used because it can monitor multiple bases simultaneously in a single run (Dizdaroglu, 1990).

Previous studies showed increased 8-hydroxyguanine (8-OHG) levels in rat cortex 15 min post injury using immunohistochemistry (Mendez et al., 2004) in young animals. Our present study using GC/MS-SIM showed 8-OHG levels were increased as a trend but not statistically significant in IP cortex of injured and sham animals compared with their CON cortex. No significant differences were observed between injured and sham animals 24 h post injury. Additionally, no significant differences were observed in 8-OHG levels in middle aged IP or CON cortex between injured and sham animals. In contrast, aged animals showed 8-OHG levels significantly increased in IP cortex of injured animals compared with their CON cortex and with sham animals. Moreover, 8-OHG levels were significantly higher in IP cortex of aged animals compared to young and middle aged animals, suggesting an age dependent increase of oxidative damage. Our lipid peroxidation study in TBI in aging showed lipid peroxidation was significantly increased in young, middle aged and aged animals in cortex 24 h post injury (Shao et al., 2006). The differences in lipid peroxidation and DNA damage in young and middle aged

animals suggest lipid damage increases more rapidly than DNA damage. In aged animals, severe oxidative damage can lead to both DNA and lipid oxidation. In hippocampus, extensive DNA damage was observed even though it is subjected to less mechanical injury compared to cortex. Our data suggest an age dependent increase of DNA damage with significant elevations of 8-OHG in injured IP hippocampus of middle aged and aged animals compared with young animals. In addition, 8-OHG levels in IP hippocampus in middle aged and aged animals were significantly increased compared to CON hippocampi. These data are consistent with our previous study of lipid peroxidation in TBI in aging (Shao et al., 2006). The presence of increased levels of DNA base adducts and lipid peroxidation byproducts in aged animals may help explain why aged animals exhibit increased neuron and memory loss following TBI (Ward et al., 1999). DNA damage may be particularly problematic because it can lead to alterations in transcription through a G:C to T:A mutation (Barzilai and Yamamoto, 2004), and changes in gene expression (Shimamura et al., 2004). Although DNA repair in nuclei can be mediated by MutT homolog (MTH1), MutT or oxoguanine glycosylase (OGG1) (Nakabeppu et al., 2004), extensive ROS may overwhelm the repair capacity. Although fapyguanine, 8-hydroxyadenine, fapyadenine, and 5-hydroxycytosine levels were not significantly different in aging in TBI, there was a trend toward increased levels of these oxidized bases in aging.

4.2.3 Antioxidant Enzyme Activities in TBI in Aging

Previous studies that secondary oxidative damage can regulate gene expression and inflammatory response in the hippocampus, especially in genes related to metabolism and antioxidant enzymes (Long et al., 2003). However, proteins are the units that function directly in cell defense, and gene transcription change may not necessarily reflect antioxidant enzyme defense capacity since oxidative damage may inactivate enzymes following TBI. In order to quantify cellular defense capacities, protein activity is most direct measure for the study. There are several antioxidant enzymes in neurons that decrease oxidative stress, including Cu/ZnSOD, MnSOD, catalase, GPx, GSSG-R, and GST. Although these enzymes work in concert to protect the cell, cell death can result if ROS production exceeds the defense capacity. We hypothesize that oxidative

damage, lipid peroxidation and DNA oxidation, following TBI and the differences in oxidative damage in TBI in aging may result from deficits in antioxidant capacities.

Superoxide dismutases function to convert superoxide ion to less active hydrogen peroxide and avoid the formation of further ROS, and have been widely investigated in neurodegenerative diseases. Transgenic mice overexpressing SOD showed increased tissue sparing and improved neurological function compared with their WT littermates following TBI (Maier and Chan, 2002). In addition, Dekosky et al (DeKosky et al., 2004) reported that SOD activity decreased significantly 6 h after TBI and 20% of activity was lost 24 h and 30% lost 7 days post injury in young rats subjected to severe injury in the anterior hippocampus. Our data failed to show a significant age-dependent change in Cu/ZnSOD activity for any age or time point studied. Although MnSOD does not change significantly in young and middle aged animals 24 h post injury, it decreased significantly (20%) in aged animals compared to middle aged animals. In contrast to the study of Dekosky et al. (DeKosky et al., 2004), we did not observe significant losses of SOD even 7 days post injury in young animals. However, we observed a 30% loss in IP hippocampus MnSOD activity in middle aged and aged animals. Statistical analysis showed an age-dependent decrease of MnSOD activity in aging. The decrease of the mitochondrial MnSOD could compromise the cell's ability to remove superoxide generated after the TBI and lead to mitochondrial damage (Xiong et al., 2005), increased lipid peroxidation (Awasthi et al., 1997), and DNA damage, which could lead to cell apoptosis and result in neuron loss and cognitive deficits in older animals.

In the antioxidant defense system, glutathione peroxidase follows SOD and reduces hydrogen peroxide to water with consumption of glutathione. Table 2 shows that GPx increased in young IP hippocampus 1 and 7 days post injury with little change in middle aged or aged animals. The current data in this dissertation show a significant age dependent decrease of GPx activity observed 24 h post injury in middle aged and aged animals compared to young animals. At 7 days post injury, GPx activities were lower in middle aged and aged IP hippocampus compared with young IP hippocampus, although the differences did not reach statistical significance. These data are consistent with those of Dekosky et al. (DeKosky et al., 2004) who showed increased GPx activity in hippocampus in young TBI animals 7 days post injury. Our data further show that GPx

protein expression increased significantly (data not shown) in IP cortex and hippocampus, which is consistent with the theory that this enzyme is upregulated by oxidative stress (Baud et al., 2004). However, higher abundance of this enzyme did not improve neuron antioxidant capacity in middle aged and aged animals. Young animals showed elevated GPx activities compared with older animals that may help limit further oxidative damage.

