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Dietary Selenium Supplementation: Effects on Neurodegeneration Following Traumatic Brain and Spinal Cord Injury

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DIETARY SELENIUM SUPPLEMENTATION: EFFECTS ON NEURODEGENERATION FOLLOWING TRAUMATIC BRAIN AND SPINAL CORD INJURY

DISSERTATION

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

By
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2015

ABSTRACT OF DISSERTATION

DIETARY SELENIUM SUPPLEMENTATION: EFFECTS ON NEURODEGENERATION FOLLOWING TRAUMATIC BRAIN AND SPINAL CORD INJURY

Traumatic brain and spinal cord injury continue to be substantial clinical problems with few available treatment strategies. Individuals who are at a greater risk for sustaining a central nervous system (CNS) injury, such as professional athletes and military personnel, may benefit from a prophylactic supplement that would intervene in the neurodegenerative pathways immediately following injury. The high demand for selenium within the central nervous system, as well as the synthesis of selenoproteins by neurons and astrocytes suggests a critical role of selenium within the brain and spinal cord. Studies were designed to test the efficacy of enriched dietary selenium status in providing neuroprotective benefits in rodent models of spinal cord and traumatic brain injury. Levels of selenium storage within the CNS are increased relative to the amount of selenium present in the diet, indicating that selenium compounds effectively cross the blood brain barrier.

In a model of moderate severity spinal cord contusion injury, dietary selenium supplementation reduced the number of days until recovery of independent bladder function following injury. These benefits did not translate to improvements in locomotor function during open field testing or reduction in overall lesion volume in the injured animal groups. Examination of gene expression changes 24 hours after spinal cord injury revealed that dietary selenium enrichment increased expression of genes involved in DNA repair, mitochondrial respiration, and transcriptional regulation. By expanding the scope of these studies to include models of traumatic brain injury, these data show the importance of selenium in the cortex as well. In particular, when compared to diets deficient in selenium, higher levels of dietary selenium improve spatial memory performance and mitochondrial respiration. The results of this dietary study show modest improvements following both traumatic brain and spinal cord injury and suggest that while selenium enrichment may not have a profound effect on the secondary injury cascade immediately following injury, the presence of adequate dietary selenium is critical for mitochondrial respiration.

Together the results of these studies suggest that dietary supplementation may play a subtle role in injury mechanisms within the CNS and warrant further investigation.

KEYWORDS: Brain Injury, Spinal Cord Injury, Selenium, Gene Expression, Mitochondrial Respiration

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5/11/2015
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1.1 Overview of Selenium

1.1.1 Selenium supplementation

Selenium in its elemental state is a non-metal element with the same valency as sulfur, allowing for it to be incorporated in place of sulfur in certain amino acids. Selenium is also an essential nutrient for human health and must be obtained through dietary sources. Natural intake occurs predominately through grains and meat[1]. Selenium is present in the soil in varying levels, dependent upon climate and specific soil composition. Plants take up selenium directly from the soil as either the inorganic salt, selenite, or as selenate, a more readily incorporated form of selenium. Plant species incorporate soil selenium as the amino acids selenomethionine and selenocysteine, or as selenate. Following uptake and incorporation in plants, herbivores receive their necessary levels of selenium from plants grown in agricultural areas rich in selenium. Within mammalian tissues, elemental selenium is stored in the form of different selenoamino acids within specific selenoproteins. The approximately 25 characterized selenoproteins all possess different cellular localization specific to that particular selenium species[2].

In modern times, selenium deficiency is somewhat rare due to increased transport and consumption of foods from various regions. However, isolated cultures that predominately eat plant and animal species grown locally can be at risk for selenium

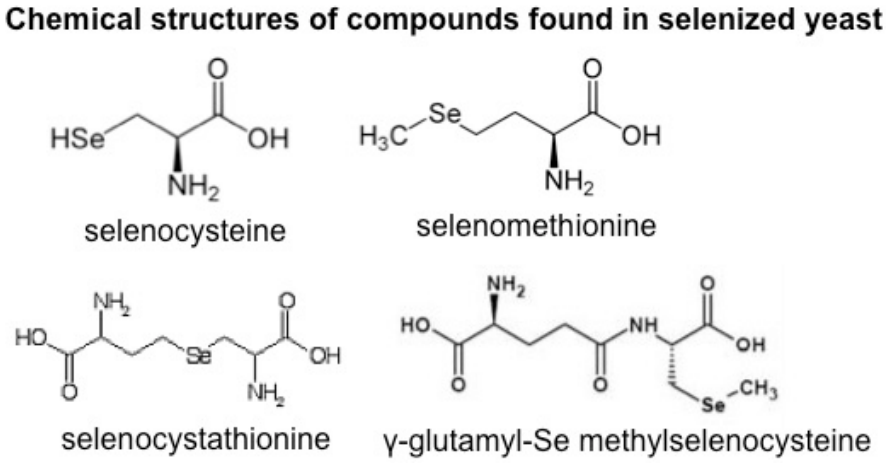
deficiency if the regional soil lacks adequate selenium levels. One of the first clinically documented cases of selenium deficiency occurred in the 1970s in an isolated, rural area of China with selenium deficient soil conditions. Keshan disease is characterized as a cardiomyopathy linked to deficient selenium dietary conditions, and initiated by a high viral load of the coxsackie virus (CVB3). Increasing dietary selenium levels raises immunity and reverses the clinical pathology[3]. Keshan disease is still prevalent in certain areas of China[4]. While most countries do not have high incidences of selenium deficiency, certain populations may be receiving a sub-optimal nutritional dose. The minimum dietary intake required to prevent disease and maintain selenoprotein activity is estimated to be between 20-50μg/day, with supranutritional protective levels estimated to be around 120μg/day[5]. Deficient conditions are typically measured using plasma selenium concentrations, however some clinicians also use activity of certain selenoproteins as a more accurate measure of selenium status in humans[6]. During dietary deficient conditions, the brain and spinal cord prioritize the need for selenium[7], suggesting a critical role within the central nervous system (CNS). Even though the current recommended dietary allowance (RDA) is 55μg/day for adults[8], a reference dose (RfD) that allows for a larger intake still within safe ranges indicates total safe selenium consumption as high as 350μg/day[9]. Some studies suggest that selenium deficiency may contribute to disease susceptibility. Deficient conditions result in difficulty in processing viral load, specifically by regulating expression of cytokines and chemokines with influenza infection[10], while supplementation with selenium decreased overall HIV viral load[11].

Toxicity, or selenosis, is characterized by brittle nails and hair loss, but may also result in skin lesions, nausea, fatigue, and a distinct garlic-like odor on the breath[12]. The tolerable upper intake level (UL) set by the Food and Nutrition Board of the Institute of Medicine for selenium consumption in adults is 400μg/day[8]. However, toxicity is largely dependent upon the form of selenium. Some residents in a region of high selenium soil content in South Dakota were reported to consume up to 700μg/day from locally raised livestock high in selenium with no adverse consequences[13]. A commonly known form of selenium, selenium disulfide in anti-dandruff shampoos, utilizes cytotoxic properties as an anti-fungal agent topically to control the scalp issues. However, taken orally, selenium disulfide has an average lethal dose (LD_{50}) lower than that of the selenium present in multivitamins[14]. Natural forms of selenium, such as selenomethonine and selenocysteine, show a significantly smaller level of toxic potential when compared to inorganic selenium salts, such as sodium selenite or selenate[15]. Selenized yeast, which incorporates inorganic selenium as selenomethionine and selenocysteine, also has higher bioavailability and decreased toxicity issues. The brand of selenized yeast utilized in these studies, Sel-Plex®, has a lower level of both acute and subchronic toxicity in mouse, rat, and canine models when compared to inorganic sodium selenite[16]. Specifically, the LD_{50} of acute administration of Sel-Plex is ≥2500mg/kg in rats and the overall no observed adverse effect level (NOAEL) is 30mg/kg/day whereas similar levels of inorganic sodium selenite resulted in occasional mortality and clinical chemical changes. The predominate form of selenium used to supplement infant formulas, as well as mutlivitamins on the market, are typically either sodium selenite or sodium selenate.

Thus, it is critically important to consider the average lethal dose and the tolerable upper intake level of selenium formulations before utilizing them in clinical studies.

Selenized yeast has also come to the forefront of supplementation studies as an effective method for delivering high levels of bioavailable selenomethionine. Yeast is grown in the presence of selenium-enriched media. Selenized yeast incorporates inorganic selenium (selenite) from supplemented media into the cell wall of the yeast and converts it into the seleno amino acids, selenomethionine and selenocysteine[17]. Along with selenomethionine and selenocysteine, selenized yeast has a unique chemical profile of other selenoproteins and selenometabolites. Selenized yeast is composed of approximately 70-90% L(+)selenomethionine, 10% selenocysteine, and a small percentage each of selenocystathionine, γ-glutamyl-Se methylselenocysteine, and potentially other predicted selenospecies (Figure 1.1)[18, 19].

A



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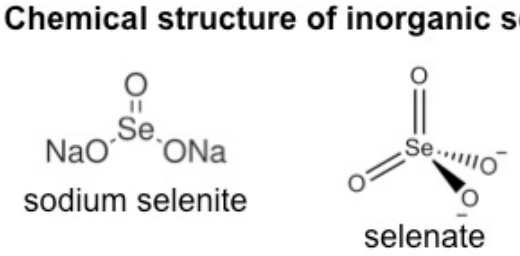


Figure 1.1: Chemical structures of selenium compounds. Different components of both selenized yeast (**A**) and inorganic selenium compounds (**B**). Selenized yeast is composed of a unique chemical profile of different selenium containing compounds including the amino acids selenocysteine and selenomethionine, as well as various derivatives such as selenocystathionine, and γ-glutamyl-Se methylselenocysteine. Inorganic selenium compounds are typically supplemented into multi-vitamins or livestock diets in the form of sodium selenite or selenate.

1.1.2 Selenium metabolism

Once selenium sources reach the digestive system, transport systems for seleno-amino acids utilize Na^+ dependent transport, much like is utilized for other amino acid absorption[20]. Selenite diffuses directly through the endothelial membrane of the small intestine. These differences in absorption may explain the critical differences observed in toxicity between these two formats. Selenoprotein P (SelP) is the primary transporter for selenium in the plasma for delivery to tissues throughout the body[21]. Once selenium reaches the target tissue, it can either be converted into selenide, the precursor for selenoprotein biosynthesis or, in the case of selenomethionine, incorporated in place of methionine in normal body proteins (Figure 1.2). The transfer RNA for methionine ($tRNA^{Met}$) cannot distinguish between methionine and selenomethionine. This allows for selenomethionine to be stored in high levels in tissues and thus readily available for conversion back into selenide for selenoprotein synthesis as needed. In particular, the brain stores large amounts of selenomethionine, emphasizing the importance of selenium within the CNS[22].

When selenium is needed from tissue stores, selenomethionine is converted into the intermediate selenocystathione, and then into selenocysteine. Selenocysteine, either from conversion of selenomethionine or from dietary sources, is converted into selenide by selenocysteine β-lyase or bacterial methionase in the intestine[23]. Selenide is then made into selenophosphate and through the selenocysteine synthetase can be attached to the selenocysteyl-tRNA ($tRNA^{Sec}$) for selenoprotein biosynthesis. The SECIS must be present upstream (within the 3' UTR) of the of the UGA codon for translation to occur. Mutations present in the SECIS are detrimental for incorporation

of selenium into selenoproteins. The SECIS binding protein (SBP2) has been found through different cross-linking studies to be critical for selenoprotein synthesis, in addition to the selenocysteyl-tRNA and the SECIS[24]. By binding to the SECIS, SBP2 prevents the translational machinery from reading the UGA as a stop codon and translation of Sec can proceed.

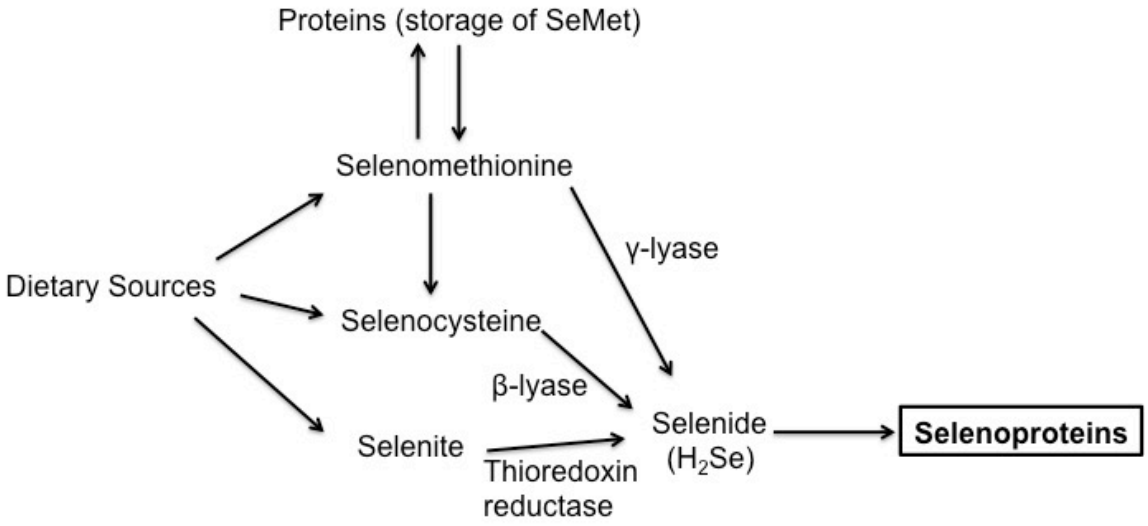


Figure 1.2: Selenium metabolism. Selenium is typically obtained from dietary sources in one of three formats, selenomethionine, selenocysteine, or the inorganic selenite (can also be in the form of selenate). Selenomethionine can be easily incorporated into bodily tissues in place of methionine, as $tRNA^{(Met)}$ does not distinguish readily between methionine and selenomethionine. This serves a store for selenium in tissues in cases of deficiency. Selenomethionine can also be converted to selenocysteine or broken down by γ-lyase into methyl-selenol (CH_3SeH), which can then be converted into selenide. Selenocysteine is converted into selenide by the enzyme selenocysteine β-lyase. Selenite is converted into the intermediate selenoglutathione by thioredoxin reductase and then into selenide. Selenide (H_2Se) is the precursor for all selenoprotein synthesis. Adapted from Schrauzer GN[19].

1.1.3 Selenoproteins

Selenoprotein biosynthesis requires dietary intake of selenium. Certain selenoproteins, such as selenoprotein P (SelP), transport selenium in the plasma to tissues for protein synthesis. Once converted to the precursor compound selenide, protein synthesis can then begin. The mRNA sequence of a selenoprotein utilizes a UGA codon, which normally functions as a stop codon, and also requires a selenocysteine (Sec) insertion sequence (SECIS). This insertion sequence is a stem-loop structure that is coded in the 3' untranslated region upstream of the coding mRNA[25]. Synthesis also requires the presence of a special tRNA, tRNA-Sec. In the amino acid cysteine, the sulphur atom is replaced by selenium. The selenocysteine residue functions as the crucial component in oxido-reductase activity in selenoprotein enzymes[26]. Based on genome searches using both the UGA codon and the upstream SECIS, approximately 25 selenoproteins have been identified[2]. Selenoproteins function as antioxidant enzymes, in redox signaling, thyroid hormone metabolism, transport and storage of Se, and putative protein folding[27]. While some groups of selenoproteins have well characterized functions, many need further study and these may play a role in cellular response to oxidative stress.

One of the predominant selenoprotein families critical for human health are the glutathione peroxidase (GPx) enzymes. Of the 7 characterized GPx enzymes, 5 are selenium dependent[27]. Glutathione peroxidase enzymes have potent antioxidant capability by catalyzing the reduction of hydroperoxides. GPx1, considered to be the classic glutathione enzyme, is found in the cytosol of cells, and has been shown to be protective against oxidative stress[28]. The other seleno-specific glutathione enzymes

include GPx2 (intestinal GPx), GPx3 (highly expressed in plasma), GPx4 (ubiquitously expressed and critical for sperm formation), and GPx6 (olfactory specific)[29]. As discussed, selenium is an essential component of several antioxidant enzymes and the glutathione peroxidase family enzymes are critical components of the human antioxidant response.

The group of thioredoxin reductases (TrxR) also plays a major role in the antioxidant defense system in human health. These enzymes can affect cell survival and apoptosis by scavenging reactive oxygen species [1]. The thioredoxin system acts on lipid hydroperoxides, a damaging by-product that is formed following brain and spinal cord injury. TrxRs play a crucial role in redox signaling by regulating thioredoxin, a central molecule in the redox pathway.

Selenoprotein P is another selenoprotein of importance in human health. SelP has up to 10 SeCys residues, allowing it to bind multiple selenium molecules at a time. Thus, SelP is the predominate transporter of selenium to peripheral tissues, including the central nervous system, and thus is highly expressed in the plasma[21]. Due to the high levels in plasma, SelP protein levels have been used frequently as a biomarker for selenium status[30]. SelP has dual functions and also has antioxidant properties, providing protection in the plasma from oxidation and nitration[31]. SelP knockout mice exhibit neurological deficits including axonal degeneration, impaired spatial learning, and altered hippocampal synaptic function[32, 33]. Additionally, SelP expressed in astrocytes is a unique membrane bound protein, distinct from the glycosylated and

secreted forms of SelP recognized in the plasma and extracellularly in other tissues[34]. This membrane bound SelP helps to protect astrocytes from oxidative damage, even selectively retaining selenium levels in cases of deficiency.

Selenoprotein S (SelS) has been identified as an important protein in protein folding and is localized within the endoplasmic reticulum[35]. SelS is also associated with inflammatory response. When examined in models of TBI, SelS was upregulated in astrocytes in response to injury. Prior to injury, expression of SelS protein is noted in naïve neurons, but not in astrocytes. This increase in SelS after injury suggests a potential protective role of this selenoprotein in response to induction of harmful inflammation that occurs after CNS injury[36].

Additionally, selenoprotein O (SelO) has been recently characterized as another selenoprotein that plays a role in redox signaling[37]. However, this particular protein is unique in that it appears to localize specifically to mitochondria. SelO expression was preserved under selenium deficient conditions, suggesting that this protein is critical for cellular function and must be maintained even in deficient conditions[37, 38]. The implications of a selenoprotein that functions as a mitochondrial specific redox regulator are particularly interesting in models of CNS trauma. Mitochondrial dysfunction generally precedes cellular death following injury and is thus an important target for investigation.

Table 1.1: Currently identified selenoproteins. Many selenoproteins have been identified and the function of these proteins has been identified and largely has been identified with antioxidant mechanisms and redox signaling in the cell. However, the function of several selenoproteins remains to be elucidated. Additional putative selenoproteins have been identified but are not yet characterized.

Protein	Function or potential function
GPx1	Antioxidant function
GPx2	Antioxidant function
GPx3	Antioxidant function
GPx4	Antioxidant function
GPx6	Antioxidant function
SelK	Antioxidant function
SelR	Antioxidant function
SelW	Antioxidant function
TrxR1	Redox signaling
TrxR2	Redox signaling
TrxR3	Redox signaling
SelO	Redox signaling, mitochondrial specifc
DIO1	Thyroid hormone metabolism
DIO2	Thyroid hormone metabolism
DIO3	Thyroid hormone metabolism
SelP	Transport and storage of selenium
SelS	Protein folding, ER specific
SelM	Potential for protein folding
SelN	Potential for protein folding
SelI	Unknown
SelH	Unknown
SelT	Unknown
SelV	Unknown

1.1.4 Selenium and modulation of gene expression

Selenium acts as a micronutrient that is an essential component for multiple different selenoproteins. In addition to being incorporated as altered amino acids into the active site of antioxidant enzymes, different levels of dietary selenium also have an effect on overall gene expression[39-42]. Changes in mRNA expression can be tissue specific, with suboptimal conditions decreasing expression levels of selenoproteins in different tissues, driven by post-transcriptional modifications. Regulation allowing translation of $tRNA^{(Sec)}$, and thus synthesis of selenoproteins, instead of reading the UGA sequence as a stop-codon is dependent upon the SECIS, located in an intron immediately upstream of the Sec codon[43]. Mutations within the SECIS will result in the Sec mRNA (UGA) being read as a termination codon instead of the Sec amino acid[44].

Selenium may also play a role in transcriptional regulation. In particular, the selenoproteins in the Trx family control cell signaling pathways via transcription factors[45]. TrxR enzymes are also part of the oxidoreductase family and can reduce oxidized Trx, selenite, and lipid hydroperoxides[27].

Several studies have examined the effect of different forms of selenium on overall gene expression. Studies examining specific selenoglycoproteins in tumor progression, showed that selenized yeast induced changes in gene expression in NF-κB and associated pathways, implying a larger role of selenium in transcriptional regulation[46]. Barger et al. also showed differential effects specific to the form of selenium supplementation in mouse models[39]. Selenium supplementation, regardless

of form, reduced the mRNA expression of Gadd45b, and reduced protein levels specifically in animals receiving selenized yeast. Expression of Gadd45b is associated with the onset of DNA damage and thus decreased expression is evidence of a protective response. Treatment with selenium, concurrent with the addition of lead, an environmental toxin, altered specific expression patterns[40]. Gene regulatory pattern analysis showed specific effects with organic selenium (selenized yeast and selenomethionine) in pathways associated with DNA repair, particularly those involved with base excision repair. These changes in gene expression also carried over to afford protection of cells from DNA damage after supplementation with organic forms of selenium.

1.1.5 Selenium and immune system function

Selenium is a critical component for healthy immune function. However, the exact mechanism is currently unknown. It is hypothesized that the antioxidant activities exert protective effects over immune function as well as in response to certain injuries[47]. Increasing levels of dietary selenium, when compared to selenium deficiency, help to improve the two components of adaptive immunity, humoral and cell-mediated immunity[48]. A study examining adults with selenium deficiency (below 1.2μmol/L) showed impaired immune function in response to an injection of a live-attenuated poliovirus. Selenium supplementation, in the form of sodium selenite, restored plasma selenium levels, cytosolic GPx activity, and increased production of cytokines[49]. The increase in selenium levels improved overall viral handling in these subjects. Different sources of selenium also improve the overall response against

certain forms of cancer. In particular, selenized yeast was used in a large scale clinical trial examining cancer prevention (Nutritional Prevention of Cancer Study Group) and has been shown to improve outcomes in carcinoma patients[50]. Selenium may function as an antioxidant through protection of DNA[51] or through cell cycle mediation[52]. Studies have also established the interaction of selenium with neutrophil function. Selenium deficiency leads to decreased GPx1 activity in neutrophils[53].

1.1.6 Selenium and the central nervous system

The well-studied antioxidant and immune functions of selenoproteins establish a crucial role in the central nervous system. While many studies have examined the therapeutic efficacy of selenium as preventative against cancer, other sources have laid the groundwork for the importance of selenium in central nervous system tissues. Several studies have emphasized the importance of antioxidants in the brain and spinal cord, particularly following neurotrauma[54]. Additionally, ebselen, a selenized yeast supplement provides neuroprotective effects in rodent cerebral artery occlusion and Alzheimer's Disease models[55-57]. Yeo et al. showed selenium provided neuroprotection *in vitro* from reactive oxygen species (ROS) induced damage in both traumatic brain and spinal cord injury[58, 59]. However, these studies used an injection of sodium selenite directly onto the epicenter of injury, immediately following injury. Sodium selenite, as discussed previously, has a high risk of toxicity. The form of selenium used, as well as the method of drug delivery, makes translation of these findings to the clinical setting unreasonable.

Selenium levels are also associated with various neurodegenerative pathologies. Patients with Alzheimer's disease (AD) have consistently lower selenium levels in the temporal lobe[60]. Additionally, supplementation with selenized yeast reduces overall amyloid plaque deposition and DNA/RNA oxidative damage in a mouse model of AD[34]. In neurons located in the substantia nigra in patients diagnosed with Parkinson's disease, expression of SelP is reduced[61]. SelP plays dual roles in transporting selenium to the CNS (and other tissues) and as an integral membrane protein in astrocytes[36, 62], suggesting a critical role for SelP in neuronal function. Additionally, recent studies have shown that selenium is protective against neuronal DNA damage in a rodent model of induced Parkinson's disease[63]. Other selenoproteins, such as GPx4, also protect the CNS from oxidative damage. Increased expression of GPx4 protects cortical neuronal cultures from cell toxicity induced by oxidative injury[64].

While the brain only contains approximately 2% of the total body selenium storage, in cases of selenium deficiency, the central nervous system prioritizes selenium retention over other tissues in the body[7]. Selenium levels are affected by ApoER2 expression. The major selenium transport protein, SelP, binds to ApoER2. In an ApoER2 knock-out model, mice without this transport protein exhibit lower brain and testicle selenium levels[65]. Once mice were kept on a selenium deficient diet, severe neurological dysfunction and death resulted. These studies further emphasize the importance of appropriate selenium levels for optimal function of the CNS.

