Dietary and Intraperitoneal Administration of Selenium Provide Comparable Protection in the 6-Hydroxydopamine Lesion Rat Model of Parkinson's Disease

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Significant research implicates the involvement of free radicals in the manifestation of Parkinson's disease. The antioxidant, selenium is a vital dietary component for mammals. It is present in the active center of glutathione peroxidase, an antioxidant enzyme that scavenges peroxides and protects membrane lipids and macromolecules from oxidative insult. The purpose of this research was to determine an effective means of delivering selenium as well as an appropriate time frame for antioxidant administration that would elicit a protective response in rats challenged with an intranigral 6-hydroxydopamine (6-OHDA) lesion. In the first part of this study, Fischer 344 rats were placed into one of four groups: selenium enhanced diet, control diet, intraperitoneal injection of selenium as Na_2SeO_3 or intraperitoneal injection of distilled water. All treatments were delivered prior to an intranigral 6-OHDA lesion. Animals were euthanized two weeks post lesion and their brains processed for tyrosine hydroxylase (TH) immunocytochemistry. Average dopamine neuron survival in the substantia nigra of control animals was less than 22%; whereas nigral dopamine neuron survival in the selenium fed group was 49.7% and 56.0% in the selenium injected group. Based on these results, a subsequent study was designed utilizing the selenium enhanced diet method of antioxidant administration. To examine the neuroprotective effect of long-term selenium treatment, pregnant Fischer 344 rats were exposed to either selenium enhanced or control rat chow. Their pups were treated with the same diet as their mothers and lesioned with 6-OHDA at two months of age. Animals were euthanized and their brains were processed for TH immunocytochemistry. Nigral dopamine neuron survival for the selenium treated animals was significantly protective (59%) when compared to the control chow fed animals (29.6%). However, when compared to the short-term exposure of selenium rat chow in the previous study, there was no significant increase in neuroprotection.

Key Words: Antioxidant; Free Radical; Neuroprotection; Dopamine; Substantia Nigra

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Introduction

Several neuroprotective strategies in animal models of Parkinson's disease are based on the hypothesis that free radicals derived from oxidation reactions contribute to the pathogenesis of this disease (Olanow, 1992, Jenner et al., 1992, Lev et al., 2003, Ebadi et al., 1996). These free radicals are highly reactive oxygen species capable of damaging a variety of essential biological molecules such as nucleic acids, membrane lipids and proteins. The substantia nigra of the midbrain is known to be particularly susceptible to free radical damage due to its high levels of iron and dopamine metabolism with the subsequent production of hydrogen peroxide. Hydrogen peroxide is not a free radical itself, but can generate the leading agent responsible for oxygen toxicity in the brain known as the hydroxyl radical (Gutteridge and Halliwell, 1996, Olanow, 1993).

As a natural protective mechanism, the brain contains antioxidant mechanisms capable of discontinuing the chain reaction of free radical formation. Antioxidants may donate an electron to the free radical, but are not converted

to free radicals themselves because they have the ability to remain stable and unreactive in their oxidized form (Halliwell, 1991). They also stabilize transition metals by binding to them and preventing the donation of an unpaired electron; thereby inhibiting the reactions responsible for generating free radicals (Halliwell, 1991, Daniel et al., 2004). Antioxidants known as free radical scavenging enzymes such as superoxide dismutase, catalyzes the alteration of the superoxide radical to hydrogen peroxide. Glutathione peroxidase and catalase consequently scavenge hydrogen peroxide to prevent its conversion into the hydroxyl radical (Olanow, 1992). Under normal physiological conditions, a balance exists between free radical formation and antioxidant reactivity. Once a discrepancy favoring the formation of free radicals occurs, neurons experience oxidative stress and subsequent degeneration. Based on this premise, it is reasonable to postulate that antioxidant therapy may offer protection against free radical damage within neurons and reduce the progression of disorders such as Parkinson's disease.

The antioxidant, selenium (Se) is an essential dietary component for mammals. It is present in the active center of glutathione peroxidase which scavenges peroxides and protects membrane lipids and macromolecules from oxidative damage (Chen and Berry, 2003). Recently, this antioxidant has been shown to be effective in diminishing oxidative stress in several animal models of neurodegeneration. Santamaria et al (2003) reported that intraperitoneal injections of sodium selenite (0.625 mg/kg) for five days decreased the lipid peroxidation and increased the glutathione peroxidase activity in rats challenged with the excitotoxin, quinolinic acid. Kim et al (2000) reported that a ninety day dietary selenium regimen (0.2 ppm Se) decreased methamphetamine neurotoxicity utilizing a glutathione peroxidase mediated antioxidant approach in C57BL/6J mice. When administered in the form of sodium selenite (0.5mg Se/kg) in drinking water for one week, this antioxidant prevented the depletion of dopamine and its metabolites resulting from methamphetamine induced neurotoxicity in C57Bl/6N mice (Imam et al., 1999). When further examining the scope of its protection in the dopamine system, rats deficient in selenium displayed greater oxidative damage of dopamine neurons following 1-methyl-4-phenylpyridinium (MPP+) toxicity than those animals deficient in vitamin E (Vizuete et al., 1994). Zafar et al (2003) also demonstrated that rats pretreated with sodium selenite (ranging from 0.1-0.3 mg/kg) for seven days displayed a dose dependent decrease in the loss of dopamine neurons in the substantia nigra and improved behavior function in animals exposed to an intrastriatal 6-hydroxydopamine (6-OHDA) lesion.