Previous studies show GST overexpression is neuroprotective in cell culture (Xie et al., 2001). Glutathione transferase can alleviate damage from lipid peroxidation by-products (HNE and acrolein) by catalyzing their conjugation with glutathione (GSH). GST depletion could result in increased protein modification and lead to more protein dysfunction, which could contribute to further oxidative damage. Our data show significant GST activity loss in IP hippocampus 7 days post injury for middle aged and aged animals, but not for young animals. However, there is no significant difference in aging in IP hippocampus. Glutathione reductase, which functions to regenerate reduced glutathione, has been studied in neurodegenerative disease (Lovell et al., 1995), but not in traumatic brain injury. Our study shows a significant decrease in GSSR-R in middle aged and aged IP hippocampi at 7 days post injury, but not for young animals.

Antioxidant enzyme activities have not been studied in cortex in TBI even though oxidative stress has been well documented in the last several decades. Our data show MnSOD in IP cortex was depleted in an age dependent manner 24 h post injury and that the decrease correlated with increased tissue loss. Diminished antioxidant activity may compromise defense capacities against oxidative stress (Azbill et al., 1997), and may contribute to neuron death in the impact zone. At 7 days post injury, MnSOD activity in IP cortex significantly decreased in young, middle aged and aged animals, but did not show an age dependent difference. Activities of Cu/ZnSOD were decreased in IP cortex of all three age groups 7 days post injury, but they did not show significant age dependent differences. GPx activity did not change significantly 24 h post injury in any age group studied, although young and middle aged animals showed increased activity as a trend at 7 days post injury. There were no age dependent differences at any time point for this antioxidant enzyme. GST activity decreased 24 h post injury in IP cortex in each age group with more pronounced decreases occurring in middle aged animals 7 days post

injury. GST depletion in cortex may result in intensive biochemical modification of biomolecules by toxic aldehydic byproducts from lipid peroxidation, suggesting middle aged animals may suffer further damage. GSSG-R decreased significantly in middle aged and aged IP cortex compared to young animals 24 h post injury, although no significant differences were observed 7 days post injury. GSSR-R depletion 24 h post injury could contribute to depletion of glutathione, a critical low molecular weight (LMW) antioxidant, and contribute to cell death and tissue loss in later days.

4.3 Mitochondrial Proteomics in TBI

In TBI, there was significant tissue loss in injured cortex 1 week post injury, but not at 24 hours post injury. However, there is significant oxidative damage at 24 h post injury, showing increased HNE, acrolein, and oxidized DNA bases. The oxidative damage in neuron is closely connected with mitochondria because mitochondria are the major source of ROS. In order to examine changes of mitochondria before the tissue loss, we examined changes of mitochondrial protein expression in cortex 1 h and 24 h post injury. Additionally, hippocampal mitochondrial protein levels were also evaluated at the same time points.

4.3.1 Mitochondrial Proteomics of Cortex

Previous studies show that ATP depletion, increased oxidative stress, cytochrome c release, and compromised mitochondrial function follow TBI (Xiong et al., 1997b; Sullivan et al., 1998; Lewen et al., 2001; Lifshitz et al., 2004). Lifshitz et al. showed significant morphologic and integrity damage, and reduced mitochondrial proteins isolated from mitochondria in injured cortex and hippocampus at 3 h and 24 h post injury using a fluid-percussion model (Lifshitz et al., 2003; Lifshitz et al., 2004). Mitochondrial dysfunction involves multiple proteins, which indicates a very complicated process (Nicholls and Budd, 2000). Although reduced mitochondrial protein was isolated from brain tissues of animals following TBI, individual mitochondrial protein expression levels in isolated mitochondria were not investigated. Mitochondrial proteomics could investigate multiple proteins involved in the dysfunction. In the present study, mitochondrial protein expression in IP and CON tissues of animals subjected to TBI was quantified relative to animals subjected to sham surgery. The majority (75.7%) of the proteins identified were mitochondrial according to the Swiss-Prot database, and included

proteins involved in energy metabolism, small molecule transport, and mitochondrial outer membrane permeability.