1.2 Traumatic Brain Injury and Spinal Cord Injury

1.2.1 Overview of Traumatic Brain Injury

Traumatic brain injury (TBI) continues to be a prevalent clinical problem, with young adult males as the most vulnerable population due to their active lifestyle and a higher prevalence of military service[66]. Due to the diverse types of TBI presentations, confounding patient symptoms, and a complex secondary injury cascade, there have not been any successful pharmacological interventions for TBI to date. Attempts to target individual components of the multifactorial secondary injury cascade have resulted in only modest improvements in experimental animal studies and lack of significant results in clinical trials[67]. It is increasingly recognized that a treatment to intervene in the progression of the secondary injury cascade will need to have a broad spectrum approach.

In response to the initial mechanical injury of a TBI, the secondary injury cascade is activated in the following first hours and days. While it is difficult to prevent the initial injury, the molecular events that eventually lead to neuronal death provide a viable target for pharmacologic intervention. Mechanisms involved in the secondary injury cascade following TBI include hypoxia, Ca^{2+} dysregulation, mitochondrial dysfunction, free radical production, lipid peroxidation, and eventual cell death. Many studies have examined antioxidants and free radical scavengers as potential pharmacologic interventions[68]. Oxidative damage following injury has a significant effect on mitochondrial respiration.

As the location of the electron transport chain and the site for the manufacture of the main form of energy, ATP, mitochondria are considered the powerhouse of the cell. Over 90% of cellular ATP is synthesized in the electron transport chain (ETC). Damage to CNS mitochondria leads to a host of cellular problems, including a halt in ATP production, calcium dysregulation, and an increase in ROS production that ultimately leads to neuronal cell death. In the secondary injury cascade, degradation of the cellular membrane results in an influx of intracellular calcium. This calcium is typically buffered by mitochondria, which function as a calcium sink for neurons. However, an overload of calcium within the mitochondria leads to cellular membrane break down and the opening of mitochondrial permeability transition pore (MPTP)[69]. These events will eventually result in neuronal cell death. To further understand the interaction between injury and mitochondrial respiration, methods have been developed to examine the different components of the ETC. Endogenous and exogenous substrates are added directly to isolated mitochondria and the resulting consumption of oxygen in the system is measured. In normal conditions, the ETC utilizes a proton electrochemical gradient to drive the phosphorylation of ADP into ATP. Electrons are passed down the different complexes of the ETC, beginning with either complex I or complex II, and then subsequently transferred to complex III and finally complex IV. Complex IV will ultimately pass the electrons to oxygen, thus consuming oxygen in the system and allowing for a measurable readout of ETC function (Figure 1.4)[70, 71]. The electrochemical gradient is created by the flux of protons out of the mitochondrial matrix and into the intermembrane space. Energy

release from electron transfer between complexes of the ETC creates the necessary drive to move protons against their concentration gradient.

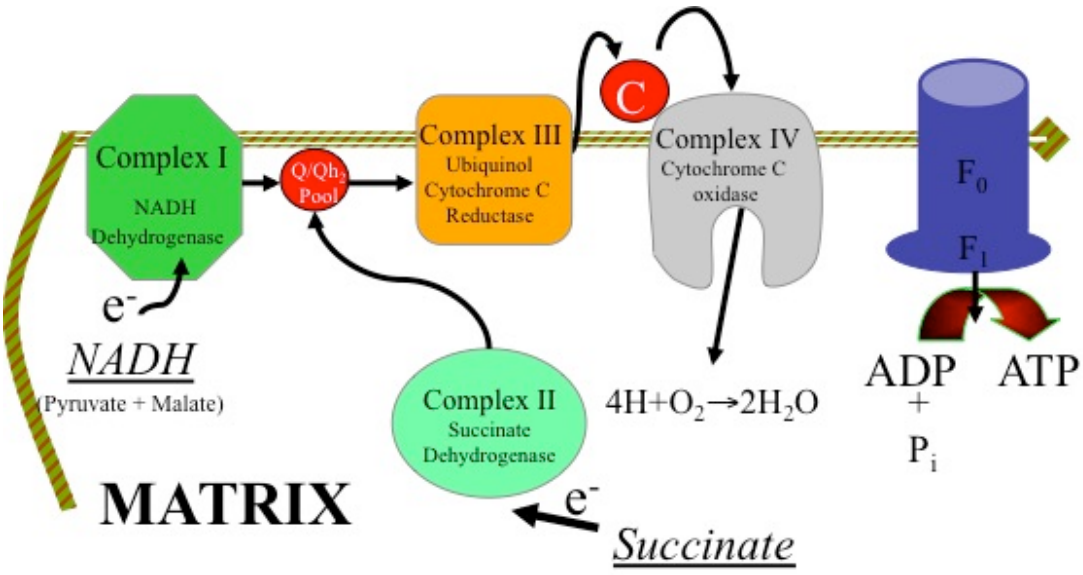


Figure 1.3: Electron transport chain. A representative diagram outlining the movement of electrons, oxygen consumption, and ultimately phosphorylation of ADP into ATP in the electron transport chain (ETC). Electrons are passed between the different complexes of the ETC and eventually transferred to oxygen by complex IV. Electrons can be passed into the ETC through either complex I or complex II. Each transfer of electrons to the subsequent complex releases energy that is used to drive protons against their concentration gradient and into the intermembrane space. The movement of protons across the mitochondrial matrix membrane creates the electrochemical gradient critical for ATP production. Protons eventually pass back into the mitochondrial matrix through the ATP synthase (F_0/F_1), driving the phosphorylation of ADP into ATP. Assays designed to measure the efficiency of mitochondrial respiration utilize endogenous and exogenous substrates added to isolated mitochondria to manipulate different complexes of the ETC. With this data, pathologies in specific steps within the ETC can be studied directly. Adapted from Sullivan et al, 2007[72].

1.2.2 Overview of Spinal Cord Injury

Spinal cord injury (SCI) is a prevalent clinical problem, with approximately 270,000 patients living with SCI in the United States alone[73]. A neuroprotective intervention that functions as a pretreatment paradigm may prove especially interesting for high risk groups of people. The primary mechanical injury results in either a contusion, complete transection of the spinal cord, or a compression injury. The severity of the primary injury, along with the spinal level of the injury, determines the clinical outcome for the patient. The financial and psychological costs are extreme for patients surviving with spinal cord injury. Much like the secondary effects described in traumatic brain injury, a similar cascade of neurodegeneration begins after the primary mechanical injury. Many of the molecular events associated with the secondary injury cascade, in particular free radical production, are common pathways between traumatic brain and spinal cord injury. Despite differences and difficulties with each injury paradigm, the similar aspects of the secondary injury cascade are of particular interest for pharmaceutical intervention.

1.2.3 Antioxidants and neurotrauma

The initial mechanical trauma in traumatic brain and spinal cord injury is followed by a wave of neurodegenerative events that eventually lead to pathological dysfunction. In particular, oxidative stress is a key contributor to neurodegeneration following injury. Antioxidants have frequently been a target of efforts to use pharmaceutical intervention as a treatment strategy for neurotrauma[54]. Antioxidant therapies can function to sequester reactive oxygen and nitrogen species or inhibit their production. Traditional endogenous antioxidants play an important role in regulating free radicals

after injury. Antioxidant enzymes, including superoxide dismutase (SOD), catalyze the conversion of oxygen radicals into hydrogen peroxide (H_2O_2). Glutathione peroxidase (GPx), a well-characterized family of selenoproteins, then converts harmful hydrogen peroxides into non-harmful by products. Some of the pathological outcomes of oxidative damage include mitochondrial dysfunction[74], neuronal death, and damage of synaptic proteins in the hippocampus[75].

Because of the high level of polyunsaturated fatty acids (PUFA) in the CNS, neurons are highly susceptible to damaging to lipid peroxidation. As free radicals attack these PUFAs, cellular membrane integrity is disrupted and will eventually lead to cytotoxic effects. Lipid peroxidation results in production of the two aldehydic compounds, acrolein and 4-hydroxynonenal. Levels of 4HNE, and thus evidence of lipid peroxidation, have been shown to peak at 24 hours following spinal cord injury[76]. 4-HNE is evident as early as 1 hour after traumatic brain injury with sustained levels as far as 96 hours post injury[77]. Antioxidant therapies have shown success in pre-clinical trials and limited efficacy in clinical trials[54]. The development of reactive oxygen species and resulting damage following injury, suggest that modulation of the antioxidant system may prove a valuable target for neuroprotection following traumatic brain and spinal cord injury.

1.2.4 Nutriceuticals in the treatment of neurotrauma

The use of dietary supplementation in the treatment or prevention of neurodegenerative disorders has been investigated in a limited fashion in previous studies. The Institute of Medicine published a report detailing the importance of proper nutrition in traumatic brain injured patients[78]. While energy requirements are critical following injury, levels of micronutrients are also important. In particular, this review of nutritional status in TBI patients emphasizes the value of supplementation with creatine, N-3 fatty acids, flavonoids, progesterone and vitamin D. In a rodent model of traumatic brain injury, creatine was shown to protect against tissue damage and deficits in mitochondrial membrane potential in injured animals[79]. In a weight-drop model of traumatic brain injury, *N*-acetylcysteine (NAC) when combined with selenium treatment increased protective cytokine levels as well as decreased harmful lipid peroxidation[80]. In a patulin induced brain damage model, selenium supplementation was also shown to be neuroprotective by reducing protein carbonyl levels, reactive oxygen species generation, and by increasing both the activity and expression of several GPx proteins[81].

Following brain injury, selenoproteins may play a role in providing critical selenium to neurons and astrocytes at the blood brain barrier. As discussed, Burk et al. showed that a knockout of apolipoprotein E receptor-2 (ApoE2) results in dysfunction of selenoprotein levels in the brain[65]. Further studies show that both SelP and apolipoprotein E receptor-2 mediate selenium uptake at the blood brain barrier, especially in conditions of selenium deficiency[62]. Another selenoprotein,

selenoprotein S (SelS) is secreted from astrocytes and expression of SelS is greatly increased in reactive astrocytes after brain injury[36]. Overexpression of SelS influences the inflammatory markers in astrocytes. Additionally, Yeo et. al, also established through several studies that sodium selenite injections are neuroprotective in both traumatic brain and spinal cord injury[58, 59]. However, these studies present limitations, including the risk of toxicity in treatments with inorganic formulations of selenium.

These various studies lay the foundation for the importance of examining the neuroprotective effects of dietary selenium in models of traumatic brain and spinal cord injury. While a few studies have investigated the potential protective properties of selenium as an important component of antioxidant systems and as a regulator of gene expression, no work has been done to date with dietary organic selenium supplementation in neurotrauma models. Due to the vast spectrum of neurodegenerative events that occur as a part of the secondary injury cascade, traumatic brain and spinal cord injury have proven very difficult diseases to target with pharmacological intervention. While unconventional in the field, a dietary pretreatment with selenium may be able to provide multiple benefits in the event of an injury. Selenized yeast should increase tissue storage of selenomethionine, allowing for a readily available source of selenium for selenoprotein synthesis following injury. It also may be able to modulate gene expression patterns, as those involved with DNA repair[40], transcriptional regulation[39], and inflammation[41] thus providing neuroprotection following injury. While the functions of many identified selenoproteins remains unknown, the critical need for selenium and beneficial effects

with supplementation support further investigation into its effects within the CNS following injury.

CHAPTER 2

Dietary supplementation with organoselenium following spinal cord injury

2.1 Introduction

Spinal cord injury (SCI) is a prevalent clinical problem, with approximately 273,000 patients living with SCI in the United States alone[73]. Young adults represent a particularly vulnerable population due to their active lifestyle and a higher prevalence of military service. A prophylactic supplement to help protect in the event of acute neurotrauma may be particularly beneficial to this high risk group.

We investigated selenium supplementation as a prophylactic treatment for reducing damage and improving functional outcomes following spinal cord injury. Selenium is essential for the formation of selenoproteins throughout the body, particularly in the CNS. However, different forms of selenium are associated with varying toxicity levels[82]. Organic selenium supplements have much lower levels of toxicity and fewer of the pathogenic effects that are commonly associated with high levels of inorganic selenium in the body[83, 84, 85, 86]. The diets utilized in this study were formulated with selenized yeast to provide an organic form of selenium supplementation. The selenium is present in the form of two amino acids, selenomethionine and selenocysteine, as well as a variety of other seleno-compounds giving this supplement a unique chemical fingerprint[18]. Selenomethionine can be incorporated in place of methionine in tissues, allowing for storage of selenium in the event of a dietary deficiency.

The brain and spinal cord prioritize the retention of selenium when dietary levels are deficient[87], utilizing the selenium transporter in the body, selenoprotein P (SelP), to supply physiological selenium to the brain[88, 89]. Selenoproteins are well characterized for their role in redox regulation[2, 90, 91] and anti-inflammatory pathways[92, 93]. In particular, several antioxidant enzymes, including glutathione peroxidases and thioredoxin reductases, are selenoproteins which require the presence of selenocysteine for protein production. Reactive oxygen species initiate lipid hydrolysis, breakdown of cellular membranes, mitochondrial dysfunction, and thus perpetuate the damaging secondary injury cascade.[94] Increasing levels of selenium available in tissues prior to injury may prepare the CNS to quickly synthesize selenoproteins to mitigate this series of damaging events.

The objective of this study was to examine the effect of supplementation with selenized yeast on functional and pathological endpoints following SCI. Dietary selenium supplementation increased selenium tissue storage in the CNS, with no aberrant weight gain or other adverse physiological changes between treatment groups. This increase in selenium levels did not translate to a visible improvement in locomotion or lesion volume in the injured tissue, but resulted in more rapid recovery of autonomous bladder expression.

2.2 Methods

2.2.1 Animal care and diet

Female Sprague-Dawley rats were obtained from Harlan at weaning and placed immediately on either a control diet or on a selenium enriched diet (Se-yeast, Sel-Plex, Alltech, Nicholasville, KY). The control diet of normal rat chow contained standard dietary levels of selenium (0.3ppm selenium, approximately 4.5µg per day) and a yeast additive to account for any variation that may be seen due to the selenized yeast in the other diet. Caloric content was appropriately adjusted in diet composition after the addition of the supplements. The selenium enriched diet also used a standard rat chow as the diet base and incorporated a selenized yeast preparation (1.3ppm total selenium, approximately 19.5µg per day) in which the yeast is grown in the presence of selenium. The selenium, in the form of selenized yeast (Sel-Plex®), is supplemented at levels well below tested toxicity levels. Animals were fed their respective diets (n=20 per diet) *ad libitum* for 4 months. Throughout the trial, animals were weighed weekly, and immediately prior to receiving the injury, to monitor health statuses of the rats as well as to check for any significant differences in body weight of the animals at the time of injury. Animals were housed and handled within regulation, as a part of an approved protocol with University of Kentucky Institute of Animal Care and Use Committee (IACUC).

2.2.2 Spinal cord injury and post-surgical care

Following 4 months of dietary supplementation, rats were subjected to a moderate contusive SCI. Contusive SCI surgeries were performed as previously described[95]. In

brief, rats were anesthetized with an intraperitoneal injection of ketamine (80mg/kg) and xylazine (10mg/kg). The spinal cord was exposed via a T10 laminectomy. The rats received a moderate (150kdyn) contusive thoracic SCI using the Infinite Horizons SCI injury device (Precision Systems and Instrumentation). Sham animals received only anesthetics and the laminectomy procedure. Following injury (n=12) or sham surgery (n=8), the musculature and skin are closed with sutures and wound clips, respectively. As part of routine post-surgical care, bladders were manually expressed twice daily using the Credé maneuver[96] until the animal recovered autonomous bladder control. Bladder function following injury was recorded as either non-functional (full bladder) or functional (empty to half-full bladder) prior to manual voiding by the investigator[97]. The number of days until each animal exhibited autonomous bladder functional recovery was recorded. The rats also received injections of Buprenorphine (0.05 mg/kg) twice daily to manage pain and Baytril (5-10mg/kg) twice daily during this time to prevent development of post-surgical infection. Animals were maintained on their respective diets until the time of euthanasia.

2.2.3 Spinal cord and cortical selenium levels

A separate cohort of animals was also maintained on the two different diets for 4 months under the conditions as described above. Animals were fed *ad libitum* for 4 months (n=5 per dietary group). Immediately following the feeding regimen animals received the moderate contusive spinal cord injury described above. 24 hours post injury, fresh tissue from spinal cord sections both rostral and caudal to the injury site

and cortical samples were removed and flash frozen in liquid nitrogen. Selenium levels were then analyzed by liquid chromatography-mass spectroscopy (LC-MS). Values rostral and caudal to the injury site were averaged for each animal. One value was removed from spinal cord dataset due to being an extreme outlier (greater than 3 standard deviations from the mean).

2.2.4 Behavioral assessment

At three days following SCI, two investigators blinded to treatment groups assessed open field locomotor functional recovery using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale[98]. Locomotor testing was then repeated once weekly for 6 weeks following injury. At 3 days following injury there is a substantial injury effect. With a moderate injury (1.50kdyn) animals lose motor control of their hindlimbs. Over 6 weeks, rats typically demonstrate a gradual improvement in locomotion that typically plateaus around a score of 13, indicating that animals have frequent to consistent weight support in plantar stepping and frequent coordination. When discrepancy occurred between observers, the lower score was assigned to the animal's performance.

2.2.5 Tissue histology

Following the final behavioral testing, animals received a thoracotomy followed by transcardial perfusions. Animals were given an intraperitoneal injection of a fatal overdose of sodium pentobarbital and then perfused with ice-cold phosphate buffered saline (PBS), followed by fixation with a 4% paraformaldehyde/PBS solution. Spinal

cord tissues were kept at 4°C in a 4% paraformaldehyde solution for 4 hours and then transferred to a 30% sucrose solution overnight at 4°C for cryopreservation. The fixed tissues were transferred into OCT medium for cryosectioning at -25°C. Transverse spinal cord sections (20μm thick) were collected every 100μm and mounted onto slides. Sections were stained according to a modified protocol utilizing eriochrome cyanine RC and cresyl violet stained myelin and differentiated between white and gray matter[99, 100]. Lesion volume and tissue sparing were measured and calculated for sections extending 0.7mm on either side of the injury epicenter. Lesion and healthy tissue were measured using Scion Imaging analysis software (Scion Corporation, Frederick, MD). Utilizing the Cavalieri method[101], the total volume of tissue in the lesion or in spared tissue was calculated. The investigator was blinded to treatment groups until all parameters had been measured and calculated.

2.2.6 Statistical Analysis

Data represented in each figure are shown as mean±standard deviation. Using GraphPad Prism 6.0, differences in weekly weight gain were evaluated by repeated measures, one-way ANOVA encompassing the 16 week feeding period. Additionally, to ensure that there were no significant differences between the two diet groups immediately before surgery, Student's t-test (significance at $p<0.05$) was used to compare final weights. Differences in behavioral assessment between treatment groups were assessed by repeated measures, one-way ANOVA with significance assigned at $p<0.05$. Tissue selenium content, separately in spinal cord and cortex, was compared using Student's t-test between control and selenium enriched diets

($p<0.05$). Bladder functional recovery between animals on the control and selenium enriched diets was assessed using a Student's t-test (significance at $p<0.05$). Differences between injury and control in lesion volume were calculated using a two-way ANOVA (significance at $p<0.05$).

2.3 Results

2.3.1 Weekly Weights

To ascertain the effect of the selenium-enriched and control diets on body weight throughout the course of the study, animals were weighed weekly as described above (n=25 for each diet). Weight gain showed no significant differences between rats maintained on the control (+yeast) rat chow and the yeast-selenium enriched diet (Figure 2.1).

Figure 2.1: Dietary selenium enrichment does not affect overall weight gain. Rats were weighed weekly. No differences in body weight were seen between selenium enriched rats and rats maintained on control diet.

2.3.2 Selenium levels in the central nervous system

Data from liquid chromatography mass spectroscopy (LC-MS) showed that dietary enrichment with selenium increased tissue storage of selenium. In the cortex, selenium levels increased by approximately 2-fold between the control diet and selenium enriched diet ($p<0.0001$) (Figure 2.2A). Supplementation also increased selenium levels in the spinal cord ($p<0.0001$) (Figure 2.2B).

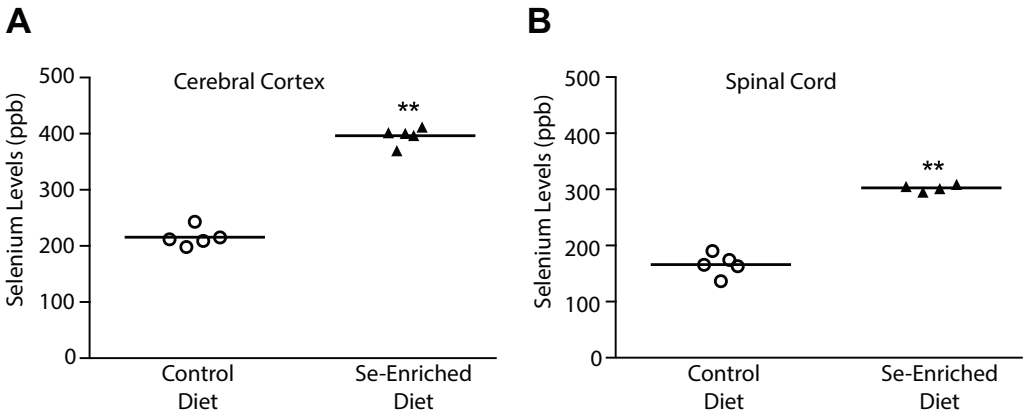


Figure 2.2: Dietary selenium enrichment increases selenium tissue storage. CNS selenium levels were analyzed by LC-MS. These results indicate that supplemented selenium is able to cross the blood-brain barrier and is available for incorporation into cortical (**A**) and spinal cord (**B**) tissue. Values are mean±SEM, $p<0.001$.

2.3.3 Bladder function

Animals (n=12 per diet) maintained on selenium enriched diets regained bladder function by 3±0.4 days after injury as compared to rats on a control diet, at 5±0.5 days (mean±SD $p<0.05$) (Figure 2.3). Animals that received sham surgeries did not lose autonomous bladder control and thus did not require manual emptying by the investigator.

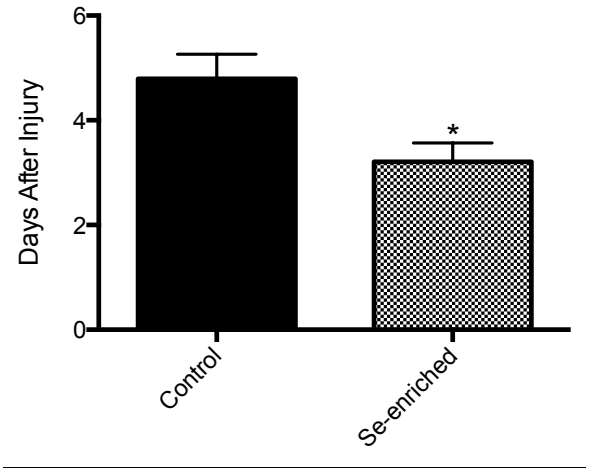


Figure 2.3: Dietary selenium supplementation improves the time to recovery of bladder function. Rats receiving selenium supplementation recovered bladder function in an average of 3 days±0.4 as compared to rats on a control diet, which recovered bladder function in 5 days±0.5. Recovery of bladder expression is an additional functional marker for improvement. Values are mean±SEM, $p<0.05$.

2.3.4 Locomotor functional recovery

Sham animals in both dietary groups showed no loss of function following sham surgery. Injured animals in both groups had a complete loss of hindlimb locomotor function immediately following surgery, confirming that the contusion injury was effective. Three days after injury, all rats exhibited slight motor recovery, as evident from the BBB scores, which reflect slight movement of two joints and extensive movement of the third joint. Over the course of the 6 week behavioral testing, all injured animals showed a steady improvement in locomotor function, plateauing around the second week with overall function that included consistent stepping, occasional to frequent coordination, and occasional correct paw placement. In the injured animals, there was no significant difference in locomotor functional recovery, over time or during the final behavioral testing, between those fed the control diet and animals fed the selenium-enriched diet (Figure 2.4).

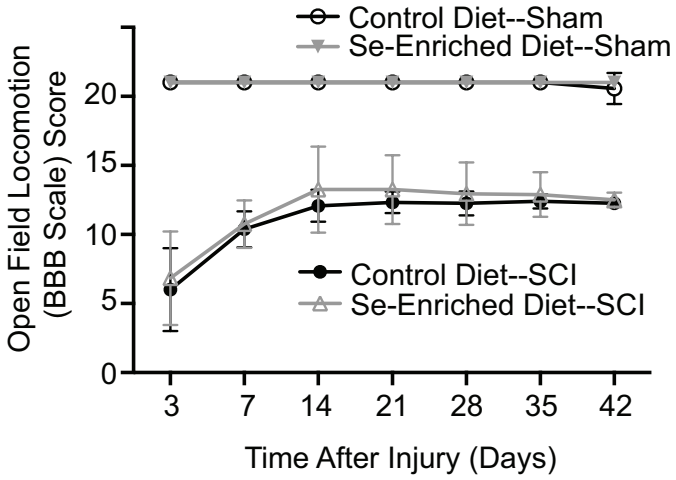


Figure 2.4: Dietary selenium supplementation does not improve locomotor functional recovery. Recovery was evaluated weekly after injury using the BBB scale. No difference in improvement was seen between the two diets in the injured rats. Sham animals showed no change in performance after laminectomy. Values are mean±SEM.

2.3.5 Tissue lesion volume measurements

Following the final behavioral testing, spinal cord tissue from each animal was fixed and cryopreserved. Staining for myelin and neuronal cell bodies showed no significant difference between the two injured groups in total lesion volume, or in the total amount of gray matter and white matter sparing after injury (Figure 2.5). Sham injured animals showed no tissue lesion.

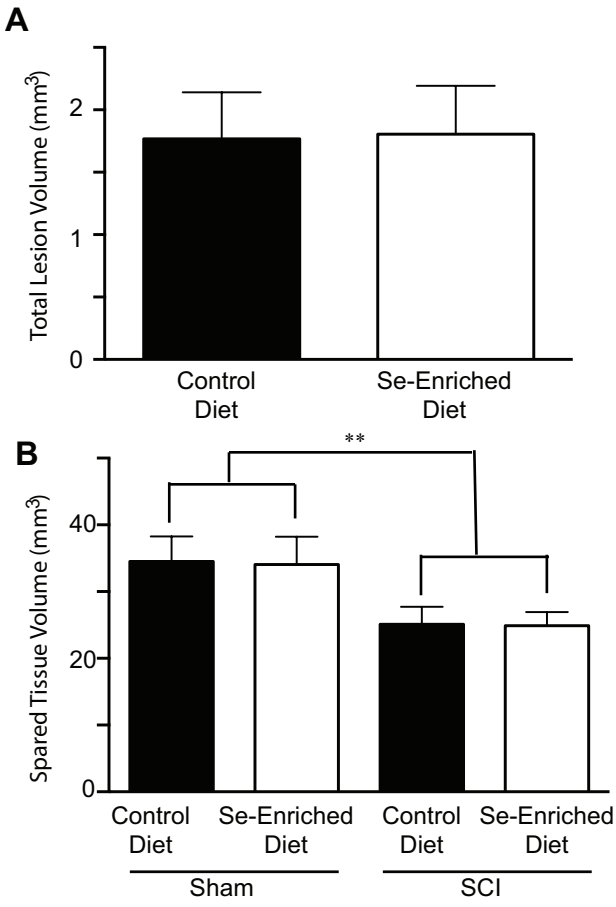


Figure 2.5: Dietary selenium supplementation does not reduce lesion volume following SCI. Spinal cords sections (20μm) were stained for cell bodies and myelin. Total lesion volume **(A)** and total tissue sparing **(B)** revealed a significant injury effect (**$p<0.01$) between sham and injured. No significant difference in lesion volume or tissue sparing was seen between the control injured and the selenium injured group. Additionally, there were no significant differences between grey matter sparing and white matter sparing.

2.4 Discussion

Dietary selenium supplementation may be particularly beneficial for CNS disorders, as the CNS maintains a high priority for selenium storage in deficient conditions[87, 102-104]. This has been examined previously for metastatic brain tumors[105], Alzheimer's Disease[106], ischemia[107], and Parkinson's Disease[108]. One previous study examined the effects of selenium on SCI[109], although the experimental conditions differed markedly. Yeo and colleagues injected sodium selenite (10-50ng/kg) mixed with matrigel directly into the lesion site immediately following a dorsal hemisection injury. This study observed a reduction in apoptotic cell death, decreased GFAP positive cells, and a dramatic improvement in locomotor function in selenium over vehicle treated rats.

Pre-injury dietary supplementation in this study showed increased CNS tissue levels of selenium, implicating increased bioavailability of seleno-amino acids for the production of selenoproteins. However, the increased selenium levels did not result in improved pathological outcomes and only minimal improvement in functional outcomes. Additionally, these effects were not evident through histological analysis of tissue sparing. In behavioral tasks, the rats receiving a selenium enriched diet did not exhibit significant improved locomotor function post-injury as compared to animals whose diet consisted of normal rat chow.

Rats in the selenium enriched diet group were able to regain autonomous control of bladder expression following spinal cord contusion injury more quickly than rats maintained on the control diet. Initiation of autonomous micturition has been

examined as a marker for recovery of sensory-motor function in previous spinal cord contusion studies[110, 111]. Neurological control of bladder function is controlled through splanchnic parasympathetic nerves (located in the sacral spinal cord S2-S4), pudendal nerves (also in the sacral spinal cord S2-S4), and thoracic sympathetic nerves (cell bodies originating at T10-L2 spinal cord levels). Sympathetic innervation of the bladder plays a crucial role in closing the internal urethral sphincter and of blood vessels in the detrusor muscle of the bladder. Although histological examination did not show an overall improvement in total lesion volume, it is possible that sympathetic neurons present at the site of injury (thoracic level T10) were protected with selenium treatment. Additionally, because selenoproteins are found highly expressed in astrocytes, the supporting glial cells may have protected neuronal cell bodies in the epicenter of injury. In rat contusion (incomplete injury) models of SCI, rats spontaneously recover voluntary bladder control in the days to weeks following injury. Human patients with incomplete SCI do not exhibit recovery of voluntary bladder control. Disruptions in bladder and bowel function are very important clinical pathologies to SCI patients, however, disconnect between clinical outcomes and those found in experimental models may limit the translation of bladder functional recovery in preclinical models to patient application.

Overall, the results do not support our hypothesis that selenium supplementation would result in improved outcomes following SCI. However, the studies that have also observed bladder functional recovery without associated improvement in locomotor behavioral tasks suggest that these improvements in bladder function may

be important markers for functional recovery[112, 113]. Shunmugavel et al. suggest the crucial importance of inflammatory pathways in mediating bladder dysfunction following SCI[114]. The involvement of selenium in modulating the inflammatory response[92, 93] may be responsible for the improvement in recovery of bladder function observed in the present study.

Current studies are examining effects of selenium-enriched and selenium-deficient diets on levels of selenoproteins and related enzyme activities in both naïve and traumatic brain injured animals. The antioxidant natures of many of the selenoproteins in the CNS, along with the results from this study suggest, that while selenium's effects may be modest in this SCI model, examining various levels of selenium to attenuate neurodegenerative pathways warrants further research.

CHAPTER 3

Dietary selenium supplementation alters gene expression following spinal cord injury

3.1 Introduction

The initial contusion of the spinal cord brought on by a mechanical injury, triggers a host of neurodegenerative molecular events, commonly referred to as the secondary injury cascade. While it is difficult to anticipate or prevent the contusion itself, the subsequent cellular and molecular changes are common targets for pharmaceutical intervention. The secondary injury cascade is characterized by lipid peroxidation[115], cytotoxic influx of calcium, mitochondrial dysfunction[116], production of reactive oxygen species, and inflammatory response. These events ultimately result in neuronal cell death and the clinical pathologies associated with spinal cord injury (SCI). In addition to these early markers of injury, spinal cord trauma also results in extensive changes in the gene expression profile as early as 4 hours and continuing through at least 24 hours post injury[117-119]. Gene ontologies associated with spinal cord injury include inflammatory cytokines, transcriptions factors, regulation of immune response, and response to oxidative stress[120]. These changes in gene expression help to complete the understanding of the secondary injury cascade and how changes in mRNA are triggered in response to injury.

With the limited success thus far with clinical treatments targeting spinal cord pathology following injury, innovative approaches that target the secondary injury cascade may be beneficial for future patients with SCI. As an essential component of several antioxidant enzymes and other selenoproteins in the body, increased dietary

selenium represents a potential strategy to combat neurodegeneration following SCI. Low tissue selenium levels or changes in selenoprotein levels are also associated with other diseases of the central nervous system (CNS) including Alzheimer's Disease[34, 60] and Parkinson's disease[61].

In addition to altering selenoprotein levels, selenium also impacts gene expression patterns, making it an interesting target for modulating gene expression in spinal cord injury models. Selenized yeast decreases expression of Gadd45b, a gene known to increase in response to DNA damage, as well as several gene ontology groupings associated with mitochondrial damage[39]. These shifts in DNA repair pathways are supported further by McKelvey et al[40] in which organic formulations of dietary selenium, including selenized yeast and selenomethionine, induce gene expression shifts in pathways associated with DNA damage. These gene expression changes also translated to protection against lead-induced DNA damage in cell culture. Additionally, specific selenoglycoproteins, extracted from selenized yeast, impact transcriptional regulation through modulating expression of nuclear factor-κβ (NF-κβ)[46].

While gene expression changes following spinal cord injury are well documented and studies have shown differential pathway shifts with formulations of selenium, the impact of selenium on mRNA expression patterns following spinal cord injury has yet to be investigated. Neuroprotective effects have been proposed for selenium in different neurodegenerative diseases but gene expression changes may provide more

of an indication through which selenium is providing neuroprotection *in vivo*. It is expected that the injury will cause vast changes in gene expression. Additionally, these studies tested the hypothesis that dietary selenium would drive additional changes in gene expression and potentially modulate expression of genes associated with pathologies of the secondary injury cascade.

3.2 Methods

3.2.1 Diet supplementation

Selenized yeast (Sel-Plex®, Alltech, Nicholasville, KY) was added to rat chow at a concentration of 1ppm selenium. Normal rat chow contains 0.3ppm of selenium, giving the selenium enriched diet a final concentration of 1.3ppm selenium (19.5µg). The control diet containing 0.3ppm of selenium (4.5µg) was representative of normal dietary levels. Yeast was also added to the control diet to account for any differences that may be seen due to the addition of yeast in the selenium enriched diet. Diets were formulated to contain otherwise similar caloric and nutritional content (Harlan Laboratories). Female Sprague-Dawley rats were obtained at weaning (21 days) and placed on either the control or selenium enriched diets (n=10 per diet). Animals were fed *ad libitum* for 16 weeks prior to receiving spinal cord injury. Throughout the study, weights and health status were monitored to check for any adverse consequences due to dietary differences. The animals were maintained on their respective diets until the terminal data collection.

3.2.2 Spinal Cord Contusion Injury and Post-Surgical Care

Rats received either a moderate (150kdyn) contusive thoracic spinal cord injury via the Infinite Horizons (IH) SCI injury device (Precision Systems & Instrumentation) or a sham laminectomy after 16 weeks on their respective diets (n=5 injured, n=5 sham within each diet). Female rats received an intraperotineal injection of ketamine/xylazine (80mg/kg and 10mg/kg, respectively). Once significant depth of anesthesia was confirmed an incision was performed at approximately T10 (thoracic spinal cord level). The exact location of T10 spinal cord level was determined and a

laminectomy removed the overlying vertebra. The animal was secured using clips on either side of the vertebral column. IH impactor was set to 150kdyn force injury and was allowed to impact the exposed spinal cord tissue. Animals were sutured and allowed to recovery on warming pads. Buprenorphine (0.05mg/kg) was administered for control of pain every 12 hours following surgery. Loss of bladder control is a typical symptom following a contusion injury of this severity. Bladders were manually expressed 2-3 times daily until rats recovered the ability to express their bladder autonomously. Additionally, animals were given baytril (5-10mg/kg) to help prevent urinary tract infection twice daily following surgery until the recovery of bladder function and urine is clear. The rats also received injections of Buprenorphine (0.05 mg/kg) twice daily to manage pain. Animals were maintained on their respective diets until the time of euthanasia at 24 hours post injury.

3.2.3 Sample Preparation and RNA Isolation

A 7mm section of spinal cord tissue directly surrounding and including the injury epicenter was dissected at 24 hours post injury. Samples were flash frozen in liquid nitrogen to preserve RNA quality. Due to the high quantity of lipids within the spinal cord white matter, total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen). Approximately 50mg of tissue was homogenized and processed according to the recommended protocol, including the optional DNase digestion. Final samples were eluted from columns with 30µL of RNase-free water. RNA quantity and quality were determined using the Nanodrop spectrophotometer and Bioanalyzer (Agilent), respectively. All samples used for microarray analysis had an RNA integrity number

(RIN) of at least 8, as is recommended for the sample purity necessary for reproducible results with the Affymetrix platform.

3.2.4 Affymetrix Gene Array

Affymetrix reagents and protocol were utilized (3' IVT Expression kit) for all steps. RNA samples (200ng) were thawed on ice and assembled with other reagents to synthesize 1-strand cDNA. Second strand cDNA synthesis created aRNA which was purified using magnetic beads. aRNA was then labeled with a biotinylated probe and 15µg of aRNA was added to fragmentation buffer. Samples were hybridized over night to a species-specific microarray chip (Rat 230 2.0 array, Affymetrix). This array chip represents over 30,000 transcripts from the rat genome. Affymetrix fluorescent scanner measures fluorescence emitted from hybridized RNA and translates this information into raw signal intensity. Software within the scanner assigns each signal intensity reading a numerical value, allowing for quantitative evaluation of gene expression levels.

3.2.5 Statistical Analysis and Template Design

The original data set included a total of 31,097 genes. From this list, the initial filtering process removed genes based on presence/absence calls, redundant gene symbols, and a lack of gene annotation. A gene is considered absent from a chip if an insufficient amount of signal intensity is detected. Expression values for a particular gene were removed from the entire data set if there are more than 17 "absent" calls out of all 20 microarray gene chips, regardless of injury/diet group. Among those

genes with redundant gene symbols, the expression data with the strongest expression across all groups was selected. Additionally, genes that do not have any annotation data were eliminated. While potentially interesting, with no annotation data available, little can be learned from these genes at the current time. This pre-statistical filtering generated a list of 14,907 genes.

Using 2-way ANOVA, a list of genes that met $p \leq 0.017$ (Bonferroni correction for 3 tests) was generated. This list included a total of 9,533 statistically significant gene expression changes. To analyze such an extensive list, templates of potential gene expression patterns were designed. Additionally, due to the concern that the extensive changes resulting from the injury effect may mask potentially subtle changes due to diet, templates pulled out gene expression changes due only to injury, exposing the dietary changes. Genes from the statistically filtered list were assigned to one of 8 constructed templates based on their expression patterns in all 4 treatment groups. These templates represent overall directions that expression could take between different groups. Genes in each of these groups were analyzed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/) to generate functional annotations of gene lists. Affymetrix probe set IDs within each template were loaded into the Functional Annotation tool, utilizing the statistically filtered list (9,533 genes) as the background for this search. Gene ontologies were selected based on the number of potential genes in that pathway, relative to the probe sets from that particular template with the pre-determined $p \leq 0.05$. The resulting gene ontologies were compiled and duplicate pathways combined to simplify analysis. Due to the

stringency of the α at each stage of statistical analysis, the final gene pathways are unlikely to be due to the large amount of multiple testing that occurs with 31,000 data points.

3.3 Results

3.3.1 Significant gene selection and template design

Analysis of gene array data began with probe sets representing 31,097 genes (Figure 3.1). Genes were selected for further analysis based on presence or absence of signal intensity in all 20 microarray chips. Genes were eliminated from further analysis if no annotation data was available or data represented a duplicate gene annotation. This initial screening yielded a list of 14,907 genes. From this list, genes were further filtered based on statistical significance. Two-way ANOVA with Bonferroni post-hoc correction indicated a p-value cut off of 0.017. Using $p \leq 0.017$, expression of 9,533 genes significantly changed in the current study.

Templates were designed based on possible directional changes in gene expression across the four groups (Figure 3.2). Template based analysis provides the unique benefit for this study of allowing for the separation of certain patterns of expressional changes. In the case of this study, and has been established by previous works, spinal cord injury creates a massive change in mRNA expression levels[117-119]. The injury has a large fold change, potentially overwhelming expression changes due to diet. Template analysis allows us to detect more subtle gene expression changes, especially those due to dietary influences. All possible changes in gene expression have been considered, however, some templates are of more interest to the current investigation than others. An algorithm assigned each gene based on its pattern of expression to a template. The first two templates (Figure 3.2 A, B) represent the overall changes either up or down regulated following injury alone. Of the total filtered list, 4301 genes were placed into the template in which all the genes were down regulated

following injury. 4812 genes were assigned to the up regulated template following injury. Two other templates that are of particular interest are those genes that are increased in the selenium injured group, suggesting a potential protective role of the dietary enrichment (Figure 3.2 I, 111 genes) and those genes that are restored to sham levels in the selenium supplemented group (figure 3.2 J, 78 genes).

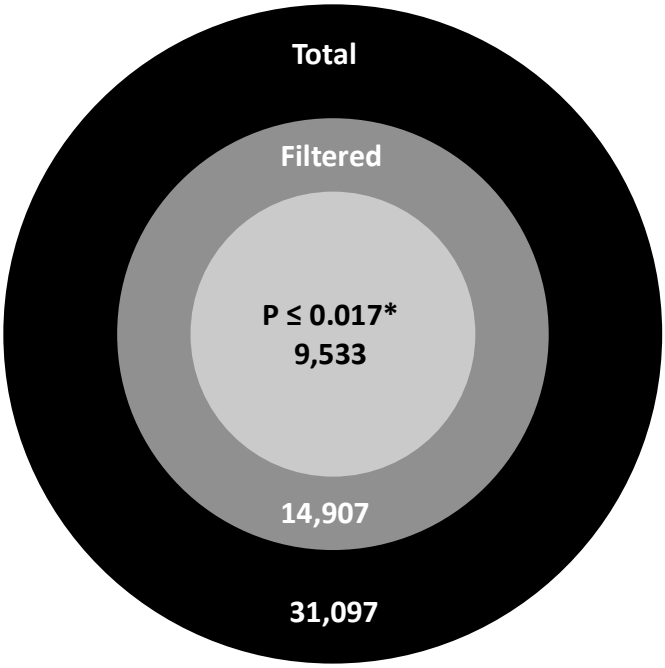


Figure 3.1: Total genes represented in microarray analyses in SCI animals. Each gene chip begins with 31,097 genes represented. Filtering for presence/absence of signal across all chips. 2-way ANOVA run for remaining genes to generate p-value for diet, injury, and interaction. Bonferroni multiple comparison correction brought significant p-value to ≤0.017 and a total of 9,533 genes.

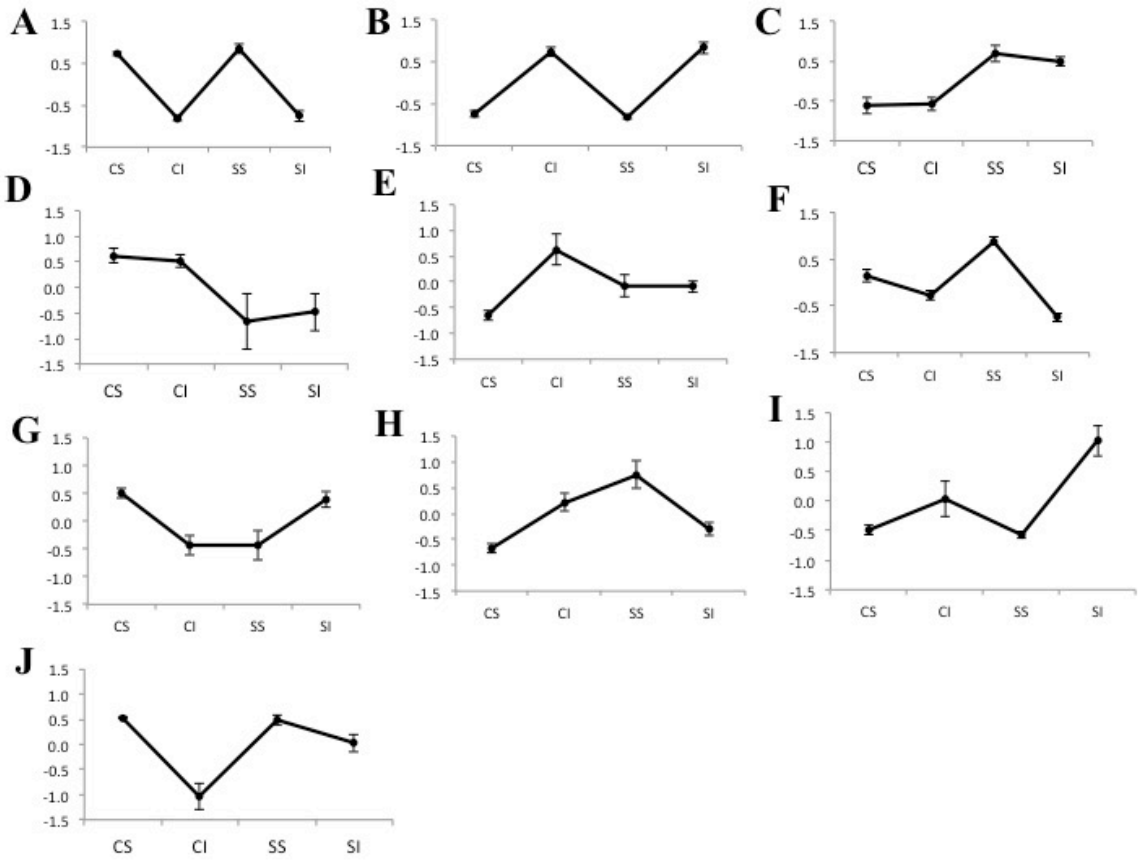


Figure 3.2: Templates representing expression patterns from microarray analysis. A) Gene expression changes representative of genes that are down regulated following spinal cord injury, regardless of diet (4301 genes). **B)** Gene expression changes representative of gene that are up regulated following injury, regardless of diet (4812 genes). **C)** Genes that are upregulated with selenium supplementation, regardless of injury (16 genes). **D)** Genes that are down regulated with selenium supplementation, regardless of injury (30 genes). **E)** Genes that are upregulated only in control injured animals (55 genes). **F)** Genes that are upregulated only in selenium sham animals (95 genes). **G)** Genes that run in opposition (7 genes). **H)** Genes that group into pattern analysis of opposition (28 genes). **I)** Genes upregulated in animals that were selenium supplemented with SCI (111 genes). **J)** Genes in this list are representative of 78 total genes that are down regulated in injured animals maintained on the control diet. Selenium enrichment in injured animals restores expression levels of these genes to sham levels.

3.3.2 Gene changes with injury

SCI causes a large change in gene expression with the majority of the genes that were a part of the final filtered list sorted into one of the two injury based templates (Figure 3.3 A, B). Figure 3.3 assigns a color relative to the amount of gene expression change. Blue indicates down regulation of gene expression and red indicates up regulation of gene expression changes. Each animal is represented by one column, each row is representative of one gene. This heat map clearly shows the strong shift in expression that is created by injury. Each heat map is coupled with its respective template for reference.

When examining the lists for assignment into specific functional annotations, the p-value cut-off was adjusted slightly for these gene lists. DAVID Bioinformatics Database only allows submission of a probe set list of 3000 genes. Therefore, the stringency was increased to $p \leq 0.0001$ to generate a list of appropriate size for analysis. This database characterized the genes into an extensive list of functional ontologies (Table 3.1). Genes that are down regulated post-injury in the current study include those involved with synaptic transmission, mitochondrial respiration and membrane integrity, ATP production, and ion transport. Genes that are up regulated post-injury include those associated with translational machinery and mRNA processing, cell cycle, nuclear transport, and lymphocyte mediated immunity.

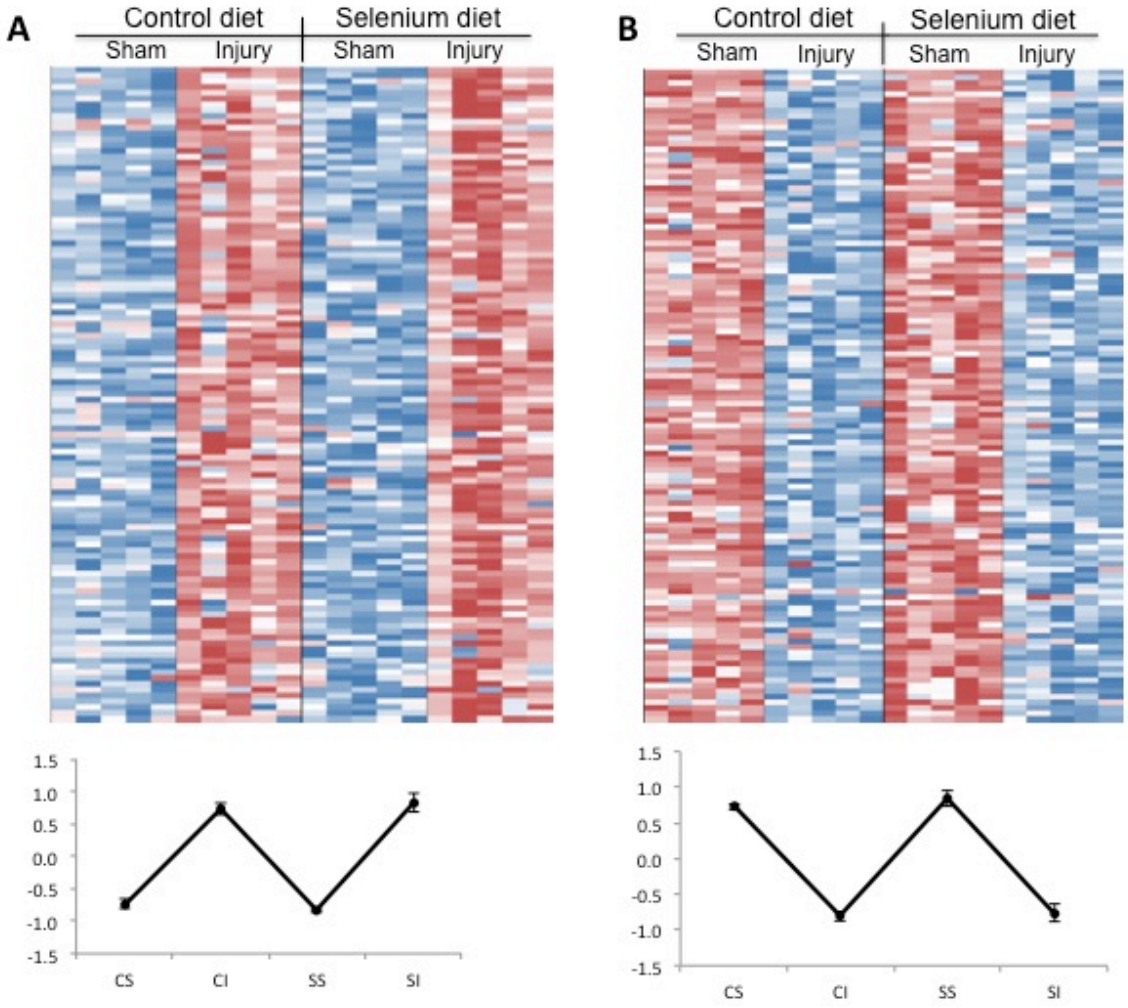


Figure 3.3: Representative heat maps and related templates for gene expression changes associated with injury. In the heat maps shown, expression values indicated by a red color are up regulated and expression values indicated by a blue color are down regulated. These images clearly show the relative gene expression changes as a result of injury.