Respiratory chain damage is widely investigated in other neurodegenerative diseases, including Huntington's disease, ALS, AD, Parkinson's disease, and ischemia (Fiskum et al., 1999). Damage to the respiratory chain following TBI is supported by studies of mitochondrial respiration in TBI, that show damage to state 3 (Vink et al., 1990; Xiong et al., 1997a; Sullivan et al., 2005). In our study, we show levels of some subunits of complex III, and IV were significantly reduced 24 h post injury. Complex III, also known as the cytochrome bc1, oxidize ubiquinol (ubihydroquinone) which reacts in one membrane phase, reduces cytochrome c in the intermembrane space (or periplasm in bacteria), and use the free energy change to drive H^+/QH_2 across the membrane from the matrix to the inner membrane space. It is suggested that this complex is one of the major sites for electron leakage. Mitochondrial cytochrome c-1 is a collapsed di-heme cytochrome (Baymann et al., 2004), that was not changed 1 h post injury in IP cortex, but was increased in CON cortex, and reduced in IP cortex 24 h post injury. Ubiquinol-cytochrome-c reductase complex core protein 2 was not changed 1 h post injury in IP, but was increased in CON cortex. Levels of the protein were decreased in both IP and CON cortices 24 h post injury. The decrease of the two complex III subunits could lead to significant electron leakage and oxidative damage to cell. Complex IV (cytochrome oxidase complex) is the site that oxygen binds with protons to form water, with the formation of a proton gradient. There are three main subunits and ten small subunits for complex IV. Among the small subunits, the subunits cox5b is small globular proteins bound to the matrix side of the core, and the globular subunit cox6b1 faces the intermembrane space (Michel et al., 1998). Subunit cox6b1 binds a zinc ion of unknown function in a tetrahedral coordination. It is suggested that the small subunits act as regulators and bind effectors and are required for complex assembly (Kadenbach, 1986). A previous study showed reduced cytochrome oxidase activity followed TBI (Hovda et al., 1991). Our data show cox5b (cytochrome c oxidase polypeptide Vb) levels decreased significantly 24 h post injury in both IP and CON cortices, but were not changed 1 h post injury in IP or CON cortex. In spinobulbar muscular atrophy disease, it has been shown that sequestration of cox5b by human androgen receptor (hAR) leads to mitochondrial

dysfunction (Beauchemin et al., 2001). In cancer cells, cox5b up-regulation increases retention of cytochrome c and enhances synthesis of ATP (Wu et al., 2000; Wu et al., 2003). So the decrease of cox5b expression after TBI in both IP and CON cortex may lead to mitochondrial dysfunction and reduced ATP production. The difference of this polypeptide levels between 1 and 24 h post injury indicates a time dependent change. In addition, another subunit of complex IV, cox6b1 (cytochrome c oxidase polypeptide VIb), decreased 24 h post injury in IP cortex. The compromised subunits of complex I, III and IV could lead to respiration deficiency that could lead to secondary respiratory deficiencies (Rotig et al., 2004) that . The respiratory deficiency could lead to significant electron leakage, and perturb proton transport and proton gradient. The proton gradient is the driving force for ATP production by complex V.

The TCA cycle involves multiple enzymes and is a key process for ATP production in mitochondria. Our data show dihydrolipoyl dehydrogenase and alpha-ketoglutarate dehydrogenase are decreased significantly 24 h post injury in IP cortex, but not in CON cortex at this time point and IP and CON cortex 1 h post injury. Dihydrolipoyl dehydrogenase is a common component in three α-keto acid dehydrogenase complexes, including pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes (Reed, 1974). This enzyme catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of the three α-keto acid dehydrogenase complexes. Reoxidation of the dihydrolipoyl group generates FADH, which can reduce NAD^+ to NADH. Alpha-ketoglutarate dehydrogenase is a component of the alpha-ketoglutarate dehydrogenase complex, along with dihydrolipoamide succinyltransferase (E2) and lipoamide dehydrogenase (E3). This complex catalyzes the overall conversion of alpha-ketoglutarate to succinyl-CoA and CO_2 with production of NADH. Decreased levels of proteins could compromise the TCA cycle and production of NADH, which could lead to ATP depletion in tissues following TBI. Previous studies show alpha-ketoglutarate dehydrogenase complex deficiencies lead to cytochrome c release (Huang et al., 2003). It was also shown this enzyme was mediator of oxidative stress and mitochondrial dysfunction (Gibson et al., 2005). So the decrease of alpha-ketoglutarate dehydrogenase

could lead to deficiency of the enzyme complex and lead to mitochondrial dysfunction, cell apoptosis and neuron degeneration.

Creatine kinase, an enzyme involved in energy transport is also a component of the MTP (Brdiczka et al., 1994). Our data show this enzyme was increased in CON cortex 1 h post injury, which suggests a compensatory response of cells to generate more ATP at this time point. Hexokinase 1 (HK1) and glyceraldehydes-3-phosphate dehydrogenase (GPDH) are key enzymes of cellular glycolysis, and are closely associated with mitochondria. HK1 is suggested to promote VDAC channel closure and prevent mitochondria mediated apoptotic cell death by inhibiting cytochrome c release (Azoulay-Zohar et al., 2004; da-Silva et al., 2004). HK1 may affect ATP production by affecting the supply of ADP (Birnbaum, 2004). Previous studies shows enhancement of glycolysis with a Pasteur effect, a shift from slow aerobic to rapid anaerobic consumption of glucose, after respiratory inhibition (Kauppinen and Nicholls, 1986). Our Western blot analyses showed increased HK1 expression following injury (data not show), which may show the Pasteur effect. However, the mitochondria-bound HK1 decreased significantly at 24 h post injury. The decrease of mitochondria-bound HK1 could lead to outer membrane permeability changes and cytochrome c release 24 h post injury in IP cortex. GPDH was thought to be a housekeeping protein, and regulate apoptosis by the inactivation of cytoprotective heat shock protein 70 (HSP70) in neurodegenerative disease and aging (Ishitani et al., 1996; Kragten et al., 1998; Chuang et al., 2005). It has been shown that more GPDH was deposited on mitochondria in cells undergoing apoptosis than cells under normal conditions (Saunders et al., 1997). Our data show GPDH levels in IP cortex increased as a trend at 24 h post injury. The increased GPDH may contribute to cell apoptosis and neuron dysfunction. Another key protein in apoptosis, VDAC, was shown decreased significantly in IP cortex 24 h post injury. Western blot analysis shows the same result as ICAT proteomics. VDAC is a key protein regulator of outer membrane permeability, which permits influx or efflux of molecules $\leq$ 1500 Da. In addition, VDAC is a also key component of the MTP formed under stress, other members including ANT in the inner membrane, cyclophilin D (CypD) in the matrix, creatine kinase in intermembrane space, and hexokinase in the outer membrane (Verrier et al., 2003). Our data show VDAC (1 & 3) levels decreased significantly in IP

cortex 24 h post injury, but not 1 h post injury. VDAC plays a key role in exchange of metabolites and metal ions between mitochondria and cytoplasm (Rostovtseva et al., 2005). The protein decrease may lead to decreased permeability resulting in slower response to calcium (Lifshitz et al., 2003). Our study identified ADP/ATP carrier protein (ANT 1), and showed a significant increase 1 h post injury, which may indicate a compensatory response to injury. Buck et al. showed increased ANT1 facilitated glutamate transport (Buck et al., 2003). The ANT1 level increase 1 h post injury in IP cortex and may indicate the neurons' response to reduce the excess glutamate released following TBI.

Carrier proteins play an important role in mitochondrial function. Cytosolic NADH is transported into mitochondria for oxidative metabolism and ATP production through two NADH shuttles, including the malate/aspartate shuttle (LaNoue and Schoolwerth, 1979). The shuttle requires aspartate/glutamate carrier, which has two isoforms (aralar 1 and citrin) (Brown, 1992). The overall structure of citrin is similar to that of aralar 1, and both have four EF-hand Ca^{2+} -binding motifs in their N-terminal domains (del Arco and Satrustegui, 1998). Previous studies show recombinant expression of aralar 1 and citrin increased ATP production by stimulation of Ca^{2+}-mobilizing agonists, in which aralar 1 and citrin function to decode calcium signals from cytosol for mitochondria (Palmieri et al., 2001; Lasorsa et al., 2003). It was also shown that aralar knock-out mice are growth-retarded, exhibited generalized tremoring, and had pronounced motor coordination defects along with an impaired myelination in the central nervous system (Jalil et al., 2005). Our data show calcium-binding mitochondrial carrier protein aralar 1 and mitochondrial glutamate carrier 1 (citrin) expression levels decreased as a trend in IP cortex 24 h post injury. The compromised protein expression could decrease NADH transport, ATP synthesis, and myelin lipid synthesis. However, the two proteins showed increased levels in CON cortex 1 h post injury, which could increase ATP production in CON cortex at this time point.

Mitochondria are dynamic organelles, and their fission and fusion could produce new mitochondria or repair damaged mitochondria. Lifshitz et al. (2003) showed that reduced mitochondrial proteins isolated following TBI in cortex. Our data showed even in intact mitochondria or slightly damaged mitochondria, protein or polypeptide levels

related to energy metabolism, transport, and mitochondrial transition pore are decreased. Lower levels of key mitochondrial proteins could lead to mitochondrial dysfunction and significant oxidative stress, which could reduce mitochondrial protein yield (Cahill et al., 1997).

4.3.2 Mitochondrial Proteomics of Hippocampus

Our proteomics study of cortical mitochondria following TBI showed significant changes in proteins involved in the respiratory chain, TCA cycle and the MTP 24 h post injury, especially in IP cortex. In cortical TBI, hippocampus receives less mechanical injury although secondary injury was demonstrated. Mitochondrial damage was evident 24 h post injury, as demonstrated by disrupted cristae, thinned outer membrane and ballooned appearance, and ATP depletion in CA3, but not CA1. In order to evaluate changes in mitochondrial protein levels in TBI, ICAT proteomics was carried out as described above. Twenty-nine proteins were identified in both IP and CON hippocampi at 1 and 24 h post injury, and 20 proteins showed significant changes. The proteins identified included proteins involved in energy metabolism, small molecule transport, and the MTP.

Previous studies showed mitochondrial respiration was suppressed in hippocampus following TBI (Lifshitz et al., 2003) resulting in decreased ATP production. Complex I is one of the major electron entry points for the electron transport chain, and the largest and most complicated respiratory complex consisting of at least 46 subunits (Fearnley et al., 2001). Complex I defects are associated with neurodegenerative diseases and aging (Lenaz et al., 1997; Schapira, 1998). NADH-ubiquinone oxidoreductase B18 subunit (NADH dehydrogenase [ubiquinone] 1 beta subunit 7), which functions to transfer electrons from NADH to ubiquinone with the generation of a proton gradient, is significantly decreased 24 h post injury in IP hippocampus, but increased in CON hippocampus. At 1 h post injury, this protein is maintained in IP and CON hippocampus. The decrease of NADH-ubiquinone oxidoreductase B18 subunit could lead to complex I dysfunction. In Complex III, our data show several protein subunits, including ubiquinol-cytochrome-c reductase complex core protein 2 and ubiquinol-cytochrome C reductase complex 11 kDa protein, increased significantly 24 h post injury in IP hippocampus, although no significant changes were observed at 1 h. These changes show a different

response compared with cortical mitochondria. The increase of protein expression could improve the availability of these polypeptides in complex assembly and improve mitochondrial function. As for complex V, ATP synthase gamma chain increased in CON hippocampus 24 h post injury. The increase of protein expression 24 h post injury may indicate a compensatory response of cells to injury. Although ATP synthase gamma chain and ATP synthase subunit alpha decreased 1 h post injury in IP or CON hippocampus, the change is very small.