Table 3.1 Gene ontologies from genes down regulated post-injury

Down regulated post-injury
synaptic transmission
neuron projection/cytoskeleton
mitochondrial inner membrane
integral to membrane
mitochondrial part (matrix)
ion transport
adult behavior (locomotor)
ATPase activity, coupled to transmembrane movement of substances
mitochondrial respiratory chain
neurotransmitter transport
regulation of neuron differentiation (neurogenesis)
NAD or NADH binding (cofactor binding)
cellular component maintenance
peroxisome
ion binding
sulfur metabolic process (glutathione metabolic process)
calmodulin binding
cellular chemical homeostasis (regulation of membrane potential)
glutamate signaling pathway
acetyl-CoA metabolic process (cellular respiration)
cation-transporting ATPase activity
regulation of catecholamine secretion
(negative) regulation of adenylate cyclase activity
homophilic cell adhesion
carbohydrate catabolic process
vesicle-mediated transport
hydrogen ion transporting ATP synthase activity, rotational mechanism
carboxylic acid transport (amino acid transport)
Ras guanyl-nucleotide exchange factor activity
microtubule motor activity
cyclic nucleotide metabolic process
activation of protein kinase A activity
phosphoinositide metabolic process
regulation of action potential
phosphoric diester hydrolase activity (lipase activity)

Table 3.2: Gene ontologies from genes up regulated post-injury

Up regulated post-injury
translational elongation
nuclear lumen
intracellular non-membrane-bounded organelle
RNA/mRNA processing
ribonucleoprotein complex biogenesis
positive regulation of I-kappaB kinase/NF-kappaB cascade
nuclear pore
proteasome complex
chromosome
ribosomal small subunit biogenesis
macromolecular complex assembly
DNA metabolic process
nucleotide binding
lymphocyte mediated immunity
helicase activity
positive regulation of transcription factor activity
mitotic cell cycle
regulation of endopeptidase activity
positive regulation of programmed cell death
chaperonin-containing T-complex
nucleotide-excision repair
positive regulation of cell migration
vasculature development
response to amino acid stimulus
nuclear-transcribed mRNA catabolic process
nuclear transport
'de novo' protein folding
erythrocyte homeostasis
peptidyl-asparagine modification
actin cytoskeleton
positive regulation of tumor necrosis factor production
regulation of RNA stability
positive regulation of myeloid cell differentiation
negative regulation of protein kinase activity
tissue remodeling

Table 3.2 (continued)
hydrolase activity, hydrolyzing N-glycosyl compounds
transition metal ion transmembrane transporter activity
biopolymer methylation
epithelial to mesenchymal transition

3.3.3 Genes up regulated in injured animals that were maintained on a selenium enriched diet

A total of 111 genes were differentially up regulated in injured animals that were maintained on the selenium diet. A heat map of these gene changes clearly shows that the animals in the selenium injured group had increased gene expression as compared to those on the control diet (Figure 3.4). Animals in the control injured group exhibited a moderate amount of increase in expression as compared to sham levels. Animal 2 in the control injured group exhibited a marked decrease in expression in the genes that filtered into this template. However, since the remainder of this sample's expression profile follows the patterns of the other templates, this data set could not be eliminated without bias.

The complete gene list of genes that filtered into this particular template is shown in Table 3.3. There are many interesting genes that are filtered into this particular template, including those associated with mitochondrial function, protein turnover, transcriptional regulation, and cell differentiation.

This complete list of genes was uploaded to DAVID Bioinformatics Software to assess any patterns in genes that were present. Only one functional annotation was returned as statistically significant ($p \leq 0.05$). Genes upregulated within the selenium injured group were associated with pathways of DNA repair and included genes such as polymerase (DNA directed) beta (Polb) and poly (ADP-ribose) polymerase 1 (Parp1).

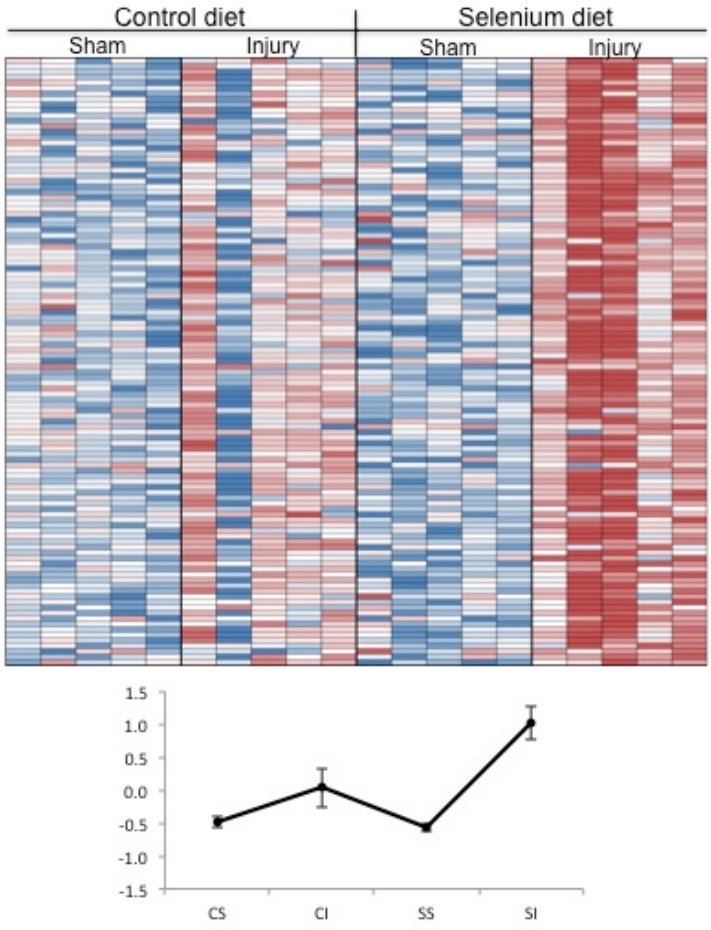


Figure 3.4: Heat map representing genes up regulated in the selenium injured group. A representative heat map from the template in which genes in animals from the selenium-enriched, injured group show increased expression. In this heat map, blue color for a gene indicates decreased expression and red indicates increased expression. Each column represents an animal in the study and each row represents a gene within this template grouping.

Table 3.3: Representative list of genes increased with selenium following injury. A functional annotation search on DAVID Bioinformatics Software indicated that there is a statistically significant alteration in expression in pathways involved with DNA repair. A complete list of the 111 genes that filtered into the increased expression in selenium-enriched template is also included (Appendix B).

Injury in diet (+), p<0.017, total of 111 genes		
Gene	**Description**	**Gene Ontology Information**
Svil	Supervillin	Regulation of actin filaments
Tgfb3	transforming growth factor, beta 3	Cell differentiation
Anxa3	annexin A3	Cell growth and signal transduction
Hspa12b	heat shock protein 12B	Cellular chaperone
Gtf2h4	general transcription factor II H, polypeptide 4	DNA repair
Recql	RecQ protein-like (DNA helicase Q1-like)	DNA repair/function
Polb	polymerase (DNA directed), beta	DNA repair/function
Parp1	poly (ADP-ribose) polymerase 1	DNA repair/function
Casp12	caspase 12	Inflammatory caspase
Mrpl12	mitochondrial ribosomal protein L12	Mitochondrial function
Acbd6	acyl-Coenzyme A binding domain containing 6	Mitochondrial function
Tfb1m	transcription factor B1, mitochondrial	Mitochondrial transcription
Snx33	sorting nexin 33	Protein sorting/targeting
Psmc4	proteasome (prosome, macropain) 26S subunit, ATPase, 4	Protein turnover
Ubap2	ubiquitin-associated protein 2	Protein turnover
Ubfd1	ubiquitin family domain containing 1	Protein turnover
Btbd12	BTB (POZ) domain containing 12	Protein turnover (substrate for ubiquitin)
Duoxa1	dual oxidase maturation factor 1	Redox regulator
Atf6b	activating transcription factor 6 beta	Transcription
Foxj2	forkhead box J2	Transcriptional activator

3.3.4 Genes down regulated in injured animals maintained on the control diet.

The template designed to examine changes in which genes are down regulated with injury but restored to sham levels with selenium enrichment resulted in a total of 78 genes sorted into this pathway. The heat map (Figure 3.5) shows a visual representation of the expression patterns of genes in this template. The animals in the injured, selenium-enriched group have expression patterns for these genes that are close to sham levels.

A total of 78 genes filtered into this template (Table 3.3) and include genes with functions associated with transcriptional regulation, mitochondrial proteins, and NF-κβ activation signaling. This gene list was uploaded to DAVID Bioinformatics Software for analysis of functional annotation. Analysis resulted in two significantly ($p \leq 0.05$) altered functional annotations, including regulation of transcription and cell cycle arrest.

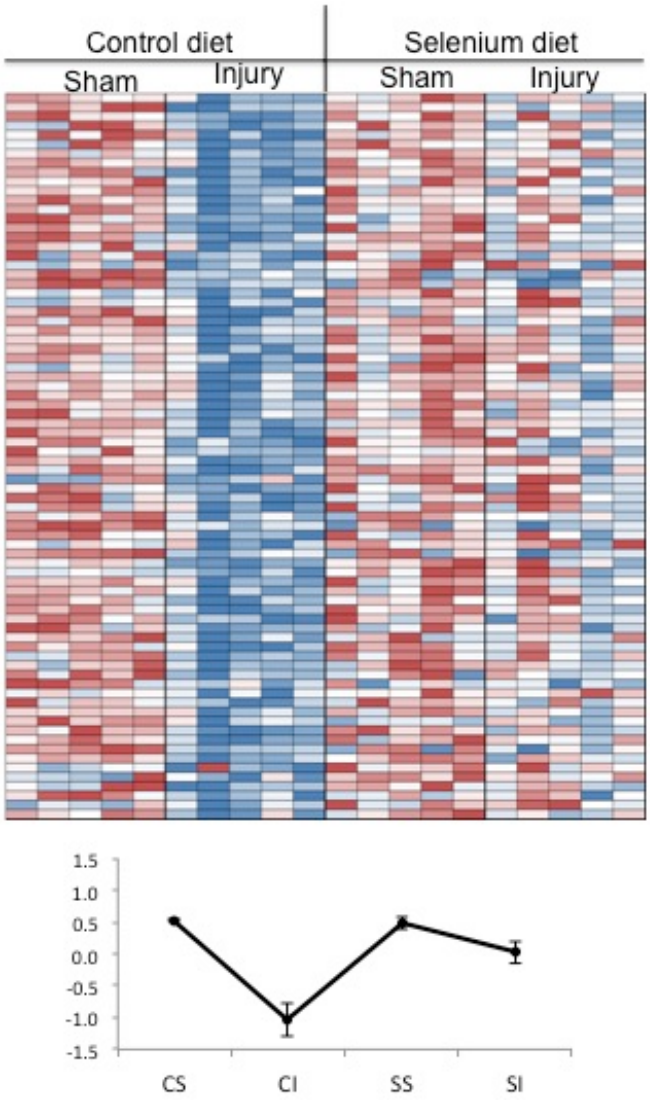


Figure 3.5: Heat map representing genes down regulated in injury and restored to sham levels with selenium supplementation. The heat map presents a visual representation of changes in gene expression in which blue indicates down regulated gene expression and red indicates up regulated gene expression.

Table 3.4: Representative list of genes down regulated in injury and restored to sham levels with selenium supplementation. Analysis of this list in DAVID Bioinformatics Software indicated 2 significant functional annotations for these genes, including transcriptional regulation and cell cycle arrest. A complete list of the 78 genes that filtered into the increased expression in selenium-enriched template is also included (Appendix C).

Injury in control (-), p<0.017, total of 78 genes		
Gene	**Description**	**Gene Ontology Information**
Aven	apoptosis, caspase activation inhibitor	Anti-apoptotic
Solh	small optic lobes homolog (Drosophila)	Calpain 15
Dmtf1	cyclin D binding myb-like transcription factor 1	cell cycle regulation
Mkks	McKusick-Kaufman syndrome	developmental dysfunction
Trmt12	tRNA methyltransferase 12 homolog (S. cerevisiae)	epigenetic regulation
N6amt2	N-6 adenine-specific DNA methyltransferase 2 (putative)	epigenetic regulation
Mmp15	matrix metallopeptidase 15	extracellular matrix
Mrpl50	mitochondrial ribosomal protein L50	Formation of mitochondrial proteins
Mrpl48	mitochondrial ribosomal protein L48	Formation of mitochondrial proteins
Tomm40b	translocase of outer mitochondrial membrane 40 homolog B (yeast)	mitochondrial translocation
Sharpin	SHANK-associated RH domain interactor	NFK-b activation - signaling
Efha1	EF-hand domain family, member A1	transcription
Zswim3	zinc finger, SWIM-type containing 3	transcription
Zfp422	zinc finger protein 422	transcription
Zdhhc8	zinc finger, DHHC-type containing 8	transcription
Zfp444	zinc finger protein 444	transcription
Zfp322a	Zinc finger protein 322a	transcription
Zzef1	zinc finger, ZZ-type with EF hand domain 1	transcription
Zhx2	Zinc fingers and homeoboxes 2	transcription
Zfhx3	zinc finger homeobox 3	transcription

3.4 Discussion

SCI affects approximately 273,000[121] patients every year and creates millions of dollars worth of health care costs. With relatively few effective treatments, a new neuroprotective strategy is needed. Transcriptional changes with selenium supplementation following injury explored some pathways that may be able to provide a small amount of neuroprotection following spinal cord injury.

Mechanical trauma to the CNS induces a sequence of molecular events known as the secondary injury cascade. Eventually, these neurodegenerative events result in neuronal cell death and pathologies associated with SCI. In addition, SCI produces a robust change in gene expression beginning as early as 4 hours post-injury and continuing into the days and weeks following the initial injury[117-120].

The gene ontologies represented in the templates for increased/decreased gene expression following SCI closely match gene ontologies observed in other studies looking at transcriptional changes after SCI. In particular, Aimone et al., showed significant changes in synaptic vesicle transport[119] in relation to disruption in synaptic plasticity. The current study supports this finding through functional pathways down regulated after injury including synaptic transmission, neurotransmitter transport, and vesicle-mediated transport (Table 3.1).

The similarities between the current study and other published studies support the methods used for performing the contusion SCI as well as the analysis of gene array data. However, the gene expression changes induced by injury alone were not the

main focus of this study. The importance of selenium for an optimally functioning CNS and previous publications that show the role selenium plays in modulating gene expression support the original hypothesis of this study that selenium will drive gene expression changes associated with the secondary injury cascade.

Template design as utilized in this analysis of microarray data provides both great benefit and a potential caveat. In the case of this study, when one particular overwhelming shift in gene expression is expected, more subtle changes in expression can be lost in the analysis. Even a moderate SCI produces pronounced changes in gene expression, with high fold changes, while the more subtle effects of diet are difficult to determine. These templates filter out all genes that are changed predominately by injury. This leaves smaller groups of genes that may show a subtle shift in gene expression, but represent significant changes due to dietary treatment in this injury model and may have been otherwise difficult to detect with traditional pathway analysis. This design, does however, limit the scope of the analysis of genes that are expected with selenium supplementation. Changes in selenoproteins, such as glutathione peroxidase for example, are expected to increase with selenium supplementation. These genes are predominately changed with injury and thus are included in the injury alone templates. Despite the caveats, this method provides an unbiased analysis of genes with subtle expressional pattern shifts, which is critical when evaluating such a large data set.

The two templates discussed in detail here are those genes with expression patterns that are upregulated in selenium enriched animals following injury and those that are down regulated in control injured animals, but brought back to sham levels with selenium treatment. Several genes associated with mitochondrial function upregulated with selenium dietary enrichment following SCI. These genes are particularly interesting in a model of CNS trauma due to the critical importance of mitochondria following injury. Mitochondrial dysfunction occurs as the result of membrane damage and calcium dysregulation in the cell and is an important trigger for apoptotic pathways following SCI. An increase in synthesis of mRNA for these mitochondrial proteins may provide neuroprotection following CNS trauma. Additionally, genes associated with transcriptional changes, NFK-b regulation, cell cycle regulation and mitochondrial proteins are down regulated following injury, but restored to sham levels with selenium supplementation. The changes in mitochondrial genes support the genes up regulated with selenium supplementation and the improvement in transcriptional regulation suggests a potential role in selenium restoring normal protein production following injury.

Further studies are needed to determine the exact mechanism through which selenoproteins are modulating expression of these different pathways. Changes in mRNA levels of these genes provide only a small picture of the cellular changes that this particular form of selenium is creating within the CNS. Selenium has been shown to activate the transcriptional regulator, NF-κβ[46], as well as modulate histone acetylation in controlling inflammatory gene expression[41]. These targeted changes of

transcriptional machinery may provide a clue as to how different seleno compounds are regulating shifts in gene expression patterns.

Another important caveat to consider when examining gene expression data in spinal cord injury models is the well characterized response to injury that involves an influx of cells into the site of primary injury[122]. In future experiments, performing cell-sorting with samples to specifically choose neuronal or glial specific cells, may provide interesting information and allow for distinction of gene expression changes from invading macrophages or leukocytes.

CHAPTER 4

Long term dietary selenium supplementation may provide neuroprotective benefits for mitochondrial respiration

4.1 Introduction

The neurological devastation brought on by traumatic brain injury (TBI) is costly both financially and in terms of quality of life for the estimated 3.17 million patients with long term disabilities associated with TBI every day[123]. Certain groups of individuals run a higher risk for sustaining a traumatic brain injury. In particular, with recent military involvement in conflicts in Iraq [Operation Iraqi Freedom (OIF)] and Afghanistan [Operation Enduring Freedom (OEF)], soldiers are increasingly returning from deployment with combat injuries classified as TBIs[66]. The prevalence of mild traumatic brain injuries among athletes in contact sports has also become a focus of growing concern, with as many as 3.8 million sports concussions occurring each year[124]. This substantial incidence of TBIs, particularly in high-risk groups, emphasizes the importance of developing novel therapies to combat the neurodegeneration and pathologies following TBI. In the hours and days after a mechanical injury to the CNS, a host of secondary molecular events occur, which, if unchecked will lead to neuronal cell death. This secondary injury cascade includes molecular problems such as calcium dysregulation, mitochondrial dysfunction, oxidative damage, lipid peroxidation, and excitoxicity. As an essential dietary nutrient, critical for the function of several antioxidant enzymes, dietary selenium enrichment may serve as an effective pretreatment to minimize CNS damage in the event of trauma.

Selenium modulates neurodegeneration in many other disorders of the CNS, making it an interesting therapeutic target for TBI. Increased dietary selenium levels reduced RNA and DNA oxidative damage in an Alzheimer's Disease model[34] and decreased DNA damage and movement disorders in mouse models of Parkinson's Disease[63]. A form of selenium yeast, ebselen, has also proven neuroprotective following ischemic stroke in rodents[56]. Selenium functions as a co-factor for several antioxidant selenoproteins, including glutathione peroxidase. Additionally, other selenoprotein families have important immune modulating and antioxidant effects[49], thereby providing potential for these neuroprotective effects to extend into neurotrauma as well.

Selenium was enriched at different levels in rat diets prior to injury to examine a potential neuroprotective role for dietary selenium. In the present study, dietary selenium enrichment was present in the form of selenized yeast. The formulation of selenium is critical when considering increasing selenium levels in both animal models and in translation to human clinical trials. Inorganic forms of selenium, such as sodium selenite and selenium sulfides, common in most over the counter multivitamins and anti-dandruff shampoos, respectively, can cause toxicity in high concentrations[14]. Selenium was incorporated into the diets in the form of selenized yeast (Sel-Plex®, Alltech, Nicholasville, KY), which like other organic forms of selenium, has a lower risk of toxicity issues than the inorganic salt forms[15, 16]. Selenized yeast has been used previously in studies examining the cancer preventative properties of selenium[50], as well as in rodent ischemic stroke models[55].

In this formulation, yeast is grown in the presence of elemental selenium and results in the presence of several different selenium species. When analyzed by mass spectrometry selenized yeast contains approximately 20% selenomethionine[125], 20% selenocystine and Se-methylselenocysteine, and 40-50% of other unique selenium compounds[126]. By adding selenium in the form of seleno-amino acids, selenium can be stored easily in tissues as selenomethionine (in place of methionine) and provide a readily available source of selenium for the formation of selenoproteins. The unique selenium compounds specific to selenized yeast may be crucial to defining the neuroprotective benefits over other formulations of selenium[18].

In the secondary neurodegenerative cascade of events that follow the initial mechanical trauma, mitochondrial dysfunction is one of the primary pathologies that precipitates neuronal death[127]. As the primary site for ATP production in the neuron, mitochondrial damage results in a loss of critical energy production as well as failure to buffer intracellular calcium levels. A dysregulation of calcium buffering within the neuron can inhibit ATP synthesis, lead to the release of the apoptotic signaling factor, cytochrome c, increase production of reactive oxygen species (ROS), and eventually lead to cell death[128, 129]. Through work with isolated mitochondria, endogenous (and exogenous) substrates can be added directly to cortical mitochondrial to mimic various states of electron transport chain (ETC) respiration (Figure 4.1). These assays measure oxygen consumption rate (OCR) and can provide information about overall mitochondrial health following injury. The Seahorse Bioscience system measures oxygen levels within a closed chamber was modified to allow for multiple sample

testing in one plate (Seahorse Bioscience®). Because they are crucial for energy production and dysfunction can trigger cell death, mitochondria are a particularly attractive target for intervention after traumatic brain injury.

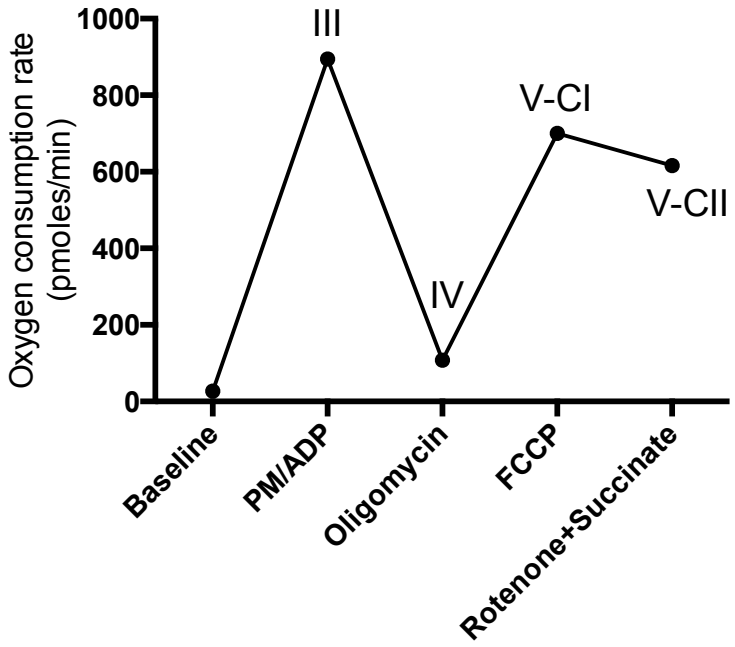


Figure 4.1: Representative trace of oxygen consumption in isolated mitochondria. Oxygen consumption is measured in response to various endogenous and exogenous substrates (Seahorse Bioscience). Isolated mitochondria are kept in a chamber with a controlled level of oxygen. Oxygen consumed is representative of the efficiency of the electron transport chain (ETC). Adding the substrates pyruvate and malate, along with ADP, allow the ETC to consume oxygen through the production of ATP from ADP (III or State III respiration). Oligomycin is then added to artificially stop the ATPase, thus effectively halting ATP production, the ETC, and oxygen consumption (IV or State IV respiration). Following that, the protonophore, FCCP, is added (V-C I or State V-complex I respiration) which allows for free passage of H^+ ions across the membrane and thus maximally driven respiration. Finally, rotenone, a Complex I inhibitor, and succinate, the endogenous substrate for respiration through Complex II, are added to test respiratory capacity through Complex II of the ETC (V-C II or State V-complex II driven respiration). Changes in rates of oxygen consumption in response to these different substrates correlate directly to problems with these specific components of the ETC, pinpointing the location of mitochondrial damage following injury.