The TCA cycle involves multiple enzymes and is a key process for ATP production in mitochondria. Citrate synthase, dihydrolipoyl (dihydrolipoamide) dehydrogenase, and glutamate dehydrogenase were significantly increased in IP hippocampus 24 h post injury. Citrate synthase is the first enzyme that controls the entrance of carbon into the TCA cycle (Krishnan et al., 2003). The increase of this enzyme may suggest upregulation of the TCA cycle in an effort to speed up energy metabolism. Increases of dihydrolipoyl dehydrogenase could lead to increased NADH production in IP hippocampus. Aconitate hydratase and malate dehydrogenase increased in CON hippocampus 24 h post injury. Increased aconitate hydratase could show increased TCA energy metabolism in CON hippocampus 24 h post injury. Isocitrate dehydrogenase [NAD] subunit alpha increased 1 h and 24 h post injury in CON hippocampus. Increases of malate dehydrogenate and isocitrate dehydrogenase [NAD] subunit could increase NADH production. Aspartate aminotransferase, another enzyme identified in this study is thought to be expressed in liver and heart, was shown increased levels at 24 h post injury in brain hippocampus. The increase of the protein suggests a compensatory response of mitochondria, and tries to produce more substances for ATP production.

Our study also identified proteins involved in glycolysis, protein transport and folding, and formation of the mitochondrial transition pore (MTP). Hexokinase 1, a member of the glycolitic pathway and the MTP, was decreased in IP cortex 1 h post injury. In contrast, it increases to control levels 24 h post injury. Similar trends were observed in CON hippocampus at this time point. The increase of HK1 could reduce cytochrome a release and inhibit cell apoptosis. These data suggest the presence of mitochondrial recovery or repair following TBI. A carrier protein, adenine nucleotide

translocator 1 (ANT1), functions to transport ATP and ADP in oxidative phosphorylaion. Our data show ANT1 levels increase in IP and CON hippocampus 24h post injury, which suggests increased mitochondrial metabolism at this time point in both IP and CON tissues. Voltage-dependent anion-selective channel protein 1 (VDAC1) and voltage-dependent anion-selective channel protein 3 (VDAC3) are key proteins regulating permeability of the outer membrane of mitochondria (Rostovtseva et al., 2005). Our data showed the two protein expression levels decreased slightly in IP hippocampus 1 h post injury, and maintained in IP hippocampus at 24 h post injury. VDAC3 levels go up in CON hippocampus at 24 h post injury. The decrease of the proteins may delay response to calcium in IP hippocampus 1 h post injury. The increase of pore proteins in CON hippocampus 24 h post injury may increase mitochondrial response to calcium. Heat shock protein, HSp60, is increased in CON hippocampus 24 h post injury. Previous studies show HSP60 levels increased in cerebrospinal fluid following TBI (Lai et al., 2006).

Mitochondrial precursor protein import receptor was shown to be significantly increased in CON hippocampus 1 h post injury. The increased protein level could indicate cells are trying to increase uptake of nuclear coded protein levels in mitochondria, and mitochondrial repair and recover.

Lifshitz et al. showed mitochondrial damage and diminished calcium response in hippocampus using fluid-percussion injury model (Lifshitz et al., 2003). In cortex, our data show protein levels related to the respiratory chain, TCA and carrier proteins and MTP proteins decreased, which agree with the damage described by Lifshitz et al. Our data from hippocampus show that those proteins in isolated mitochondria increased following injury. It is interesting to note that the mitochondria in hippocampus showed an earlier response to TBI than cortex even though it receives less mechanical injury. However, Lifshitz et al. showed hippocampal mitochondria received less damage than cortical mitochondria. It is possible that the mitochondrial repair mechanism by multimitochondrial fusion lead to an increase in levels of some proteins in isolated intact hippocampal mitochondria (Busch et al., 2006; Chan, 2006). The increased levels of critical proteins in intact mitochondria may speed up ATP production in cells and compensate the consequence of some mitochondrial damage. Mitochondrial

channel protein levels were maintained and other energy metabolic related enzyme increased in our study. However, Lifshitz et al. showed reduced CsA-sensitive and CsA-insensitive swelling and deficits of the respiratory chain following fluid-percussion injury. Our data showed increased oxidative stress in hippocampus following TBI. It is possible that oxidative damage in mitochondria lead to dysfunction of channel proteins involved in mitochondrial membrane permeability (Singh et al., 2006).

4.4 Protective Property of Creatine in TBI

The aging study in TBI shows significant oxidative damage. These data suggest treatment of TBI patients with antioxidants may be helpful in recovery. Creatine is shown to have antioxidant properties, and also enhance energy storage and stabilization of the mitochondrial permeability transition pore (MTP) (O'Gorman et al., 1997; Sullivan et al., 2000; Wyss and Kaddurah-Daouk, 2000). Previous studies show creatine protects against glutamate, 1-methyl-4-phenylpyridinium (MPP^+) and β-amyloid toxicity, and blocks oxidative damage and inhibits cytochrome c release (Klivenyi et al., 1999; Matthews et al., 1999; Brewer and Wallimann, 2000; Brustovetsky et al., 2001; Scheff and Dhillon, 2004; Zhu et al., 2004; Bender et al., 2005). The present data suggest creatine reduces oxidative stress and increases antioxidant enzyme capacities in TBI.

In TBI, the hippocampus undergoes significant neuron degeneration and significantly increased lipid peroxidation 1 and 7 days post injury. In cortex, primary mechanical brain injury causes cell death in the impact zone that likely involves complicated processes such as oxidative stress that are evident 1 and 7 days post injury. Previous studies show creatine supplementation significantly reduces free fatty acid levels in TBI and inhibits oxidative injury in animal models of amyotrophic lateral sclerosis (ASL) (Klivenyi et al., 1999; Scheff and Dhillon, 2004). Our data show levels of HNE were significantly reduced in IP hippocampus and cortex of creatine treated animals compared to animals with a regular diet. Previous studies also show creatine supplementation increased creatine and phosphocreatine (Pcr) stores in brain (Dechent et al., 1999). Creatine kinase, creatine and phosphocreatine play a key role in energy buffering and transport, and can help reduce glutamate toxicity and maintain Ca^{2+} homeostasis, which may block oxidative processes (Hemmer and Wallimann, 1993; Wallimann and Hemmer, 1994; Bender et al., 2005). In addition, creatine can stimulate

oxidative phosphorylation and inhibit mitochondrial transition pore opening, which can increase bioenergetic synthesis and may prevent leakage of ROS from mitochondria (Wallimann et al., 1992; Kay et al., 2000; Meyer et al., 2006).

Our study shows creatine can improve antioxidant capacities, which can contribute to reduced oxidative stress. Previous studies show SOD activity decreases following TBI. The experiments conducted in this study show that Cu/ZnSOD activity was significantly increased to near CON levels in IP cortex and hippocampus of creatine treated animals compared with regular diet animals. It is possible that increased phosphocreatine level in cytosol helps to direct protein repair by such enzymes as HSP70 and disulfide isomerase following injury to maintain the enzyme pool with normal function and lead to higher enzyme activity (Lu et al., 2001; Hansel et al., 2002; Kregel, 2002). MnSOD was significantly increased to CON levels in IP hippocampus of creatine treated animals compared with controls, but no significant difference was observed in IP cortex. The mitochondrial proteomics study showed mitochondria were severely damaged in cortex, which could result in ATP shortage in the mitochondria for MnSOD folding and repair. Unfortunately, phosphocreatine in cytosol may not help since the disruption is primarily mitochondrial. Mitochondria in hippocampus receive less damage, and ATP production and enzyme repair may be maintained. The increase of SOD improves the cell's ability to remove superoxide generated after TBI and reduce lipid peroxidation. Previous studies show Vitamins C and E reduce oxidative stress and increase antioxidant capacities (Murugesan et al., 2005). Because creatine can function as a direct antioxidant (Lawler et al., 2002) it can neutralize ROS and reduce oxidative damage in a similar fashion as Vitamins E and C.

Glutathione peroxidase reduces hydrogen peroxide to water with consumption of glutathione. Previous studies show GPx activity increased following TBI since this enzyme is greatly induced by oxidative stress (DeKosky et al., 2004). Our data show GPx activity decreased in IP tissues in animals with creatine supplementation compared to controls. Aging studies show dietary restriction extends life by reducing oxidative stress. Meanwhile, dietary restriction reduces GPx activity in aging (Baek et al., 1999). Reduced GPx could be an indicator of less oxidative stress, although diminished activity may accelerate oxidative damage in IP tissues. Lindenau et al. (Lindenau et al., 1998) reported

that GPx was localized predominantly in microglia in CNS, and was shown to be upregulated in microglia and astrocytes, but not in neurons after CNS lesion. Based on previous studies showing that TBI initiated an inflammatory response and led to neuron damage (Lu et al., 2005), our data suggest that creatine may inhibit microglia and astrocyte activation, and GPx expression.

Glutathione transferase can alleviate oxidative damage by catalyzing conjugation of glutathione (GSH) with nucleophilic lipid peroxidation products, especially aldehydic by-products (HNE and acrolein). In this study, GST activity increased significantly in IP hippocampus of animals provided with a 2% creatine diet. GST activities showed a significant dose dependent increase among the three groups. An increase in GST activity suggests creatine improves the animal's capacity to decrease oxidative damage. In contrast to cortex, GST activity did not change in the hippocampus. Glutathione reductase, which functions to regenerate reduced glutathione, showed reduced activity following TBI in our previous study. Our current study shows a significant increase in GSSG-R in IP cortex of creatine supplemented animals. An increase of GSSG-R activity suggests the cortex may have a greater capacity to maintain the reductive environment following TBI compared to control, which can protect cells from damage.

Creatine supplementation significantly reduced lipid peroxidation, especially the amount of HNE, which is the major products of lipid peroxidation and is believed to be an important marker in oxidative stress (Esterbauer et al., 1991). Although no statistically significant differences in acrolein levels were observed in cortex and hippocampus, the trend showed levels of acrolein were reduced in animals provided a creatine supplemented diet (Lee et al., 2006). Animals provided creatine supplementation did not show alterations in oxidative damage or antioxidant capacity in CON cortex or hippocampus suggesting creatine functions in injury. In addition, 2% creatine supplementation did not show more protection than 1%, except for GST in IP hippocampus in present study.

CHAPTER FIVE

Conclusion

TBI is a major public health concern in industrialized nations primarily due to motor vehicle accidents. TBI is a tragedy for patients and their families, and costs about $20 billion for treatment annually in the US. Additionally, improved living conditions have led to extended longevity of humans, and a significant increase of the number of people age 65 and over. Elderly subjects 75 years or older, show higher death rates following brain injury due to motor vehicle accidents or falls.