The detrimental impact of the secondary injury cascade typically results in neuronal death. Ultimately, this neuronal death leads to pathological functional changes. Improving these functional deficits is crucial in the lives of patients living with TBI and thus is also an important parameter to investigate. Different animal models of TBI have been linked to spatial memory deficits[130, 131]. As this is also a common clinical symptom, the Morris Water Maze task was developed and used in a wide variety of studies to test cognitive problems in various rodent models[132]. In addition, memory deficits have been linked to development of cortical lesion, particularly in the controlled cortical impact (CCI) injury model.

This study examines the potential for increased dietary selenium to protect various states of mitochondrial respiration in the injured cortex, prevent neuronal cell death, and ultimately improve memory deficits following TBI.

4.2 Methods

4.2.1 Animal care and diet

Male Sprague-Dawley rats were purchased from Harlan and placed immediately on one of four diets. Selenized yeast was formulated within the rat diet for a final concentration of either 1.3ppm selenium (approximately 19.5μg/day) or 2.3ppm selenium (approximately 34.5μg/day). Normal rat chow containing 0.3ppm of selenium (4.5μg/day) was utilized as the control diet. The control diet is comparable to a normal rat chow with a yeast additive to account for any differences caused by the yeast in the selenium compound. The selenium enriched diets are labeled as 1ppm (SP1) or 2ppm (SP2) of additional selenium in the form of selenium yeast. Yeast was grown in the presence of selenium; allowing selenium to be taken up into the cell walls of the yeast and incorporated as the amino acids selenocysteine and selenomethionine, as well as other small seleno-compounds. The selenium deficient diet is formulated using a torula yeast base. Because selenium is present in most plant sources, normal plant-based rodent diets will contain a small amount of selenium. By using torula yeast as a base for the diet, this controls the growing environment and ensures the diet will be completely deficient in selenium. Animals were fed *ad libitum* for 2, 4, 8, or 16 weeks, depending on the length of feeding required for each experiment. Diets were formulated by Harlan and selenized yeast, in the two different concentrations, was supplied to Harlan as Sel-Plex® (Alltech, Nicholasville, KY).

4.2.2 Selenium tissue levels

Diets were fed *ad libitum* to male Sprague-Dawley rats for either 2 weeks, 4 weeks, 8 weeks, and 16 weeks prior to tissue collection. Naïve cortex samples were collected

for tissue analysis. Prior to sampling, animals were transcardially perfused with approximately 250mL of phosphate buffered saline to eliminate selenium contamination from blood in the tissues. Selenium levels were analyzed (Alltech) by liquid chromatography-mass spectrometry (LC-MS) in samples taken from the left cortex (n=6 per diet). Animal weights and caloric content consumed were also monitored for significant changes due to the different dietary formats.

4.2.3 Controlled cortical impact injury

After being maintained on their respective diets for 16 weeks, rats received a moderate (1.75mm depth) unilateral, controlled cortical impact (CCI) injury[133] or a sham craniotomy. In the second major cohort of animals tested, the dosage period was shortened to 4 weeks of dietary supplementation (or deficiency) and animals received a severe (2.2mm depth) unilateral, CCI or a sham craniotomy. Animals were first anesthetized with 3% continuous isoflurane, followed by shaving the scalp, and securing the animal in a stereotaxic frame. After sterilizing the scalp, an incision was made over the site of the intended craniotomy. A 6mm craniotomy was created using a hand trephine, lateral to the central suture, centered between lambda and bregma, and positioned directly over the left parietal cortex. The injury was performed with a pneumatically controlled impactor with an intended depth of either 1.75mm or 2.2mm (depending on the experimental paramaters), a dwell time of 500msec, and a velocity of 3.5m/s as previously described[129] (Precision Systems and Instrumentation, Fairfax Station, VA). Immediately following the injury, the craniotomy was covered, the scalp sutured, and the scalp injected with bupivicaine/epinephrine to minimize

discomfort due to the incision. Sham controls received a craniotomy, anesthesia, and bupivacaine/epinephrine solution without the cortical contusion injury.

4.2.4 Behavioral assessment of functional deficit

Spatial memory after injury was assessed using the Morris Water Maze task. The Morris Water Maze (MWM)[132] was developed as an open field behavioral task designed to test spatial and working memory in rodent models. Since its development, the MWM is a widely utilized and highly cited method to determine cognitive deficits in the trauma field. This task is an open-field swimming task in which a sunken platform is placed in a particular quadrant of the testing pool. To discover the location of the platform, animals must rely on spatial memory and visual cues on the walls surrounding the pool. Testing in the water maze began at 10 days post-injury. This lapse of time after injury is necessary for sufficient recovery following surgery. Animals underwent 4 trials each day for 4 consecutive days. During each trial, animals had 60 seconds to find a sunken platform. If the platform was not discovered, rats were led to the platform. The fifth day consisted of a probe test in which the platform was removed and the number of times the animal crossed the location of the (now) missing platform were recorded. Both time and distance (path length) to the platform were recorded as functional measures of spatial memory over the testing days and the number of times to cross the platform location during the probe trial.

4.2.5 Mitochondrial respiration

Mitochondria were isolated from a separate cohort of animals using a differential preparation method at 24 hours after injury, with slight alterations from Patel et al[72,

[134]. Animals were euthanized with CO_2 inhalation and the brain was rapidly removed. Following a brief rinse of the surface of the cortex with ice cold isolation buffer (1 mM EGTA, 215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, pH 7.2 adjusted with KOH), a cortical punch was taken from the area immediately surrounding the injury epicenter in the ipsilateral cortex. A cortical punch was also taken from either the sham cortex or the contralateral cortex. The sham or contralateral cortices were utilized to control for any plate-to-plate differences within the assay. The tissue was homogenized in 1mL of cold isolation buffer using a downs homogenizer. Isolation buffer was added to samples to a total of 2mL and spun at 1300 x g for 3 minutes at 4°C. Supernatant was transferred to new tubes and filled to 2mL with isolation buffer. The pellet was resuspended in 500µL of isolation buffer and spun again at 1300 x g for 3 minutes at 4°C. The resulting supernatant was transferred to a new tube and filled to 2mL with isolation buffer. The two sets of supernatants from the two different spins were then spun at 13,000 x g for 10 minutes. The supernatant was discarded and the resultant pellet was resuspended in 400µL of isolation buffer. Samples were then placed in a cell disruption nitrogen bomb to release mitochondria contained within synaptosomes at 1200 psi for 10 minutes. Total mitochondria were then spun at 10,000 x g for 10 minutes. The resulting pellet was resuspended in 100µL of isolation buffer without EGTA (as listed above without EGTA added).

Mitochondrial viability was assayed by measuring respiration rates using the Seahorse XF Extracellular Flux Analyzer (Seahorse Bioscience). Each plate contains

24 wells, allowing for up to 8 samples to be run in either duplicate or triplicate on each plate. To control for plate-to-plate variation in signal by the Seahorse Bioscience, each sample was normalized to the sham control within the same plate. A total of 15μg of isolated mitochondria was added to each well in the plate. Any preparations that resulted in a respiratory control ratio (RCR) of <5 for the contralateral or sham samples were not included in further analysis. The RCR is the ratio of State III/State IV oxygen consumption rates.

4.2.6 Immunohistochemistry

Animal cohorts that were used in behavioral testing were euthanized after the final behavioral assessment at 15 days post injury. Animals were injected with an overdose of sodium pentobarbital (150mg/kg, i.p.). Once an appropriate depth of anesthesia was determined based on a non-response with toe-pinch, a thoracotomy was performed. Animals were transcardially perfused with ice-cold 1X phosphate-buffered saline (PBS) (approximately 200mL), followed by cold 4% paraformaldehyde (approximately 250mL). Following perfusion, whole brain was removed and placed into a post-fixative solution of 4% PFA for 24 hours at 4°C. Then brains were transferred to 30% sucrose for 3 days for cryoprotection. Brains were sectioned using a sliding microtome in 50μm sections in the coronal plane. Sections were kept in a series of 10, ensuring that each set of sections was representative of tissue at 500μm apart. Sections were stored in a cryoprotective solution (30% ethylene glycol, 30% sucrose in 1X PBS) until staining. Sections were mounted to slides (Fisher SuperFrost Plus, Fisher Scientific) and dehydrated first with chloroform + 95% ethanol (EtOH). Sections were then rehydrated using increasing

dilutions of EtOH with water. Slides were then stained with cresyl violet stain and serially dehydrated with increasing concentrations of EtOH, followed by Citrisolv. Slides were cover slipped with Permount (Fisher Scientific) and allowed to dry overnight prior to imaging. Spared tissue was evaluated as a percentage of the healthy tissue on the contralateral side.

Tissue sections analyzed for the presence of gluial fibrillary acidic protein (GFAP), a marker of astrocytic activation, were transferred from cryoprotectant solution into TBS. Free floating sections were blocked for 1 hour with 1% normal horse serum at room temperature and then incubated in the primary antibody for GFAP (Mouse anti-GFAP, 1:50,000, Millipore) overnight at room temperature. After washes in Tris-buffered saline (TBS), anti-GFAP was bound to the secondary antibody conjugated to biotin (1:5000 Jackson Immunoresearch) for 1 hour at room temperature. Slices were treated with peroxidase conjugated streptavidin (1:1000, Jackson Immunoresearch) and then detected using 3,3'-diaminobenzidine (DAB, Jackson Immunoresearch) for 5 minutes. Slides were allowed to dry overnight and then dehydrated with increasing concentrations of EtOH, then xylene, and cover slipped with Permount (Fisher Scientific).

4.2.7 Glutathione peroxidase activity

Glutathione peroxidase (GPx) enzyme activity was determined using a glutathione peroxidase assay kit (Cayman Chemical). In naïve animals, the left cortex was homogenized in 1mL of ice-cold buffer (50mM Tris-HCL, pH 7.5, 5mM EDTA, 1mM DTT). In injured animals, a portion of the ipsilateral and contralateral cortex

homogenized for mitochondrial analysis was set aside for GPx activity. These samples were further homogenized with the homogenization buffer buffer containing (1mM DTT). The samples were then centrifuged at 10,000 x g for 15 minutes at 4°C. Samples were loaded onto a 96-well plate along with other buffers provided with the kit. The assay was activated with cumene hydroperoxide and immediately read for absorbance at 340nm every minute for 5 total minutes. The assay measures the oxidation of NADPH to $NADP^+$, which results in a decrease in absorbance at 340nm. Each sample was run in duplicate or triplicate on the plate. The overall rate of change in absorbance values were calculated using the following equation (*A* indicates absorbance):

$$\Delta A/\min = \frac{|A(Time\ 2) - A(Time\ 1)|}{Time\ 2(min.) - Time\ 1\ (min.)}$$

The change in absorbance was averaged across all wells for each sample. The rate of change (*ΔA/min*) for the background wells was then subtracted from values for the samples. The following formula was used to calculate the GPx activity:

$$GPx\ activity = \frac{\Delta A/min}{0.00373\mu M^{-1}} x \frac{0.19mL}{0.02mL} x\ sample\ dilution = \frac{nmol}{min}/mL$$

The constant ($0.00373\mu M^{-1}$) is the NADPH extinction coefficient and was applied to all samples according to the above formula. After the GPx activity was calculated for each sample, the activity was also adjusted for protein concentration of the sample.

4.2.8 Statistics

Data presented in each figure are shown as mean±standard error of the mean (SEM). For all experiments, significance was set as α=0.05. Using GraphPad Prism 6.0, differences between mitochondrial respiration data were calculated using either a Student's t-test (Figure 4.1) or a one-way ANOVA (Figure 4.6) depending on the number of variables being tested. Each state of mitochondrial respiration was analyzed separately as each is a separate measure, using distinct substrates. Data generated from MWM behavioral testing was evaluated using either a 2-way, repeated measures ANOVA when examining the time and distance to the platform over the 4 training days or a 2-way ANOVA when analyzing the significance in the 4^{th} day of training and probe test data (Figure 4.2 and Figure 4.8). Differences in cortical tissue sparing and GFAP between control and SP1 animals were calculated using a Student's t-test (Figure 4.3). Tissue selenium content was analyzed using a repeated measure, one-way ANOVA for the time course supplementation data, or a one-way ANOVA for the selenium levels for each diet after 4 weeks of supplementation (Figure 4.4). A one-way ANOVA was used to analyze differences between overall caloric content and final weight gain of animals maintained on the 4 different diets, with multiple comparisons to the selenium deficient diet (Figure 4.5). Significance of data on GPx activity was calculated using either a one-way ANOVA for naïve animals and for samples taken from injured isolated mitochondrial preparations (Figure 4.7A, B). No statistical analysis was performed on data for GPx activity in the injured cortex as the sample size was too small to accurately run statistics (Figure 4.7C, D).

4.3 Results

4.3.1 Mitochondrial respiration (16 weeks of dietary supplementation)

When examining mitochondrial respiration 24 hours post-injury, those animals on the selenium enriched diet showed improvement in the different states of respiration tested. When the endogenous substrates, pyruvate/malate and ADP, were added to isolated injured mitochondria a significant improvement in OCR in mitochondria isolated from animals maintained on the selenium enriched diet (Figure 4.2A). Mitochondrial samples were then manipulated with oligomycin and FCCP. These substrates mimic maximal mitochondrial respiration, State V complex I driven respiration. When normalized to sham controls, animals maintained on the selenium enriched diet showed significant increase in oxygen consumption as compared to control animals (Figure 4.2B). Substrates rotenone and succinate were then added to the samples. Rotenone acts to inhibit complex I in the electron transport chain, allowing succinate to supply complex II (mitochondrial respiration as State V, complex II). Mitochondria isolated from animals maintained on the selenium supplementation showed improved State V, complex II respiration following injury when compared to control animals (Figure 4.2C).

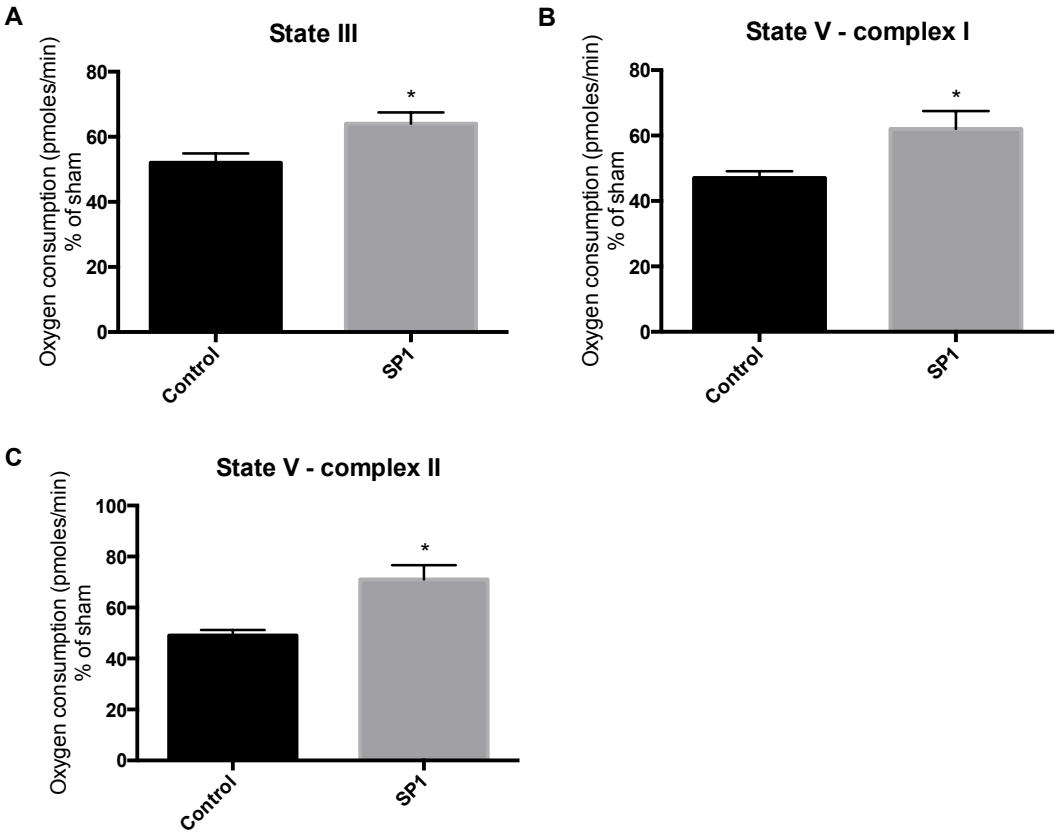


Figure 4.2: Mitochondrial respiration following injury in animals maintained on different dietary levels of selenium. Animals were fed diets for 16 weeks (n=4 for all injured/sham). OCR is expressed as a percentage of sham levels within the same plate to account for plate-to-plate variability. **A)** Dietary enrichment with selenium (SP1) improved State III mitochondrial respiration following the addition of ADP and pyruvate/malate. **B)** Increased selenium improved State V-complex I driven mitochondrial respiration following the addition of FCCP. **C)** Animals with increased selenium exhibited improved mitochondrial respiration through complex II in State V-complex II respiration after the addition of rotenone and succinate. Error bars represent SEM, *$p \leq 0.05$

4.3.2 Spatial memory following injury (16 weeks of dietary supplementation)
Behavioral training began 10 days following injury and included 4 days of training followed by a probe test on the 5th day. Both the time and distance to the sunken platform were recorded as a measure of spatial memory and learning within the Morris Water Maze (MWM) task. Sham animals showed no difference in spatial memory between different dietary levels of selenium. Injured animals on the selenium enriched diet (SP1) showed an overall trend over the four days towards shorter time and distance to platform discovery when compared to the control diet injured group (Figure 4.3A and 4.3B). When examining the final day of training (Day 4), the selenium enriched group had a significantly shorter distance to platform discovery (Figure 4.3D). While the time to the platform was not significantly shorter between injured animals on the selenium diet and control diet injured animals, the data shows a trend toward improvement in the selenium enriched group (Figure 4.3C). On the fifth day of MWM testing, the platform was removed as part of the probe test. The total number of times the animal crossed the original location of the platform was recorded as a final measure of spatial memory. The data presented represents the total number of platform crossings within the first 15 and 30 seconds after being placed in the pool. Probe data indicates a trend towards improvement after injury in animals supplemented with selenium after both 15 and 30 seconds but does not reach statistical significance (Figure 4.3E and 4.3F).

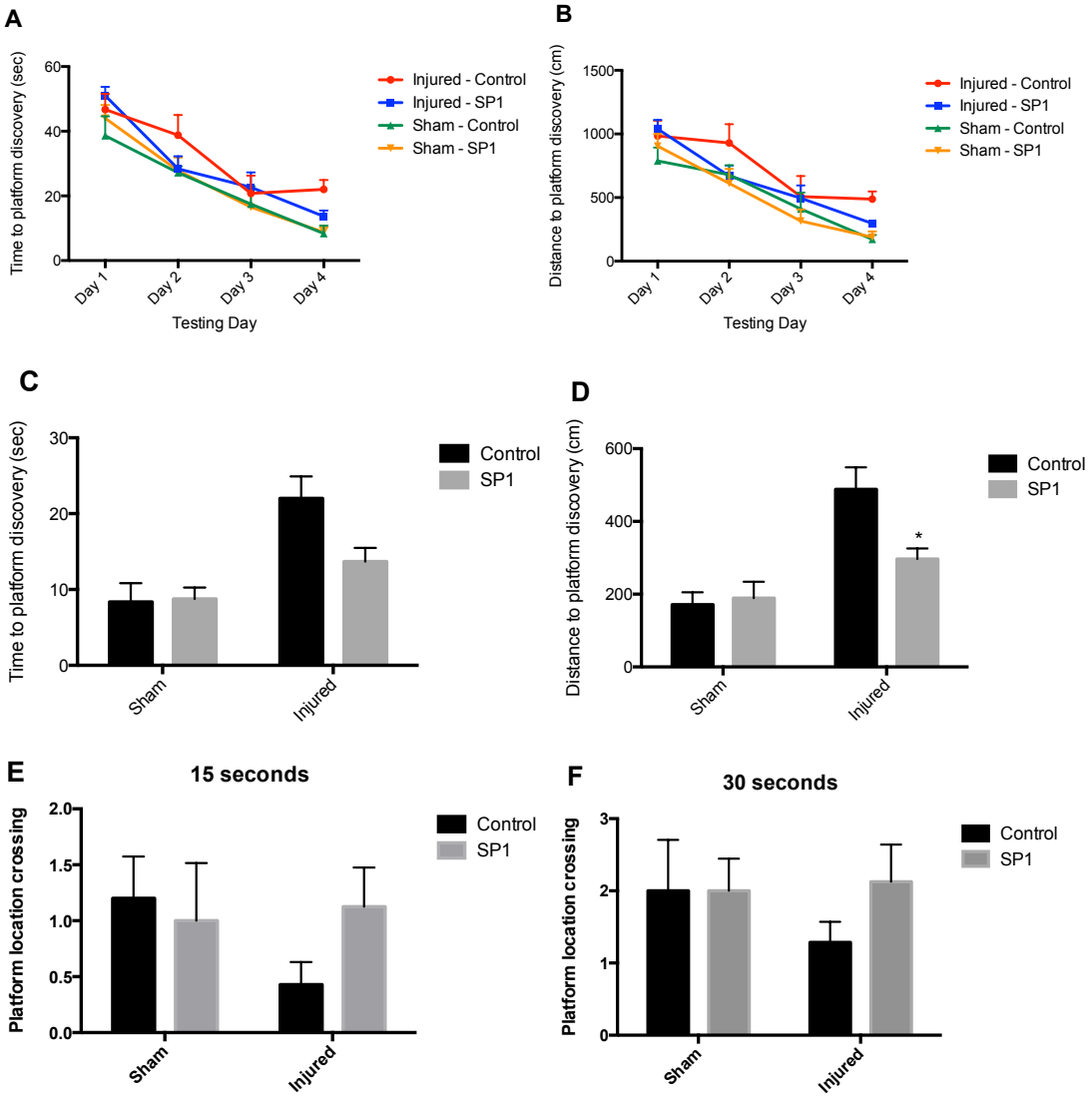


Figure 4.3: Selenium improves performance in behavioral testing after injury. Overall performance on MWM task during four training days and final probe test (n=7 control injured group, n=8 SP1 injured group, n=6 sham groups). One animal in control injured group was euthanized due to complications from surgery. **A, B)** Sham animals showed no difference in the time or distance to the platform over the four training days. In injured animals, those supplemented with selenium exhibited an overall improvement in the time and distance to discovery of the platform over the four training days. **C)** On day 4, the time to the platform discovery appeared to improve in injured animals supplemented with selenium, however did not reach significance. **D)** On day 4, the distance to the platform was improved in injured animals with the enriched selenium diet when compared to injured animals on the control diet. Sham animals did not show any differences between dietary groups. **E, F)** During the probe test, animals on the selenium enriched diet found the location of the platform similar to sham animals within 15 and 30 seconds of being placed in the testing pool. However, due to variability, this did not reach statistical significance. Error bars represent SEM, *p≤0.05.

4.3.3 Tissue histology

Brain tissue collected from animals that were utilized for MWM testing was examined for overall lesion volume and astrocyte activation following injury. At 15 days after the initial CCI injury, cell death begins to occur and typically a lesion results in the area of the impact. In this data, selenium enrichment did not provide any protection from cell loss leading to a lesion in the cortex (Figure 4.4A). Additionally, astrocyte activation following injury is not significantly different between the animals maintained on the two different diets (Figure 4.4B).

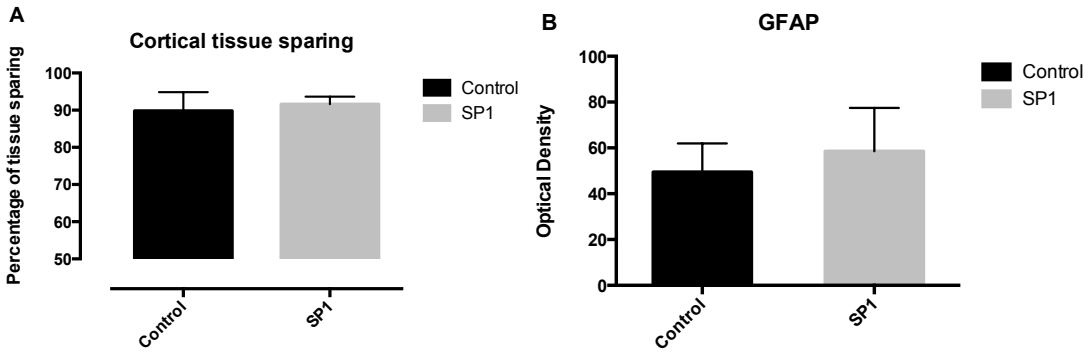


Figure 4.4: Selenium does not improve cortical tissue sparing or astrocyte activation following injury. Overall cortical tissue sparing and astrocyte activation shown in coronal sections (50μm) in the injured cortex. **A)** Animals maintained on the selenium enriched diet (SP1) did not show any improvement in cortical tissue sparing. Additionally, the moderate injury (1.75mm impact depth) did not result in a large amount of lesion in the cortex. **B)** Astrocyte activation, as measured by GFAP staining, did not show any significant differences between the two dietary groups.