TBI patients suffer a variety of potential problems because of neuron damage near and remote from the site of injury. Studies to assess neuronal damage following TBI is the key to finding effective treatments. Research over the past 30 years suggestes the pathology of TBI occurs at the cellular and molecular level. Currently, it is widely agreed that secondary injury is important in neurodegeneration following TBI. Current data suggest the primary mechanical injury is not treatable, leaving secondary injury as the best hope for treatment in TBI. Although excess excitatory amino acids, mitochondrial dysfunction, oxidative damage, adenosine and triphosphate adenine nucleotide depletion, inflammatory events, ion homeostasis disruption, proteolytic enzymes activation, DNA damage, and cholinergic receptor dysfunction have been shown to contribute to secondary injury, the exact mechanism is still unclear. We hypothesize oxidative damage and compromised antioxidant capacity contribute to neurodegeneration following TBI. This dissertation described oxidative damage and mitochondrial changes in TBI.

In human TBI, computed tomography can be performed immediately following injury to evaluate tissue damage by measuring the size of infarct or hemorrhage in the brain. At the site of mechanical injury, tissue loss or shrinkage is observed following neuronal death or damage hours, days or weeks post injury. Neurons are the basic unit of brain, and neuron loss could lead to series of problems for patients even though they survived. Severity of neuronal loss or damage indicates the more severe injury patients suffer, and the more problems patients could carry in the future. Unfortunately, neurons cannot regenerate although current studies are aimed at regeneration of neurons by transplant.

The work described in this dissertation is the first to measure tissue sparing following TBI as a function of age using Fisher-344 rats subjected to a controlled cortical impact injury. Although human subjects can suffer injury at any area of the brain and injury is probably more complicated, the animal model used in current study can mimic pathology in human brain injury. In our study, significant tissue loss was observed 7 days post injury, and showed an age-dependent increase in aging. This is the first study to show tissue sparing in TBI in aging using young, middle aged and aged animals. The increase of tissue loss in aging may lead to more problems for aged animals. These results suggest the aged brain is more fragile and aged neurons are less durable than young ones.

However, an interesting question is why the response of aged brain is different from young brain following TBI. The answer to this question could significantly improve our understanding of the mechanism of secondary injury in aging and led to improved treatment of aged patients. This dissertation showed significant lipid peroxidation and DNA oxidation in cortex and hippocampus following TBI in each age group that increased in an age dependent format. This is the first study to evaluate oxidative damage in TBI in aging. Lipid peroxidation is the consequence of oxidative damage to polyunsaturated fatty acids, leading to formation of aldehydic byproducts that modify proteins and DNA and lead to further damage to cell. DNA damage can lead to gene mutations and affect transcription and protein expression, leading to alteration in normal cellular function. Because the animal model used in current study mimics human TBI, our results suggest oxidative damage may also happen in human brain. Additionally, the data described in the dissertation showed an age-dependent increase of lipid peroxidation following TBI, which could lead to aged neurons more fragile than young neurons.

The increase of oxidative damage and its age-dependent changes following TBI suggest cellular defenses toward oxidative damage may be compromised following TBI and are probably affected by age. So we measured the antioxidant enzyme activities following TBI. We measured MnSOD, Cu/ZnSOD, GPx, GSSG-R, and GST activities, enzymes that play different rules at different stages in cellular defense. MnSOD and Cu/ZnSOD convert superoxide to hydrogen peroxide, and GPx converts hydroperoxides and to alcohols or water. GSSG-R helps to maintain the reduced glutathione pool, and

GST can detoxify xenobiotics and endogenously produced aldehydes, including lipid peroxidation byproducts. Since MnSOD is located in mitochondria where most ROS are produced, it is probably more important than other enzymes. Small changes in this enzyme may lead to mitochondrial damage by superoxide and compromise ATP production, leading to energy shortage in cells. Because most biological reactions are ATP dependent, the ATP shortage could slow down or shut down most biological process, leading to cell dysfunction. We observed significant decrease of MnSOD in cortex following injury with aged animals showing more pronounced loses than young adults. In the hippocampus, young animals maintained MnSOD activity until 24 h post injury, and decreased 7 days post injury. But for middle aged and aged animals, MnSOD decreased significantly beginning 24 h post injury. These results suggest aged animals may have diminished capacities to remove superoxide leading to increased damage in mitochondria. Cu/ZnSOD decreased in cortex 24 h and 7 days post injury. The decrease of Cu/ZnSOD activity suggests damage in oxidative defense in cytoplasm. It is interesting to note that MnSOD activity changed earlier than Cu/ZnSOD in hippocampus, and decreased at 7 days post injury but Cu/ZnSOD did not. It is possible that oxidative damage begins in mitochondria and spreads to cytosol. In cortex, young animals showed a loss of Cu/ZnSOD activity 24 h post injury, and middle aged and aged animals showed decreased Cu/ZnSOD 7 days post injury. MnSOD activity did not change in young animals 24 h post injury, and decreased 7 days post injury, suggesting different responses in cortex and hippocampus.