4.3.4 Different time periods and supplementation dosages

Due to the promising results seen after 16 weeks of selenium enrichment, the time course and dietary dosage were examined to determine the shortest length of supplementation time necessary for protective effects. Shortening the supplementation time frame helped to simplify the time constraints for laboratory experiments and may potentially be beneficial for translation of this supplement in the clinical setting. LC-MS analysis showed that selenium levels increased significantly in the CNS as early as 2 weeks following dietary enrichment (Figure 4.5A and 4.5B). For further experiments, the 4 week supplementation time period was selected. To expand the scope of our studies, we added two additional dietary dosage levels for 4 week dietary supplementation (selenium deficient and increased selenium, SP2). The selenium deficient diet decreased overall tissue storage of selenium in the cerebral cortex and the SP2 diet further increased cortical selenium levels (Figure 4.5C).

The diets were adjusted so that caloric content was similar among the different diets. However, upon close examination of the nutritional content, the selenium deficient diet contained a large difference in different micronutrient content. In order to formulate the diet into pellets, a large amount of corn oil was added to the torula yeast based diet. Additionally, high levels of choline are present in this diet (Appendix H). While the caloric content consumed by the animals was relatively equal, the differences in micronutrients within the diet as well as the large amount of corn oil confound any potential conclusions that can be drawn from this data.

The slightly different composition of the selenium deficient diet may be of concern when considering specific components, but the overall weight gain and calories consumed by animals on these diets showed no significant differences (Figure 4.6A and 4.6B).

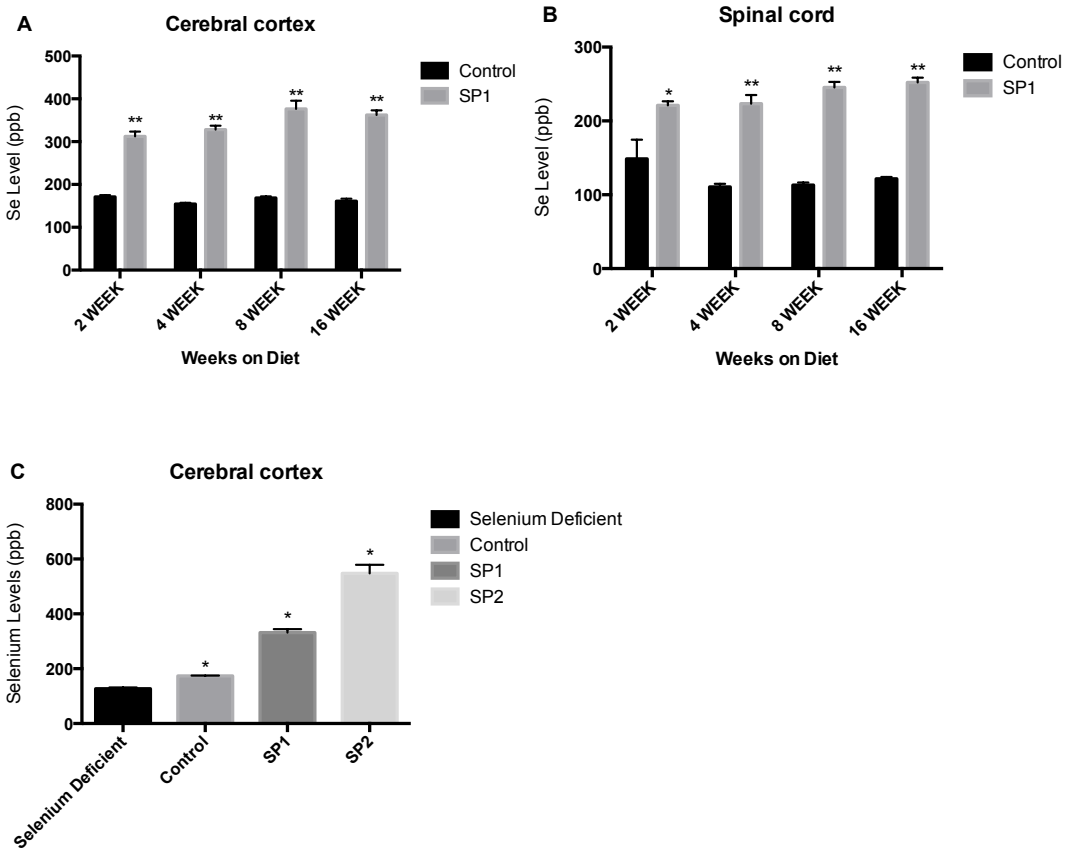


Figure 4.5: Selenium tissue levels are altered relative to time of dietary supplementation and level of selenium in the diet. A) Cerebral cortex levels at different time points of supplementation in naïve animals. (n=5) As early as 2 weeks of supplementation with increased selenium (SP1), tissue selenium levels were increased in cortex. This level continued to increase over 16 weeks of supplementation. **B)** Spinal cord selenium levels at different time points of supplementation in naïve animals. (n=5) Selenium tissue levels were increased at 2 weeks and continued to increase over 16 weeks of supplementation with SP1 diet. **C)** The time point of 4 weeks of supplementation was selected for further dietary supplementation studies. (n=6) Studies were widened to include additional dietary levels of selenium, including a diet deficient in selenium and one increased level of selenium. Tissue levels of selenium were altered relative to the amount of selenium present in the diet. The tissue levels in animals on the control, SP1, and SP2 diets were increased relative to those maintained on the selenium deficient diet. Error bars represent SEM, *$p \leq 0.05$, **$p \leq 0.001$

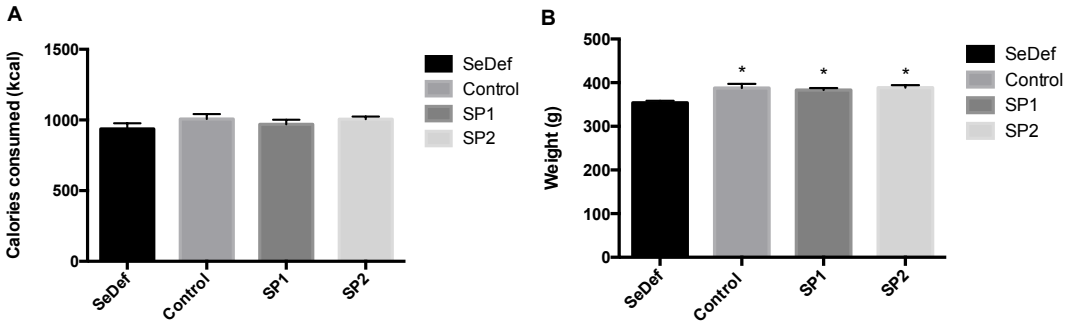


Figure 4.6: Dietary composition did not significantly alter the caloric intake, but did change weight gain. **A)** Average number of kcal consumed per cage, per week (2 animals housed in each cage). (n=3 cages, calories representative of calories for 2 animals) The different diets did not have a significant effect on the total number of calories the animals consumed. **B)** Final weight of animals prior to surgery (n=6). The weights of animals in the selenium deficient diet group were significantly different from animals in the control, SP1, and SP2 groups. However, because the weight difference was less than 20% of animals in the other group, the difference was not a concern for animal care and surgical techniques. Error bars represent SEM, *$p \leq 0.05$

4.3.5 Mitochondrial respiration (4 weeks of supplementation)

To examine the effects of the 4 different levels of dietary selenium over 4 weeks of supplementation on mitochondrial function, following injury we utilized a more severe injury (2.2mm impact depth). Due to modest mitochondrial protective effects seen at a less severe injury (1.75mm) in previous experiments, the injury severity was increased in these studies and similar parameters were tested in an effort to demonstrate a more robust effect of selenium treatment. At 24 hours following injury, cortical mitochondria isolated from the area immediately surrounding the injury (ipsilateral) showed damaged mitochondrial respiration in comparison to the contralateral side. In these experiments the contralateral cortical mitochondria was used as an in-plate control. The ipsilateral mitochondria, when expressed as a percentage of the contralateral side did not replicate the protective effects of selenium enrichment seen previously from the 16 week supplementation time period (Figure 7A, B, C). While it is clear that the selenium deficient diet exacerbated the injury effect, the additional levels of selenium did not provide additional protection from injury. The increased detriment to respiration in animals on the selenium deficient diet does, however, emphasize the importance of selenium in the CNS following injury. Interestingly, this effect persisted throughout the three different states of respiration tested (Figures 7A, 6B, 6C).

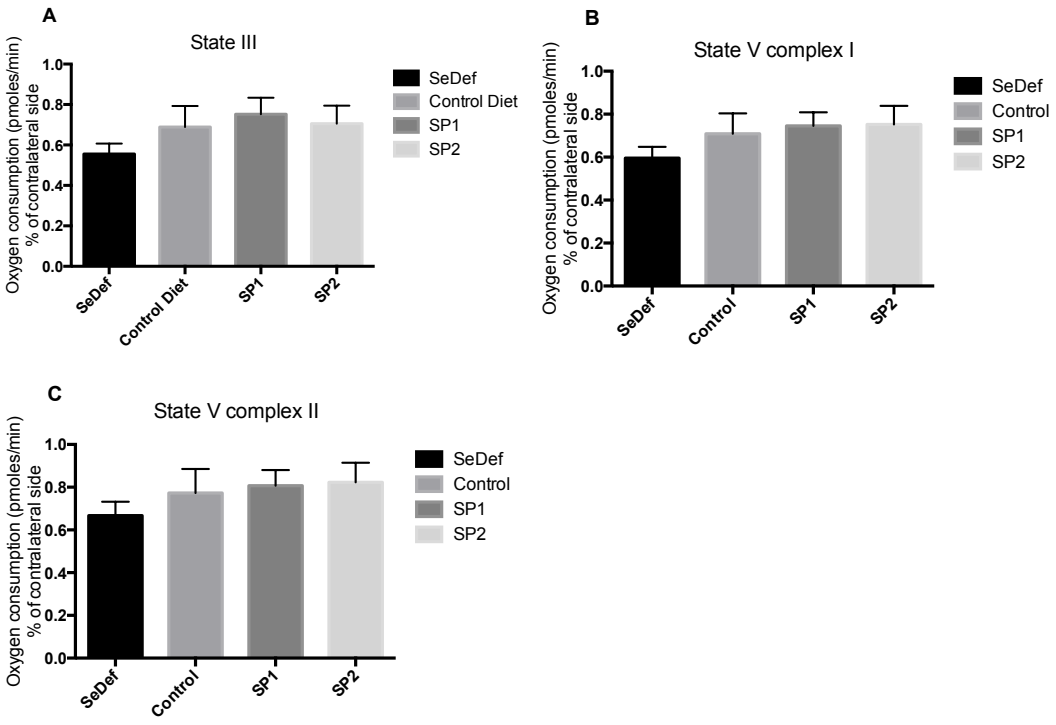


Figure 4.7: Mitochondrial respiration in injured cortex following 4 weeks of dietary supplementation. OCR is expressed as a percentage of the contralateral cortex samples within the same plate to account for plate-to-plate variability. (n=7 for all injured/sham) **A)** Selenium enrichment did not improve mitochondrial respiration through State III driven respiration (**A**), State V-Complex I driven respiration (**B**), or State V-complex II driven respiration (**C**). There is a clear trend exhibiting exacerbation of the injury effect in animals maintained on the selenium deficient diet. However, this was not statistically significant (α=0.05). Error bars represent SEM.

4.3.6 GPx activity with different levels of selenium supplementation

After being maintained on 4 different levels of selenium supplementation for 4 weeks, naïve cortex samples showed no significant changes in glutathione peroxidase (GPx) activity (Figure 4.8A). Animals receiving the selenium deficient diet, exhibited a slight decrease in GPx activity, but deficient conditions were not sufficient to impact overall GPx activity.

Following severe (2.2mm impact depth) TBI, GPx activity in crude mitochondrial preparations had very little difference in activity levels between the dietary groups (Figure 4.8B). The dietary source was not enough to drive a change in GPx activity or potentially the sample utilized for these studies did not represent total cellular GPx activity and thus is not conclusive of the effect on overall GPx activity within the injured cortex.

In a small cohort of animals, we also tested GPx activity following injury in isolated cortical samples from both the ipsilateral injured cortex as well as the contralateral cortex. Due to limitations of available diet, the number of animals included in this experiment was not large enough to run statistical analysis. These animals did however demonstrate a trend towards a rise in GPx activity following injury (Figure 4.8C). Additionally, when examining only the ipsilateral cortex, it is clear that the first level of enriched dietary selenium (SP1) increases GPx activity at the highest level in comparison to other dietary levels of selenium (Figure 4.8D).

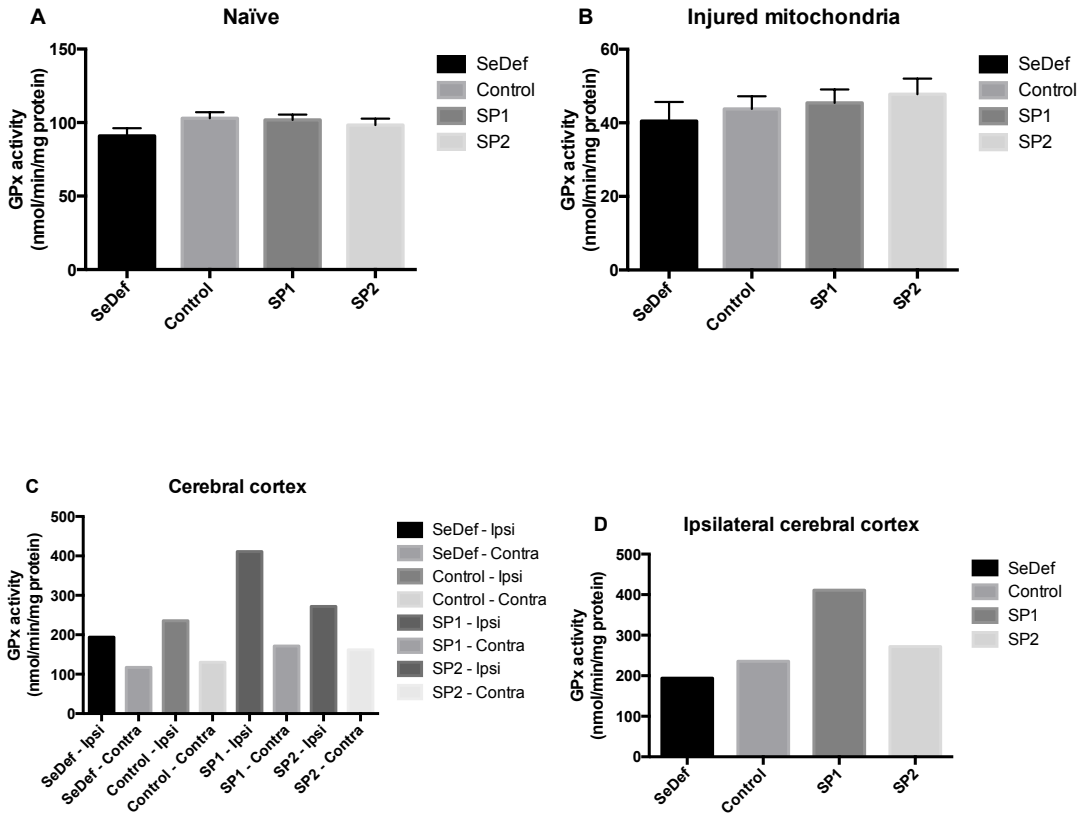


Figure 4.8: Glutathione peroxidase activity in both naïve and traumatic brain injured animals. **A)** Naïve animals showed no changes in overall GPx activity despite 4 weeks of differing dietary levels of selenium (n=6). **B)** Samples from isolated crude mitochondria following injury showed no changes relative to dietary selenium content. (n=5) **C)** All 4 dietary sources show a distinct injury effect. There is an increase in GPx activity in the ipsilateral cortex at 24 hours after TBI. Ipsilateral (ipsi) and contralateral (contra) cortical tissue levels are shown for each dietary group. (n=2) **D**) Within the cerebral cortex data (C), the ipsilateral cerebral cortex showed an increase specifically with the first level of enriched selenium dietary level (SP1). Due to a small number of animals within each group, no statistics were run on this data. (n=2)

4.3.7 Spatial memory following injury (4 weeks of supplementation)

Due to a lack of effect in comparison to the SP1 diet in mitochondrial and GPx data, the highest level of selenium enrichment (SP2) was eliminated from the behavioral studies. After 4 weeks of dietary supplementation and a severe CCI injury, animals showed an injury effect during testing in the Morris Water Maze task (Figure 4.9A, B). The selenium deficient and SP1 groups exhibited the expected injury effect in the time and distance to platform discovery. The confounding results in these behavioral studies are that the control sham group performed poorly in the 4 days of MWM training and testing. Additionally, these animals showed the lowest number of platform crossings out of all sham animals during probe testing (Figure 4.9E, F). The previously observed improvements in time and distance to platform discovery in animals maintained on the selenium enriched diet were not replicated in this current study (Figure 4.9C, D). While an injury effect is apparent between the selenium deficient and SP1 groups, any significant improvements in spatial memory between injured animals on these diets were not apparent.

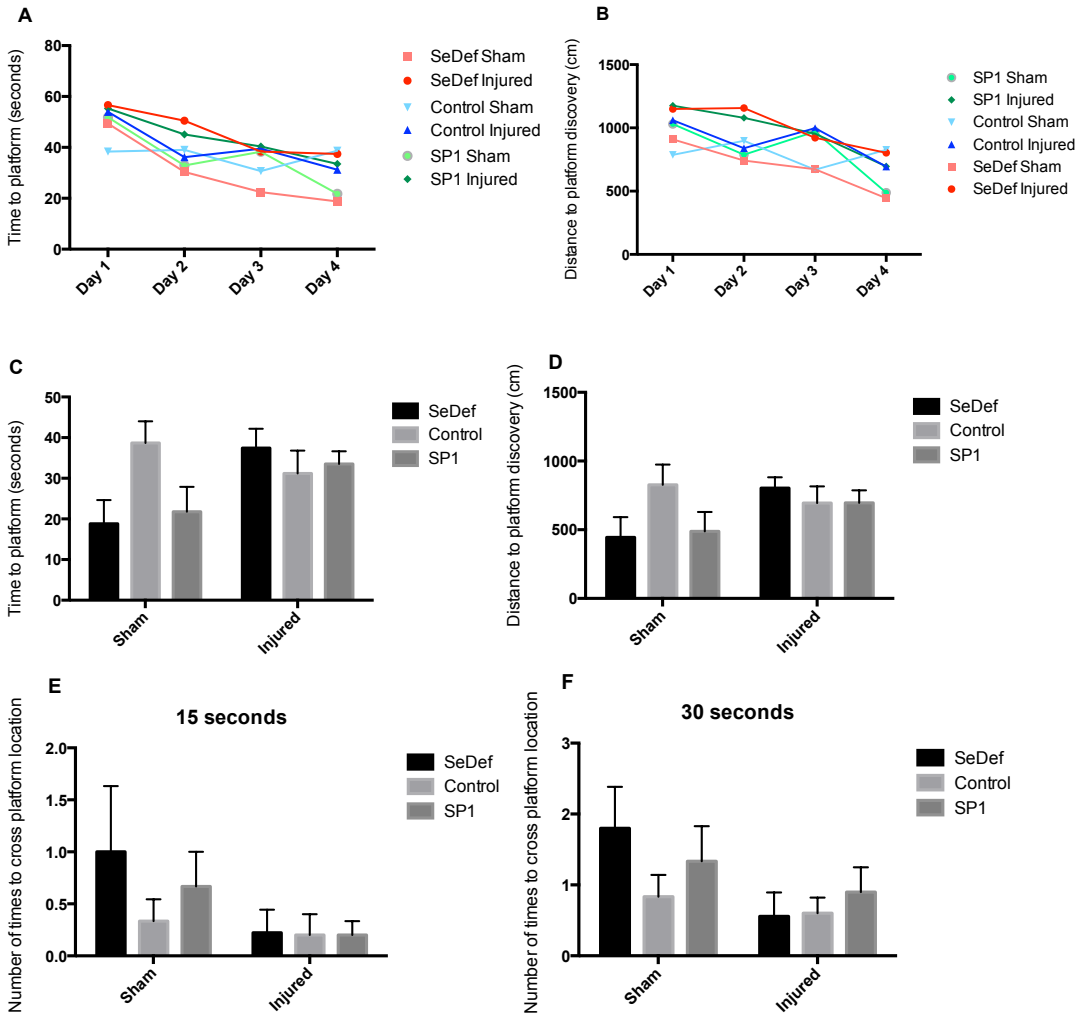


Figure 4.9: Selenium supplementation over 4 weeks does not improve spatial memory following injury. Overall performance on Morris Water Maze task is shown following TBI and 4 weeks of dietary supplementation (n=10 all injury groups, n=5 selenium deficient sham, n=6 control and SP1 shams). One animal in the selenium deficient sham group was euthanized due to complications from surgery. **A, B)** Sham animals showed an overall improvement in comparison to injured animals. The sham animals maintained on the control diet were an exception, however, and did not show improvements over the 4 days of training in the time or distance to platform discovery. **C, D)** On day 4, the time and distance to platform discovery showed a significant injury effect but no differences due to diet within the injured group. Control sham animals did significantly worse in locating the sunken platform on day 4. **E, F)** Number of times animals crossed the platform location within 15 and 30 seconds with probe testing showed an injury effect but no significant differences due to diet. Error bars represent SEM.

4.4 Discussion

Selenoproteins play a critical role in several different pathways essential for human health. Of the approximately 25 selenoproteins that have been currently identified, these proteins have roles in immune function[47], antioxidant systems[2], and selenium transport to tissues[21]. The CNS prioritizes storage of selenium in deficient conditions, underlining the importance of maintaining selenium levels in the brain and spinal cord[7]. These findings, along with many studies that have emphasized the role of selenium in neurological diseases such as AD, Parkinson's disease, and spinal cord injury, support our original hypothesis that enriching dietary selenium levels prior to injury will protect mitochondria and improve pathological outcomes following TBI, while a deficiency in dietary selenium would exacerbate observed injury effects.

Animals that were fed the selenium enriched diet (SP1) for a long supplementation period of 16 weeks showed modest improvements in both mitochondrial respiration and spatial memory deficits. The improvements in mitochondrial respiration, in particular, are suggestive of neuroprotection provided by enriched level of selenium in the diet. These effects were carried over and also seen in the spatial memory tasks at a longer time point after injury. The data indicate that long term dietary selenium enrichment may provide neuroprotection in neurodegenerative models.

To examine the length of time necessary to achieve these neuroprotective benefits, the time course studies examined selenium tissue storage over four different lengths of supplementation. Selenium levels within the CNS were increased as early as after 2 weeks of dietary supplementation. The 4-week time period was chosen for future

studies. This represents a supplementation period that will simplify future laboratory experiments and also shorten the necessary time frame for supplementation in human clinical application. Additionally, to further expand the scope examined in these studies, two other doses of selenium levels were added. The selenium deficient diet and 2ppm selenium diet (SP2), when added to the control and 1ppm selenium diet (SP1) provide data on the physiologic response from a variety of dietary doses of selenium.

Animals supplemented with the four different levels of dietary selenium for four weeks prior to receiving a CCI injury, unfortunately did not repeat the mitochondrial protective effect that was previously observed over the longer (16 week) supplementation time frame. While there is a clear trend for the exacerbation of the injury effect for animals deficient in selenium, the other groups tested did not show any improvements in mitochondrial respiration. These data suggest little neuroprotection occurs with short term selenium supplementation and no additional protective mechanisms are noted between supplemented animals and those with sufficient dietary selenium levels present.

Due to the critical nature of selenium in the formation of the selenoprotein, glutathione peroxidase, we hypothesized that dietary selenium enrichment would increase the activity of glutathione peroxidase, particularly following injury. Naïve animals on different levels of dietary selenium, including deficient conditions, did not show any changes in overall GPx activity. After injury, no changes in GPx activity

were observed in isolated mitochondrial preparation. While this preparation was a crude mitochondrial sample and may have contained a small amount of other cellular fractions, these samples may not have contained enough cytoplasmic GPx to properly ascertain how GPx activity was changed in these circumstances. A small cohort of animals showed that following TBI, GPx activity is increased in the ipsilateral cortex. GPx activity was the highest in animals receiving the SP1 diet, suggesting that this dose of dietary selenium improved the function of the antioxidant enzyme, GPx. However, due to the small size of this group of animals, further testing is required to make definitive conclusions about this data.

While tissue storage levels were increased significantly in the CNS, the lack of mitochondrial protection and behavioral outcomes following four weeks of supplementation suggests that this time frame may not be long enough to create the physiological changes necessary for neuroprotection. If selenium is modulating gene expression, down stream effects may take longer to occur, despite the increase in tissue selenium. It is also possible that the method used to measure selenium levels (LC-MS), while incredibly precise, may not be sufficient to determine physiological activity of selenium in these tissues. Further studies examining the exact speciation of selenoproteins in the supplemented central nervous system tissues may be needed to ascertain the exact mechanism through which selenized yeast is affecting mitochondrial respiration.

CHAPTER 5
Discussion and Concluding Remarks

Identification of improved treatments for CNS trauma continues to be a clinical issue of great significance. With approximately 270,000 people sustaining a spinal cord injury (SCI)[121] and 3.17 million with a traumatic brain injury (TBI)[66] each year in the United States, understanding more about the pathology of this disease is critical for millions of patients in this country and worldwide. Certain groups of people are at higher risk for neurological injuries. With a rise in TBIs among soldiers returning from recent conflicts in Iraq and Afghanistan and growing concern surrounding sports related concussions, pretreatment strategies in the form of a safe, dietary supplement, such as selenium, could be of particular interest for these groups.

The action of selenium in several antioxidant enzymes, as well as its established role in preventing neurodegeneration in other CNS disorders, led to the hypothesis that prophylactic dietary selenium treatment would attenuate molecular degeneration and provide overall neuroprotection in the event of an injury. The results from the studies described herein have only shown modest improvement in the different neurotrauma models investigated. Further studies are still warranted to ascertain whether selenium is a viable pretreatment for CNS trauma.

5.1 Pretreatment with Selenium in Spinal Cord Injury

Following 16 weeks of prophylactic treatment with selenized yeast and a moderate contusive SCI, selenized yeast did significantly improve the number of days to recovery of bladder function. However, treatment was unable to preserve locomotor

deficits or rescue the overall lesion volume normally observed after this injury model. Any neuroprotective effects that selenium may have imparted through gene expression changes or through alteration of selenoprotein status was not strong enough to prevent degradation of spinal locomotor associated neurons, but may have prevented cell death in those spinal cord cell bodies or supporting glia responsible for innervation of bladder function.

When expanded to consider the effect of selenium on transcriptional regulation, selenized yeast differentially altered gene expression patterns 24 hours after moderate contusive SCI. Analysis methods for dealing with the large quantity of data produced by microarray gene expression can vary greatly from study to study. The template analysis described here recapitulated similar gene pathways changed with injury that have been previously published supporting the validity of our analysis method. Expression patterns in which selenium supplementation increased gene expression as compared to control injured groups, included genes involved with mitochondrial respiration/function and protein turnover were significantly up regulated following injury. Additionally, expression patterns of genes that returned to sham levels with selenium supplementation included genes associated with transcriptional regulation, as well as cell cycle control. These two particular expression patterns support a potential neuroprotective effect of dietary selenium enrichment and agrees with current literature in which certain selenoproteins have recently been characterized as a redox regulator specific to mitochondria[37].

5.2 Selenium in Traumatic Brain Injury

The results from dietary enrichment with selenium prior to SCI were interesting and led to further investigation of other models of neurotrauma. In order to eliminate any potential confounding neuroprotective effects of estrogen, only male rats were contused using the controlled cortical impact (CCI) device[133]. Following 16 weeks of supplementation, selenium provided modest neuroprotection following TBI as measured by mitochondrial respiration and behavioral assessment. Selenium enrichment improved mitochondrial respiration 24 hours post-injury in testing ADP phosphorylation and during maximal electron transport (complex I and II driven) respiration, as well as improved spatial memory. Due to the extended supplementation period, we wanted to investigate the time course and dosage needed to see neuroprotective effects. Selenium levels in the CNS were increased with enrichment at 4 weeks following injury. Different dosages of selenium in the diets showed changes in selenium levels within the CNS relative to the dose present in the diet. Shortening the supplementation period to 4 weeks increased storage of selenium in the CNS, however, the modest neuroprotection seen after 16 weeks of supplementation did not recapitulate with the shorter time frame of supplementation. While the diet deficient in selenium appeared to be detrimental to mitochondrial respiration, the neuroprotective effect on injured mitochondria seen after 16 weeks with selenium enrichment was not evident after only 4 weeks of supplementation. When examining behavioral pathologies at a more chronic time point the deficient diet continued to exacerbate the injury effect, but selenium enrichment did not provide any improvements in spatial memory as compared to the control injured animals.

5.3 Future Directions

These studies indicate that while 16 weeks of selenium supplementation improves mitochondrial function and behavioral outcomes in TBI by providing modest improvements in SCI models, but 4 weeks may not be long enough to induce noticeable neuroprotective effects in animal models of neurotrauma. Transcriptional changes can occur quickly within different cellular systems, depending on the stimulus driving the change. Supplementation studies indicated increased tissue levels of selenium at early time points suggesting potential downstream effects of enriched selenium tissue levels may require longer periods of increased tissue levels. It is not surprising that SCI induces a vast response of expressional changes soon after injury. CNS trauma triggers a host of cellular responses, including influx of different inflammatory molecules. The presence of these different cell types is solitarily sufficient to create substantial changes in gene expression. Selenium, however, builds up in the tissues as short as 2 weeks after supplementation and may require an even longer to change enzyme levels or trigger downstream signaling cascades.

Selenium is typically stored in tissues in the form of selenomethionine and can be broken down into the intermediate compound, selenide, for selenoprotein synthesis. Uniquely beneficial selenoproteins may take longer to build up within tissues. Exact speciation of selenoproteins that are increased within the CNS following dietary enrichment may provide critical information in further elucidating this mechanism. Further testing is required to determine whether shorter time frames, such as 8 or 12 weeks of supplementation prior to injury, are sufficient to provide neuroprotective

benefits. Other models using various treatments for CNS disorders, such as anti-depressants, require long-term treatment before patients experience any noticeable changes in symptoms. Much like the proposed mechanism for these drugs, selenium could be inducing transcriptional changes, but the translational modifications and alterations in protein levels within the CNS may take longer to reach levels sufficient to confer neuroprotection.

An alternative hypothesis for the mechanism of action of selenium within the CNS is through modulation of gene expression pathways associated with DNA repair. One of the significantly upregulated pathways from our microarray analysis indicated that selenium enrichment increased expression of DNA repair pathways following injury. This data is interesting in light of other experiments that support the role of selenium in DNA oxidation. McKelvey and colleagues showed that organic selenium compounds (including selenized yeast as used for these studies) protected against DNA damage as well as altered expression patterns in DNA damage functional pathways[40]. Additionally, studies using AD mouse models also showed *in vvio* decreases in DNA and RNA oxidation in animals supplemented with increased levels of dietary selenium[34]. Barger and colleagues detected improvements in DNA damage gene pathways[39]. Specifically, selenized yeast reduced the expression of the DNA damage inducing gene, Gadd45b. The current study supports these previous findings and suggests that selenium may play a role in modulating transcriptional changes in gene involved with DNA repair following traumatic SCI. Up regulation of specific

genes within this pathway prior to injury could lead to an improved response in the secondary injury cascade that follows CNS trauma.

Long term dietary selenium enrichment provided modest neuroprotection following CNS injury, supporting our original hypothesis. However, a shorter supplementation time frame did not recapitulate these findings. While it appeared that the presence of selenium was beneficial for mitochondrial health in comparison to deficient conditions, there was no additional protective effect with increased levels of selenium. In shorter time frames, increased levels of selenium may not confer additional neuroprotection when compared to normal dietary selenium. Further investigation into treatment time frames for use in neurodegenerative disorders may still be warranted.

While the rationale for these studies was well supported by current literature, dietary selenium enrichment did not provide overwhelming support for the use of selenium as a prophylactic against CNS trauma. The acute nature of a CNS insult may overwhelm the potential protective effects gained from selenium supplementation. However, the modest neuroprotective role of selenium may be beneficial for chronic models of neurodegeneration. Alzheimer's disease, which is characterized by a slow onset, has shown that selenium provides protective effects in preclinical trials[34, 57]. Currently, a clinical trial is underway examining the potential neuroprotective role of selenium and vitamin E for Alzheimer's disease (clinicaltrials.gov: NCT00040378). The presence of critical nutrients, such as selenium, is known to be crucial for maintaining

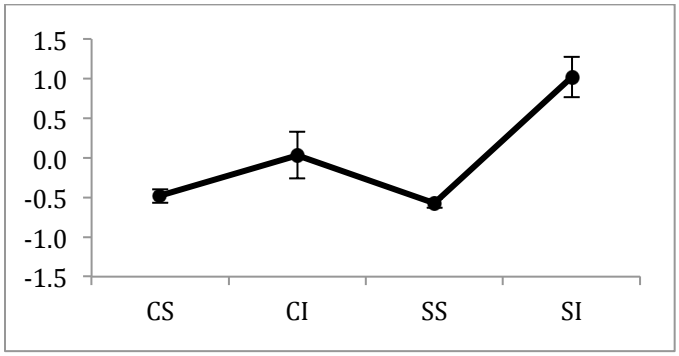
normal health and may alter the neurodegenerative pathologies that ultimately lead to disease.

APPENDICES

Appendix A: List of acronyms and abbreviations

3' UTR	3' untranslated region
AD	Alzheimer's Disease
ANOVA	analysis of variance
ApoE2	apolipoprotein E receptor 2
BBB	Basso, Beattie, Bresnahan locomotor score
CCI	controlled cortical impact injury
CNS	central nervous system
CVB3	coxsackie virus
ETC	electron transport chain
EtOH	ethanol
GFAP	glial fibrillary acidic protein
GPx	glutathione peroxidase
LC-MS	liquid chromatography mass spectrometry
LD_{50}	average lethal dose
MPTP	mitochondrial permeability transition pore
MWM	Morris Water Maze
NOAEL	No observed adverse effect level
OCR	oxygen consumption rate
PUFA	polyunsaturated fatty acids
RDA	Recommended Dietary Allowance
RfD	Reference dose
ROS	reactive oxygen species
SBP2	SECIS binding protein
SCI	spinal cord injury
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SeCys	selenocysteine (amino acid)
SeDef	selenium deficient
SelO	selenoprotein O
SelP	selenoprotein P
SelS	selenoprotein S
SEM	standard error of the mean
SeMet	selenomethionine
SP1	control diet + 1ppm selenized yeast
SP2	control diet + 2ppm selenized yeast
TBI	traumatic brain injury
$tRNA^{Met}$	transfer RNA for methionine
UL	tolerable upper intake level

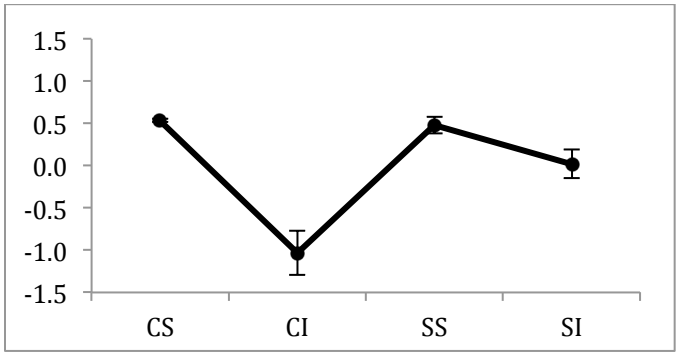
Appendix B: Genes increased in expression in injured animals on selenium enriched diets



Affymetrix probe set ID	Gene ID	Description
1390124_at	Fam98b	family with sequence similarity 98, member B
1379254_at	Tmem183a	transmembrane protein 183A
1373745_at	Gtf2h4	general transcription factor II H, polypeptide 4
1370225_at	Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4
1369327_at	Pdzd2	PDZ domain containing 2
1382751_at	Wfdc10	WAP four-disulfide core domain 10
1392719_at	Mthfs	5,10-methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)
1372942_at	Exosc5	exosome component 5
1392351_at	Fpgt	fucose-1-phosphate guanylyltransferase
1389604_at	Hspa12b	heat shock protein 12B
1376145_at	Eif2b5	eukaryotic translation initiation factor 2B, subunit 5 epsilon
1379015_at	Foxj2	forkhead box J2
1396674_at	Gpr44	G protein-coupled receptor 44
1399007_at	Cog2	component of oligomeric golgi complex 2
1395406_at	Sbno1	Strawberry notch homolog 1 (Drosophila)
1399108_at	Dis3l	DIS3 mitotic control homolog (S. cerevisiae)-like
1370087_at	Rab2a	RAB2A, member RAS oncogene family
1374715_at	Wdr70	WD repeat domain 70
1399092_at	LOC100361208	transmembrane protein 39b
1388506_at	Dsp	desmoplakin
1383729_at	LOC303566	E2F1-inducible gene
1392828_at	Med12	mediator complex subunit 12
1388156_at	Plcb3	phospholipase C, beta 3 (phosphatidylinositol-specific)
1373429_at	Atf6b	activating transcription factor 6 beta
1377532_at	Phf20	PHD finger protein 20
1377383_at	Efs	embryonal Fyn-associated substrate
1369969_at	Parp1	poly (ADP-ribose) polymerase 1
1382151_at	Trub1	TruB pseudouridine (psi) synthase homolog 1 (E. coli)
1369685_at	Twist2	twist homolog 2 (Drosophila)
1371015_at	Mx1	myxovirus (influenza virus) resistance 1
1381816_at	Rnls	renalase, FAD-dependent amine oxidase
1398943_at	Lage3	L antigen family, member 3
1368379_at	Scarb2	scavenger receptor class B, member 2
1372494_a_at	Hmg20b	high mobility group 20 B
1387605_at	Casp12	caspase 12
1382175_at	Wtap	Wilms tumor 1 associated protein
1373138_at	Nudt5	nudix (nucleoside diphosphate linked moiety X)-type motif 5
1368441_at	Msln	mesothelin
1378079_at	Golga3	golgi autoantigen, golgin subfamily a, 3
1398944_at	Acin1	apoptotic chromatin condensation inducer 1
1377801_at	Btbd12	BTB (POZ) domain containing 12
1370005_at	Cyb5b	cytochrome b5 type B (outer mitochondrial membrane)

1372152_at	Alg14	asparagine-linked glycosylation 14 homolog (S. cerevisiae)
1368355_at	Myo5b	myosin Vb
1389061_at	Nsun5	NOL1/NOP2/Sun domain family, member 5
1382083_at	Coch	coagulation factor C homolog, cochlin (Limulus polyphemus)
1393745_at	Orc3l	origin recognition complex, subunit 3-like (yeast)
1372305_at	Copz2	coatomer protein complex, subunit zeta 2
1387037_at	Cubn	cubilin (intrinsic factor-cobalamin receptor)
1393017_at	Rhpn1	rhophilin, Rho GTPase binding protein 1
1385189_at	Duoxa1	dual oxidase maturation factor 1
1374704_at	Kdelc2	KDEL (Lys-Asp-Glu-Leu) containing 2
1372431_at	Mrpl12	mitochondrial ribosomal protein L12
1395325_s_at	Mmgt1	membrane magnesium transporter 1
1390933_a_at	Rg9mtd3	RNA (guanine-9-) methyltransferase domain containing 3
1395881_at	Dap	Death-associated protein
1369714_at	Dnajc14	DnaJ (Hsp40) homolog, subfamily C, member 14
1371611_at	Ext2	exostoses (multiple) 2
1387531_at	Msra	methionine sulfoxide reductase A
1379949_at	Tfb1m	transcription factor B1, mitochondrial
1390105_at	B4galt2	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2
1371442_at	Hyou1	hypoxia up-regulated 1
1388141_at	Cetn3	centrin, EF-hand protein, 3 (CDC31 homolog, yeast)
1370651_a_at	Inppl1	inositol polyphosphate phosphatase-like 1
1375439_at	Wdr18	WD repeat domain 18
1392116_at	Sike	suppressor of IKK epsilon
1380360_at	Oma1	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)
1372323_at	Sardh	sarcosine dehydrogenase
1388959_at	Ttll12	Tubulin tyrosine ligase-like family, member 12
1390385_at	Glce	glucuronic acid epimerase
1383692_at	Prelid2	PRELI domain containing 2
1374555_at	Acbd6	acyl-Coenzyme A binding domain containing 6
1393317_at	Thumpd3	THUMP domain containing 3
1389089_at	Slc39a7	solute carrier family 39 (zinc transporter), member 7
1391768_at	Psmc4	proteasome (prosome, macropain) 26S subunit, ATPase, 4
1390141_at	Mthfd1l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
1370941_at	Pdgfra	platelet derived growth factor receptor, alpha polypeptide
1374517_at	Recql	RecQ protein-like (DNA helicase Q1-like)
1368341_at	Polb	polymerase (DNA directed), beta
1394403_at	Spata20	spermatogenesis associated 20
1375042_at	Alg8	asparagine-linked glycosylation 8, alpha-1,3-glucosyltransferase homolog
1376493_at	Commd7	COMM domain containing 7
1383391_a_at	C2	complement component 2
1378178_at	Smc6	structural maintenance of chromosomes 6
1374767_at	Npepo	aminopeptidase O
1392033_a_at	Pank2	pantothenate kinase 2 (Hallervorden-Spatz syndrome)
1376192_at	Nat9	N-acetyltransferase 9 (GCN5-related, putative)
1372320_at	Msl3	male-specific lethal 3 homolog (Drosophila)
1388913_at	Ppap2c	phosphatidic acid phosphatase type 2c
1372127_at	Ubap2	ubiquitin-associated protein 2
1377787_at	Rbm6	RNA binding motif protein 6
1370835_at	Tox4	TOX high mobility group box family member 4
1373239_at	Snx33	sorting nexin 33
1378150_at	LOC100363332	caspase recruitment domain family, member 11
1383107_at	Snrpd1	small nuclear ribonucleoprotein D1
1367859_at	Tgfb3	transforming growth factor, beta 3
1387233_at	Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7
1391455_at	Zc3h18	zinc finger CCCH-type containing 18
1386006_at	Larp7	La ribonucleoprotein domain family, member 7
1391004_at	Svil	Supervillin
1373000_at	Srpx2	sushi-repeat-containing protein, X-linked 2
1382396_at	Ubfd1	ubiquitin family domain containing 1
1378165_at	Twist1	twist homolog 1 (Drosophila)
1369412_a_at	Slc19a1	solute carrier family 19 (folate transporter), member 1
1370374_at	Steap3	STEAP family member 3
1367974_at	Anxa3	annexin A3
1368571_at	Clip2	CAP-GLY domain containing linker protein 2
1399114_at	Gtf2e2	general transcription factor IIE, polypeptide 2, beta

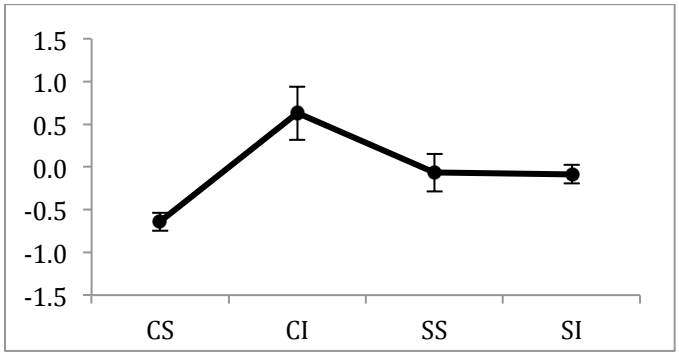
Appendix C: Genes down regulated in injury and restored to sham levels with selenium supplementation



Affymetrix Probeset ID	Gene	Description
1398660_at	Slc35f2	solute carrier family 35, member F2
1387209_at	Sec16b	SEC16 homolog B (S. cerevisiae)
1392162_at	Pspc1	Paraspeckle component 1
1370202_at	Pla2g16	phospholipase A2, group XVI
1387083_at	Ctf1	cardiotrophin 1
1382462_at	L2hgdh	L-2-hydroxyglutarate dehydrogenase
1369508_at	Kcnj15	potassium inwardly-rectifying channel, subfamily J, member 15
1371590_s_at	Ubl5	ubiquitin-like 5
1374604_at	Mfsd9	major facilitator superfamily domain containing 9
1374328_at	C2cd3	C2 calcium-dependent domain containing 3
1368069_at	Sharpin	SHANK-associated RH domain interactor
1390926_at	Zswim3	zinc finger, SWIM-type containing 3
1387105_at	Zfp422	zinc finger protein 422
1386346_at	Tmem19	transmembrane protein 19
1385296_at	Trmt12	tRNA methyltransferase 12 homolog (S. cerevisiae)
1380118_at	Fdxacb1	ferredoxin-fold anticodon binding domain containing 1
1389452_at	Zdhhc8	zinc finger, DHHC-type containing 8
1397284_at	Zfp444	zinc finger protein 444
1374115_at	Mzf1	myeloid zinc finger 1
1385899_at	Trim16	tripartite motif-containing 16
1378092_at	Mdp1	magnesium-dependent phosphatase 1
1373308_at	Sgsm3	small G protein signaling modulator 3
1395820_at	Rnf150	Ring finger protein 150
1384324_at	Rbm4	RNA binding motif protein 4
1369233_at	Kcnk10	potassium channel, subfamily K, member 10
1387137_at	Comp	cartilage oligomeric matrix protein
1375666_at	Dmtf1	cyclin D binding myb-like transcription factor 1
1393632_at	C1qtnf7	C1q and tumor necrosis factor related protein 7
1387232_at	Bmp4	bone morphogenetic protein 4
1389433_at	Mkks	McKusick-Kaufman syndrome
1394842_at	Tmem19	transmembrane protein 19
1384217_at	Zhx2	Zinc fingers and homeoboxes 2
1388660_at	Mcts1	malignant T cell amplified sequence 1
1376088_at	Slc25a19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19
1374309_at	Radil	Ras association and DIL domains
1388760_at	Slc35b4	solute carrier family 35, member B4

1367797_at	Men1	multiple endocrine neoplasia 1
1368159_at	Abcb6	ATP-binding cassette, sub-family B (MDR/TAP), member 6
1376106_at	Tmem178	transmembrane protein 178
1367953_at	Tyro3	TYRO3 protein tyrosine kinase
1371645_at	Sdf2	stromal cell derived factor 2
1374658_at	Iqsec2	IQ motif and Sec7 domain 2
1383668_at	Mmp15	matrix metallopeptidase 15
1373020_at	Magmas	mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction
1369113_at	Grem1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)
1396022_at	Zfp322a	Zinc finger protein 322a
1372105_at	Efha1	EF-hand domain family, member A1
1390098_at	N6amt2	N-6 adenine-specific DNA methyltransferase 2 (putative)
1367550_a_at	Tm2d1	TM2 domain containing 1
1368891_at	Gnpat	glyceronephosphate O-acyltransferase
1372819_at	Cog4	component of oligomeric golgi complex 4
1371548_at	Mrps25	mitochondrial ribosomal protein S25
1372927_at	Mrpl50	mitochondrial ribosomal protein L50
1374020_at	Lrrc38	leucine rich repeat containing 38
1370540_at	Nr1d2	nuclear receptor subfamily 1, group D, member 2
1374188_at	Sec62	SEC62 homolog (S. cerevisiae)
1392189_at	Rfx4	Regulatory factor X, 4 (influences HLA class II expression)
1371586_at	Mrpl48	mitochondrial ribosomal protein L48
1367815_at	Slc5a6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6
1373952_at	Prkag2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit
1389792_at	Solh	small optic lobes homolog (Drosophila)
1385810_at	Lrtomt	leucine rich transmembrane and 0-methyltransferase domain containing
1397541_at	Twsg1	twisted gastrulation homolog 1 (Drosophila)
1379484_at	Aven	apoptosis, caspase activation inhibitor
1389662_at	Wnk4	WNK lysine deficient protein kinase 4
1399125_at	Inpp1	inositol polyphosphate-1-phosphatase
1376909_at	Rasl10a	RAS-like, family 10, member A
1383049_at	Klhl8	kelch-like 8 (Drosophila)
1377961_at	Zfhx3	zinc finger homeobox 3
1389703_at	Zzef1	zinc finger, ZZ-type with EF hand domain 1
1389044_at	Gbf1	golgi-specific brefeldin A resistant guanine nucleotide exchange factor 1
1378158_at	Brms1	breast cancer metastasis-suppressor 1
1371979_at	Srebf2	sterol regulatory element binding transcription factor 2
1385473_x_at	Tomm40b	translocase of outer mitochondrial membrane 40 homolog B (yeast)
1380641_at	Pcdhb6	protocadherin beta 6
1381783_at	Akap3	A kinase (PRKA) anchor protein 3
1391775_at	Zpbp2	zona pellucida binding protein 2

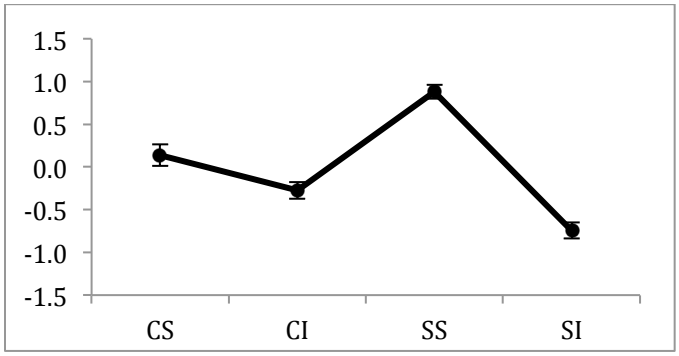
Appendix D: Genes increased with injury and restored to sham levels with selenium supplementation