In cells subjected to oxidative damage, mitochondria are thought to be critical important as the major source of ROS. Our data show a significant loss of MnSOD following TBI. An understanding of mitochondrial changes following TBI may help yield clues for TBI treatment. For example, with an understanding of what is going on in neuronal mitochondria following TBI, it might be possible to target mitochondria in patient treatment. In order to investigate changes in mitochondria following TBI, we carried out studies of mitochondrial protein. Our data show mitochondrial protein expression changes in cortex and hippocampus at 1 h and 24 h post injury. This is the first study to examine how mitochondrial protein changes in isolated mitochondria following TBI. An early time point (1 h) was selected for this study because previous

studies from other labs showed early changes in mitochondria following TBI. We used the isotope-coded affinity tag (ICAT) method to label mitochondrial proteins, and following protein trypsinization and peptide purification, peptides were sequenced and quantified using 2D LC/MS/MS. ICAT analysis of mitochondria from cortex and hippocampus showed different responses to injury. Proteins or polypeptides of proteins from the respiratory chain, and TCA cycle and channel proteins decreased significantly in cortex 24 h post injury. In contrast, increased protein levels were observed in hippocampus at this time point in both IP and CON hippocampus. A decrease of complex I to complex V polypeptides in cortical mitochondria could lead to deficiencies of there complexes, and an increase of ROS leakage, reducing the proton gradient and ATP synthesis. ROS leakage could damage unsaturated fatty acids and the enzymes in mitochondria, including MnSOD, leading to increased oxidative damage in cytosol. Decreased levels of shuttle proteins for NADH transport from the cytosol to mitochondria could lead to shortages of NADH in mitochondria for the electron transport chain. Additionally, deficiencies in the TCA cycle following TBI could result in increased NADH shortages. Voltage gated channel proteins function to regulate permeability of outer membrane of mitochondria, and the observed decrease of their expression levels in cortex could compromise metabolite exchanges between cytosol and mitochondria, including ATP and calcium. These changes in mitochondria following TBI could shut down mitochondrial function, leading to neuron dysfunction or death by triggering apoptosis or necrosis. In the hippocampus, an increase of TCA enzymes could increase NADH synthesis for the respiratory chain, and increased electron transport. However, we observed one polypeptide, NADH-ubiquinone reductase B18 subunit that was significantly decreased. Although function of this protein is unclear, the deviation of its levels from normal may diminish the respiratory chain. Channel protein and heat shock protein expression levels increase 24 h post injury in hippocampus. The increase of VDAC protein could increase the permeability of outer membrane of mitochondria and speed up metabolite and ion exchange between cytosol and mitochondria, and increase the response to calcium following injury. Although previous studies by Lifshitz eta al. (2003) showed mitochondrial shape changes and damage to the respiratory chain in hippocampus following fluid percussion injury, our data showed protein levels increase

in mitochondria. Hall et al. (2006) showed increased oxidative damage in mitochondria following TBI. It is possible that oxidative damage in mitochondria lead to mitochondrial deficits even though increased mitochondrial proteins. For future studies, we want to elucidate why the mitochondrial protein expression changes following TBI and how oxidative damage affects individual proteins in mitochondria.

The data described in this dissertation showed oxidative damage and changes in mitochondrial proteins following TBI, and these could lead to neuron dysfunction. We observed compromised antioxidant capacity in our study. If we can find a method to reduce oxidative damage through increased antioxidant defenses and protect mitochondria following TBI, it could be very helpful in the recovery of TBI patients.

Creatine is an endogenously synthesized small molecule in liver and kidney, and is released to blood stream in human and other mammals. Creatine can cross the blood brain barrier through a specific creatine transporter CRT, so its possible creatine may be a good candidate drug to reduce oxidative damage and protect mitochondria. Clinical studies in muscle disease showed creatine did not show side effects on patients (Felber et al., 2000). Previous studies have shown oral or intracerebroventricular creatine to be neuroprotective in PD, ALS, spinal cord injury and TBI. Pilot study carried out with 34 patients from 1 to 18 years old showed creatine was beneficial to TBI patients with no side effects observed in an oral administration at a dose of 0.4 gr/kg (Sakellaris et al., 2006). The administration of creatine to TBI patients improved patients in several aspects, including duration of post-traumatic amnesia, duration of intubation, intensive care unit stay, disability, general recovery, self care, communication, locomotion, sociability, personality/behavior and neurophysical, and cognitive functions. Significant improvement was recorded in the categories of cognitive, personality/behavior, self care, and communication. Unfortunately, the mechanism by which creatine is unclear. To determine if creatine can reduce oxidative damage, we provided animals a creatine enriched diet prior to injury. The results described in this dissertation showed animals with creatine supplementation had significantly reduced lipid peroxidation following TBI. But how does creatine work to reduce oxidative damage? Further studies showed SOD increased in animals with a creatine diet. GSSG-R and GST also increased in IP tissues of animals with creatine supplementation. So we believe creatine can reduce

oxidative damage through increased antioxidant defenses, thus protect neurons. Of course, further studies are needed to elucidate how creatine administration affects antioxidant enzyme activity.

Altogether, creatine, an endogenously synthesized small molecule, showed benefitial function in primary clinical studies with small populations of patients. Previous studies in other research groups show creatine can protect mitochondria through regulation of MTP. Our data show creatine can reduce oxidative damage by increased antioxidant enzyme activities. This is the first study to evaluate how creatine affects antioxidant defense *in vivo*. This new study improved our understanding the function of creatine, and further approved the beneficial effects of creatine supplementation to patients with brain injury. We believe creatine may be a potential therapeutic for TBI patients. Although, more clinical studies are needed, including post injury treatment studies, pharmaceutical studies for the optimum dose.

Appendix

Nomenclature

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CON, contralateral; Cu/ZnSOD, copper/zinc superoxide dismutase; FA, fapyadenine; FG, fapyguanine; GPx, glutathione peroxidase; GSSG-R, glutathione reductase; GST, glutathione transferase; HNE, 4-hydroxynonenal; IP, ipsilateral; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; PD, Parkinson's disease; TBI, traumatic brain injury; MPT, mitochondrial permeability transition; 8-OHG, 8-hydroxyhuanine; 8-OHA, 8-hydroxyaenine; 5-OHC, 5-hydroxycytosine; CCI, cortical controlled injury

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