Affymetrix Probeset ID	Gene	Description
1394731_at	Csnk1g3	casein kinase 1, gamma 3
1368866_at	Eif2c2	eukaryotic translation initiation factor 2C, 2
1382523_at	Dpm1	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
1384957_at	Atf6	activating transcription factor 6
1393822_at	Tmcc3	transmembrane and coiled-coil domain family 3
1370485_a_at	Bcl2l1	Bcl2-like 1
1393738_s_at	Mfhas1	malignant fibrous histiocytoma amplified sequence 1
1398648_at	Mfhas1	malignant fibrous histiocytoma amplified sequence 1
1397302_at	Zmynd11	zinc finger, MYND domain containing 11
1382103_at	Pgm3	phosphoglucomutase 3
1379409_at	Atxn7	Ataxin 7
1375121_at	Smad6	SMAD family member 6
1395261_at	Snrnp70	small nuclear ribonucleoprotein 70 (U1)
1368961_at	Mmp23	matrix metallopeptidase 23
1375085_at	Slc25a35	solute carrier family 25, member 35
1396078_at	Krit1	KRIT1, ankyrin repeat containing
1384348_at	Tcf23	transcription factor 23
1369347_s_at	Prom2	prominin 2
1391303_at	Purb	purine rich element binding protein B
1392542_at	Cdc42se2	CDC42 small effector 2
1389364_at	Ndfip2	Nedd4 family interacting protein 2
1387484_at	Tgfbr3	transforming growth factor, beta receptor III
1377919_at	Arhgap10	Rho GTPase activating protein 10
1395441_at	Pcbp4	poly(rC) binding protein 4
1391787_at	Traf3ip3	TRAF3 interacting protein 3

1395015_at	F8	coagulation factor VIII, procoagulant component
1374021_at	Wipi1	WD repeat domain, phosphoinositide interacting 1
1384050_at	Bub3	budding uninhibited by benzimidazoles 3 homolog (S. cerevisiae)
1371103_at	Rab6a	RAB6A, member RAS oncogene family
1387638_a_at	Ctla4	cytotoxic T-lymphocyte-associated protein 4
1387410_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2
1387533_at	Pspn	persephin
1394518_at	Adamts9	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9
1370974_at	Vps54	vacuolar protein sorting 54 homolog (S. cerevisiae)
1389088_at	Adnp	activity-dependent neuroprotector homeobox
1393593_at	Mar6	membrane-associated ring finger (C3HC4) 6
1381804_at	Bcl6b	B-cell CLL/lymphoma 6, member B (zinc finger protein)
1369912_at	Crk	v-crk sarcoma virus CT10 oncogene homolog (avian)
1386721_at	Znf503	zinc finger protein 503
1385502_at	Trim21	Tripartite motif-containing 21
1391128_at	Bcl9l	B-cell CLL/lymphoma 9-like
1375228_at	Brd2	bromodomain containing 2
1397473_at	Dtnbp1	distrobrevin binding protein 1
1393843_at	Fem1b	fem-1 homolog b (C. elegans)
1382203_at	Gdf1	growth differentiation factor 1
1390531_at	Pric285	peroxisomal proliferator-activated receptor A interacting complex 285
1384773_at	Ubtd2	ubiquitin domain containing 2
1367770_at	Degs1	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)
1389184_at	Rpp30	ribonuclease P/MRP 30 subunit (human)
1376294_at	Smcr7	Smith-Magenis syndrome chromosome region, candidate 7 homolog (human)
1379668_at	Alg11	asparagine-linked glycosylation 11, alpha-1,2-mannosyltransferase homolog (yeast)
1369571_at	Golph3	golgi phosphoprotein 3 (coat-protein)
1369862_at	Pim1	pim-1 oncogene
1394749_at	Atg7	ATG7 autophagy related 7 homolog (S. cerevisiae)

Appendix E: Genes increased in sham animals on selenium enriched diets

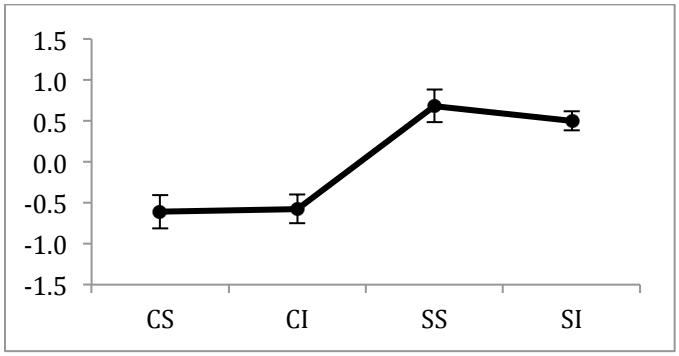


Affymetrix Probeset ID	Gene	Description
1372243_at	Cab39	calcium binding protein 39
1369179_a_at	Pparg	peroxisome proliferator-activated receptor gamma
1388058_at	Taf6	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor
1394838_at	Aoc3	amine oxidase, copper containing 3 (vascular adhesion protein 1)
1389037_at	Rit1	Ras-like without CAAX 1
1369634_at	Slc4a1	solute carrier family 4 (anion exchanger), member 1
1387043_at	Lypd3	Ly6/Plaur domain containing 3
1378435_at	Art4	ADP-ribosyltransferase 4
1368755_at	Clec4f	C-type lectin domain family 4, member f
1368102_at	Hsd11b2	hydroxysteroid 11-beta dehydrogenase 2
1371066_at	Snrk	SNF related kinase
1384890_at	Ezh1	enhancer of zeste homolog 1 (Drosophila)
1369192_at	Cdkn1b	cyclin-dependent kinase inhibitor 1B
1368879_a_at	Gnao1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O
1368144_at	Rgs2	regulator of G-protein signaling 2
1396749_at	Cabp1	calcium binding protein 1
1369232_at	Kcnk10	potassium channel, subfamily K, member 10
1398948_at	Tax1bp1	Tax1 (human T-cell leukemia virus type I) binding protein 1
1369510_at	Gapdhs	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
1376953_at	Unc5cl	unc-5 homolog C (C. elegans)-like
1372036_at	Cd2bp2	Cd2 (cytoplasmic tail) binding protein 2
1386883_at	Gsk3a	glycogen synthase kinase 3 alpha
1370983_at	Pou6f1	POU class 6 homeobox 1
1396711_at	Obsl1	Obscurin-like 1

1368859_at	Ppm1a	protein phosphatase 1A, magnesium dependent, alpha isoform
1397866_at	Serpinb6b	serine (or cysteine) peptidase inhibitor, clade B, member 6b
1369809_at	Htr1a	5-hydroxytryptamine (serotonin) receptor 1A
1394764_at	Lmtk2	lemur tyrosine kinase 2
1391075_at	Rgs17	regulator of G-protein signaling 17
1395232_at	Dcaf6	DDB1 and CUL4 associated factor 6
1384645_at	Toag1	tolerance-associated gene 1
1398614_at	Ptch1	patched homolog 1 (Drosophila)
1376992_a_at	Ccdc84	coiled-coil domain containing 84
1369660_at	Defb1	defensin beta 1
1370268_at	Kcna5	potassium voltage-gated channel, shaker-related subfamily, member 5
1398289_a_at	Crhr1	corticotropin releasing hormone receptor 1
1369016_at	Cdon	Cdon homolog (mouse)
1393337_at	Tcfcp2l1	transcription factor CP2-like 1
1395990_at	Srcap	Snf2-related CREBBP activator protein
1370753_at	Olr1078	olfactory receptor 1078
1386280_at	Mettl7b	methyltransferase like 7B
1379550_a_at	Gtf2ird1	GTF2I repeat domain containing 1
1384975_at	Ermp1	endoplasmic reticulum metallopeptidase 1
1384933_at	Slc18a2	Solute carrier family 18 (vesicular monoamine), member 2
1393436_at	Scgb1c1	secretoglobin, family 1C, member 1
1375494_a_at	Nlgn3	neuroligin 3
1392340_at	Klhl3	kelch-like 3 (Drosophila)
1397211_at	Grb10	growth factor receptor bound protein 10
1387828_at	Agap2	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2
1382024_at	Dnajb6	DnaJ (Hsp40) homolog, subfamily B, member 6
1397716_at	Klhdc2	Kelch domain containing 2
1369359_at	Il9r	interleukin 9 receptor
1369188_at	Fbxo32	F-box protein 32
1369864_a_at	Sds	serine dehydratase
1389834_at	Nudt8	Nudix (nucleoside diphosphate linked moiety X)-type motif 8
1398267_at	Slc22a7	solute carrier family 22 (organic anion transporter), member 7
1388613_at	Isca1	iron-sulfur cluster assembly 1 homolog (S. cerevisiae)
1369287_at	Syt9	synaptotagmin IX
1396158_at	Rem1	RAS (RAD and GEM)-like GTP-binding 1
1398314_at	Hoxd3	homeo box D3
1387672_at	Gnmt	glycine N-methyltransferase
1379456_at	Mcart1	mitochondrial carrier triple repeat 1
1379203_at	Tfdp2	transcription factor Dp-2 (E2F dimerization partner 2)
1379264_at	Znrf2	zinc and ring finger 2
1392079_at	Akap7	A kinase (PRKA) anchor protein 7

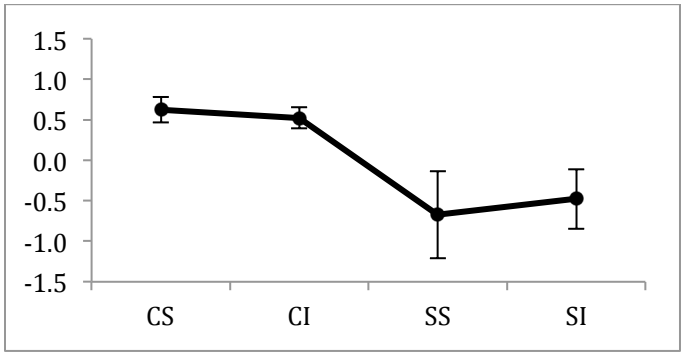
1392976_at	Tpm2	tropomyosin 2, beta
1368077_at	Fbp1	fructose-1,6-bisphosphatase 1
1387322_at	Sema6b	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6B
1368788_at	Chad	chondroadherin
1368334_at	Grb7	growth factor receptor bound protein 7
1369017_at	Kcnh6	potassium voltage-gated channel, subfamily H (eag-related), member 6
1384978_at	Klk1c10	T-kininogenase
1388238_at	Defa24	defensin, alpha, 24
1393474_at	Sult2b1	sulfotransferase family, cytosolic, 2B, member 1
1376274_at	Btbd10	BTB (POZ) domain containing 10
1387267_at	Ntf3	neurotrophin 3
1393390_at	Shd	Src homology 2 domain-containing transforming protein D
1389496_at	Akap7	A kinase (PRKA) anchor protein 7
1387999_at	Slc18a1	solute carrier family 18 (vesicular monoamine), member 1
1372280_at	Asb2	ankyrin repeat and SOCS box-containing 2
1392474_at	Ulk2	Unc-51 like kinase 2 (C. elegans)
1380453_at	Zswim5	zinc finger, SWIM domain containing 5
1371165_a_at	Atp2a3	ATPase, Ca++ transporting, ubiquitous
1368837_at	Arid4b	AT rich interactive domain 4B (Rbp1 like)
1385130_at	Fezf2	Fez family zinc finger 2
1387215_at	Agxt	alanine-glyoxylate aminotransferase
1370129_at	Mgea5	meningioma expressed antigen 5 (hyaluronidase)
1387512_at	Zfp238	zinc finger protein 238
1387388_at	Chp	calcium binding protein p22
1385740_at	Lrrc27	leucine rich repeat containing 27
1384397_x_at	Crb3	crumbs homolog 3 (Drosophila)
1378278_at	Pou2f2	POU class 2 homeobox 2
1369446_at	Cry2	cryptochrome 2 (photolyase-like)

Appendix F: Genes increased with selenium supplementation, regardless of injury



Affymetrix Probeset ID	Gene	Description
1379197_at	Cacna1h	Calcium channel, voltage-dependent, T type, alpha 1H subunit
1369011_at	Apoa5	apolipoprotein A-V
1395026_at	Fmo4	flavin containing monooxygenase 4
1389884_at	Ccdc88b	coiled-coil domain containing 88B
1393689_at	Ndufaf1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1
1395579_at	Dhx32	DEAH (Asp-Glu-Ala-His) box polypeptide 32
1373313_at	Sel1l	Sel-1 suppressor of lin-12-like (C. elegans)
1368205_at	Cfi	complement factor I
1368317_at	Aqp7	aquaporin 7
1387250_at	Pla2g10	phospholipase A2, group X
1388478_at	Tbl1x	transducin (beta)-like 1 X-linked
1389115_at	Evpl	envoplakin
1369385_at	Afap1	actin filament associated protein 1
1374119_at	Elf3	E74-like factor 3
1393651_at	Galnt10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)

Appendix G: Genes decreased with selenium supplementation, regardless of injury



Affymetrix Probeset ID	Gene	Description
1384133_at	Kif1a	kinesin family member 1A
1391625_at	Wasl	Wiskott-Aldrich syndrome-like
1399132_at	Cul3	cullin 3
1387117_at	Zranb2	zinc finger, RAN-binding domain containing 2
1388064_a_at	Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3
1375641_at	Arpc5l	actin related protein 2/3 complex, subunit 5-like
1369095_at	Ppp1r9a	protein phosphatase 1, regulatory (inhibitor) subunit 9A
1389912_at	Ensa	endosulfine alpha
1377798_at	Tchp	trichoplein, keratin filament binding
1380121_at	Nek7	NIMA (never in mitosis gene a)-related kinase 7
1369046_at	Syt6	synaptotagmin VI
1391820_at	Tanc2	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2
1369177_at	Pi4k2a	phosphatidylinositol 4-kinase type 2 alpha
1368450_at	Myo5a	myosin VA
1386547_at	Tanc2	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2
1393324_at	Jam2	junctional adhesion molecule 2
1398533_at	Cyfip2	cytoplasmic FMR1 interacting protein 2
1382981_at	Ahi1	Abelson helper integration site 1
1389874_at	Zrsr1	zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1
1391285_at	Zfyve9	zinc finger, FYVE domain containing 9
1368750_a_at	Pde4d	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)
1376347_at	Kdm5b	lysine (K)-specific demethylase 5B
1394626_at	Hmbox1	homeobox containing 1
1376843_at	Bmpr2	bone morphogenetic protein receptor, type II (serine/threonine kinase)

1384376_at	Dnajb14	DnaJ (Hsp40) homolog, subfamily B, member 14
1385970_at	Ctr9	Ctr9, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)
1376739_at	Ddx24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24
1369310_at	Basp1	brain abundant, membrane attached signal protein 1
1369008_a_at	Olfm1	olfactomedin 1
1395399_at	Zcchc17	zinc finger, CCHC domain containing 17

Appendix H: Dietary nutrient information

	Diet			
Nutrient	**Selenium Deficient**	**Control**	**SP1**	**SP2**
Kcal/g	4.3	3.1	3.1	3.1
Protein (% by weight)	15.3	16.2	16.2	16.2
CHO (% by weight)	57.9	52.8	52.8	52.8
Fat (% by weight)	14.9	3.6	3.6	3.6
Selenium (ppm)	0	0.3	1.3	2.3
Micronutrients (g/kg)				
Choline	2.8	1.03	1.03	1.03
Niacin	0.03	0.075	0.075	0.075
Pantothenic Acid	0.016	33	33	33
Vitamin B6	0.007	0.018	0.018	0.018
Vitamin B1	0.006	0.017	0.017	0.017
Vitamin B2	0.006	0.015	0.015	0.015
Folate	0.002	0.004	0.004	0.004
Biotin	0.0002	0.0004	0.0004	0.0004
Vitamin B12	0.025	0.00008	0.00008	0.00008
Vitamin E	0.1	0.11	0.11	0.11
Vitamin K	0.003	0.05	0.05	0.05

Additional additives	**Selenium Deficient**
Sucrose (g/kg)	537
Corn oil (g/kg)	140
Torula yeast (g/kg)	300

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133. Lighthall JW. Controlled cortical impact: a new experimental brain injury model. Journal of neurotrauma 1988;5:1-15.
134. Patel SP, Sullivan PG, Pandya JD, et al. N-acetylcysteine amide preserves mitochondrial bioenergetics and improves functional recovery following spinal trauma. Experimental neurology 2014;257:95-105.

VITA

Carolyn Anne (Crowdus) Meyer

CITIZENSHIP

United States of America

EDUCATION

University of Kentucky College of Medicine, Lexington, KY
- Doctor of Philosophy, Anatomy and Neurobiology, August 2015.
- Graduate Certificate in Anatomical Sciences Instruction, August 2015.

University of Kentucky College of Agriculture, Lexington, KY
- Bachelor of Science, Agricultural Biotechnology, May 2008

RESEARCH EXPERIENCE

Research Assistant/Graduate Student
University of Kentucky College of Medicine, Department of Anatomy and Neurobiology and Spinal Cord and Brain Injury Research Center (SCoBIRC)
Mentor: James W. Geddes, PhD
Dates: August 2010 – August 2015

Research Laboratory Technician
Center for Animal Nutrigenomics and Applied Animal Nutrition, Alltech, Nicholasville, KY
Supervisor: Ronan F. Power, Ph.D.
Dates: July 2008 – July 2010

Research Intern
Center for Animal Nutrigenomics and Applied Animal Nutrition, Alltech, Nicholasville, KY
Supervisor: Ronan F. Power, Ph.D.
Dates: May 2008 – July 2008

Undergraduate Research Assistant
Gluck Equine Research Center, University of Kentucky
Mentor: Daniel K. Howe, Ph.D.
Dates: August 2004 – May 2008

TEACHING EXPERIENCE

Teaching Assistant
University of Kentucky, College of Medicine, Department of Anatomy and Neurobiology
Supervisor: April Hatcher, Ph.D.
Dates: January 2015 to May 2015
Course: Physician Assistant (ANA611) and Physical Therapy (ANA811) Regional Human Gross Anatomy

Instructor
Area Health Education Center's (AHEC) Summer Enrichment Program, University of Kentucky Outreach Program
Supervisor: Carlos Marin
Dates: June – July (2012, 2013, 2014, 2015)
Course: Anatomy and Physiology Instructor

Teaching Assistant
University of Kentucky, College of Medicine, Department of Anatomy and Neurobiology
Supervisor: Magdalena Muchilinski, Ph.D.
Dates: 2011, 2012, 2013, 2014
Course: Anatomy and Physiology (ANA 109/110)

Private tutor
Anatomy and Physiology (ANA 109/110) – 2014, 2015

AWARDS AND HONORS

BGSFN Poster Presentation Award – 2015
University of Kentucky 3 Minute Thesis® Competition, Second place – 2014
Seahorse Bioscience Travel Award – 2013
University of Kentucky Graduate School Travel Award – 2013
BGSFN Chapter Travel Award for Society for Neuroscience meeting – 2013
BGSFN Poster Presentation Award – April 2013
Neurobiology of CNS Injury and Repair Training Grant (T32 NS077889) – April 2013 – April 2015
Alltech Graduate Student Fellowship – August 2010 – April 2013

ACTIVITIES

American Heart Association Heart Walk Volunteer Coordinator – 2013, 2014, 2015
Women in Neurotrauma Research (WiNTR) Newsletter Editor – 2013, 2014, 2015
Committee for Center-Wide Initiatives, SCoBIRC – Member – 2011, 2012
Integrated Biomedical Sciences Student Advisory Committee – 2011, 2012

BGSFN Graduate Student Representative – fall 2013 – spring 2014

OUTREACH
Fayette County Public Schools Science Fair Judge – 2015
Wheelchair for a Day Fundraiser, organizer and participant – 2013
STEM Camp Neuroscience Instructor, University of Kentucky – 2013
Fayette County Public Schools Science Fair, BGSFN representative – 2013
Science Explorers with the Living Arts and Sciences Center – 2011, 2012
Athens-Chilesburg Elementary Arts and Sciences Day – 2012

PROFESSIONAL MEMBERSHIPS
Society for Neuroscience – 2013
National Neurotrauma Society – 2012, 2014
Bluegrass Society for Neuroscience (BGSFN) – 2011, 2012, 2013, 2014, 2015

ORAL PRESENTATIONS
Kentucky Spine and Head Injury Research Trust (KSHIRT) Symposium. May 2015.
Selenium Deficiency is Detrimental Following Traumatic Brain Injury.

PEER-REVIEWED PUBLICATIONS

1. Reneer DV, **Crowdus CA**, Ghoshal S, Corkins J, Hisel RD, Lusk BT, Geddes JW. 2014. Extent of cerebrovascular disruption following blast exposure is influenced by the duration of the positive phase in addition to peak overpressure. J Neurol Neurophysiol. 5:188.
2. Yu CG, Singh R, **Crowdus C**, Raza K, Kincer J, Geddes JW. 2014. Fenbendazole improves pathological and functional recovery following traumatic spinal cord injury. Neuroscience. Jan 3;256:163-9.
3. Brennan KM, **Crowdus CA,** Cantor AH, Pescatore AJ, Barger JL, Horgan K, Xiao R, Power RF, Dawson KA. 2011. Effects of organic and inorganic dietary selenium supplementation on gene expression profiles in oviduct tissue from broiler-breeder hens. Anim Reprod Sci. 125(1-4): 180-8.
4. Xiao R, Power RF, Mallonee D, **Crowdus C**, Brennan KM, Ao T, Pierce JL, Dawson KA. 2011. A comparative transcriptomic study of vitamin E and an algae-based antioxidant as antioxidative agents: investigation of replacing vitamin E with the algae-based antioxidant in broiler diets. Poult Sci. 90(1):136-46.
5. Mallonee DH, **Crowdus CA**, Barger JL, Dawson KA, Power RF. 2010. Use of stringent selection parameters for the identification of possible selenium-responsive marker genes in mouse liver and gastrocnemius. Biol Trace Elem Res. 2010 Nov 16.
6. **Crowdus CA,** Marsh AE, Saville WJ, Lindsay DS, Dubey JP, Granstrom DE, Howe DK. 2008. SnSAG5 is an alternative surface antigen of *Sarcocystis neurona* strains that is mutually exclusive to SnSAG1. Vet Parasitol. 158(1-2): 36-43.

ABSTRACTS – POSTER PRESENTATIONS

1. **Meyer CA,** Power RF, Geddes JW. 2015. Selenium deficiency is detrimental following traumatic brain injury. Bluegrass Society for Neuroscience, Spring Neuroscience Day.
2. **Crowdus CA**, Blalock EM, Yu CG, Power RF, Geddes JW. 2014. Gene expression changes in response to selenium diet in spinal cord injured rats. National Neurotrauma Symposium.
3. **Crowdus CA**, Pandya JD, Power LM, Sebastian AH, Yonutas HM, Hopkins DM, Jones J, Pauly JR, Sullivan PG, Power RF, Geddes JW. 2013. Dietary selenium supplementation improves behavioral and mitochondrial function following traumatic brain injury. Society for Neuroscience Annual Meeting.
4. **Crowdus CA,** Pandya JD, Power LM, Sebastian AH, Yonutas HM, Hopkins DM, Jones J, Pauly JR, Sullivan PG, Power RF, Geddes JW. 2013. Selenium for the treatment of traumatic brain injury. Kentucky Spine and Head Injury Research Trust (KSHIRT) Symposium.
5. **Crowdus CA,** Pandya JD, Power LM, Sebastian AH, Yonutas HM, Hopkins DM, Jones J, Pauly JR, Sullivan PG, Power RF, Geddes JW. 2013. Selenium for the treatment of traumatic brain injury. Bluegrass Society for Neuroscience, Spring Neuroscience Day.
6. **Crowdus CA,** Yu CG, Singh R, Pandya JD, Patel SP, Sullivan PG, Rabchevsky AG, Power RF, Geddes JW. 2012. Enhancing endogenous protective mechanisms following spinal cord injury. National Neurotrauma Symposium.
7. Yu CG, **Crowdus CA,** Raza K, Geddes JW. 2012. Fenbendazole improves locomotor recovery after spinal cord injury in mice. National Neurotrauma Symposium.
8. **Crowdus CA,** Yu CG, Singh R, Power RF, Geddes JW. 2012. Enhancing endogenous protective mechanisms following spinal cord injury. University of Kentucky Department of Physical Medicine and Rehabilitation Annual Resident Research Day, Cardinal Hill Rehabilitation Hospital.
9. **Crowdus CA,** Power L, Chishti A, Geddes JW, Yu CG. 2011. Calpain 1 knockout is not protective for spinal cord injury. Bluegrass Neuroscience Day, University of Kentucky.
10. Xiao, R., Power RF, Mallonee D, **Crowdus CA,** Ao T, Pierce JL, Dawson KA. 2009. Gene expression profiles provide evidence of reduced vitamin E needed by feeding EconomasE™ in broiler diets. International Poultry Scientific Forum.
11. Xiao, R., Power RF, Mallonee D, **Crowdus CA,** Ao T, Pierce JL, and Dawson KA. 2009. Transcriptional signatures associated with biological functions of Bio-Mos® and Actigen™ in broilers. International Poultry Scientific Forum.
12. **Crowdus CA** and Mallonee D. 2009. Nutrigenomics Methods: Using DNA Microarrays. Alltech 25th International Symposium. Lexington, KY.

13. **Crowdus CA,** Marsh AE, and Howe DK. 2008. Characterization of the *Sarcocystis neurona* merozoite surface antigen SnSAG5. Annual Midwestern Conference of Parasitologists.
14. **Crowdus CA** and Howe DK. 2008. Characterization of a *Sarcocystis neurona* Surface Antigen (SnSAG5). University of Kentucky Showcase of Undergraduate Scholars.
15. **Crowdus CA** and Howe DK. 2008. Characterization of a *Sarcocystis neurona* Surface Antigen (SnSAG5). National Conference for Undergraduate Research.