The present study was designed to further examine the ability of selenium to protect dopamine neurons challenged with 6-OHDA. In this project, 6-OHDA was directly administered into the substantia nigra rather than its target, the striatum. Once the cell bodies of the dopamine neurons residing in the substantia nigra are exposed to the neurotoxin, cell death begins within five minutes of exposure via an auto-oxidative process. 6-OHDA is a selective catecholamine neurotoxin responsible for the alkylation of DNA and impairment of mitochondrial ATP production within substantia nigra dopamine neurons (Sachs and Jonsson, 1975). Using this animal model of Parkinson's disease, two methods of selenium administration were examined in the first part of this study. Fischer 344 rats treated with either a three week daily administration of dietary selenium or a seven-day daily administration of selenium as selenium selenite through intraperitoneal injection prior to an intranigral 6-OHDA lesion displayed significant protection of dopamine neurons within the substantia nigra. Since the diet form of selenium administration was less stressful for the animals, a subsequent study was designed to examine the long term effects of selenium pretreatment in animals challenged with 6-OHDA. In this second part of the project, pregnant Fischer 344 rats were exposed to the selenium enhanced rat chow. Their pups were treated with the same diet and exposed to an intranigral 6-OHDA lesion at two months of age. Nigral dopamine neuron survival for the selenium treated animals was significantly

protective when compared to the control chow fed animals. However, when compared to the short-term exposure of selenium rat chow in the previous study, there was no significant increase in neuroprotection.

Materials and Methods

Animals

For part one of this study, thirty-two young adult male Fischer 344 rats (Charles River Laboratory) weighing 200-250g at the start of the experiment were used. The animals were housed one per cage in a temperature-controlled (70^{o}F) room with a 12 hour light/ 12 hour dark cycle. The rats were randomly divided into the following four groups: 1) selenium diet; 2) control diet; 3) selenium injected; 4) control injected. All animals were lesioned with 6-OHDA three weeks post treatment.

For the second part of the study, six pregnant Fischer 344 rats (Charles River Laboratory) were evenly divided into the following two groups: 1) selenium diet or 2) control diet. They gave birth to twenty-two rat pups (eleven in each group). Once the pups were weaned, they were housed one per cage in a temperature controlled (70^{o}F) room with a 12 hour light/12 hour dark cycle. The rat pups were lesioned with 6-OHDA two months later. The prelesioned selenium administration period was longer in this second study to allow the pups to mature to young adulthood prior to 6-OHDA exposure. Selenium treatment was started in utero to develop a model of lifelong antioxidant enhanced therapy. Students were directly supervised and trained in animal handling, surgery and euthanasia by Dr. Cecilia Fox. The animals used in this project were maintained according to the *NIH Guide for the Care and Use of Laboratory Animals*. All protocols for animal care and use at Moravian College are approved by its Institutional Animal Care and Use Committee.

Diet

Control rat chow (AIN-93G Purified Rodent Diet) and selenium enhanced rat chow (AIN-93 Purified Rodent Diet with 2ppm selenium) were purchased from Dyets, Inc. (Bethlehem, PA). For part one of the study, the diet treated animals received either control rat chow or selenium enhanced rat chow for three weeks ad libitum prior to 6-OHDA exposure.

For the second part of the study, three pregnant females ingested the selenium enhanced rat chow ad libitum while the three remaining pregnant females ingested the control rat chow ad libitum. Each group gave birth to a total of eleven rat pups. The females remained on their diet until the pups were weaned. The pups were then placed on the same diet as their mothers for two months.

Intraperitoneal Injections

In part one of this study, selenium through injection was administered daily as a dose of selenium selenite (Sigma - 0.5mg/kg per day, i.p.) dissolved in distilled water for a duration. of seven days. Control animals received a daily injection of distilled water during the same time period.

Drugs

6-Hydroxydopamine (6-OHDA) was dissolved in 0.9% saline at a concentration of 4mg/mL (Sigma).

Surgery

To lesion the animals with 6-OHDA, they were anesthetized with sodium thiopenthal (50mg/kg, i.p; Butler, Columbus, OH) and placed in a stereotaxic frame with the tooth bar set at- 3.3mm. The skull was exposed, and a burr hole was made using a high-speed Dremel drill.

Each injection of 6-OHDA was administered directly into the right substantia nigra using the following coordinates: AP -5.4mm; ML -2.2mm; and DV, -8.2mm from skull (Paxinos and Watson, 1986). Both selenium treated and control groups received a single intranigral injection of this neurotoxin (8μg in 2μL of saline containing 0.02% ascorbic acid).

All injections were performed using a Hamilton 10μL syringe with a 26 gauge tapered needle at a rate of 0.4 μL/min. At the completion of each injection, the needle was left in place for five minutes and then withdrawn at a rate of 2

mm/min. All surgeries were performed under aseptic conditions.

All animals surviving the surgical procedure and post surgery care were euthanized two weeks post lesion by intracardiac perfusion in the first part of the study and two months post lesion in the second part.

Tissue Preparation for Immunocyto-chemistry

Brains were fixed with 4% paraformaldehyde solution in 50mM potassium phosphate-buffered saline (KPBS). The brains were then transferred to 30% sucrose in 50mM KPBS at 4°C. Serial coronal frozen sections of 20μm thickness were cut on a sliding microtome. Six sets of sections were collected in cryoprotectant solution (100mM KPBS, 30% sucrose, polyvinylpyrrolidone {PVP-40} and 30% ethylene glycol) and stored at 20°C until immunocytochemical processing.

A series using every sixth section was stained for the primary antibody against TH (1:1000; Chemicon, Temecula, CA). Sections exposed to the primary anti-TH antibodies were incubated in biotinylated horse anti-mouse secondary antibody (1:500; Vector, Burlingame, CA). Sections were then incubated in the avidin-biotin peroxidase complex using the Elite ABC Vectastain kit (Vector, Burlingame, CA). TH immunoreactivity (TH-IR) was visualized using 3,3'-diaminobenzidine as the chromagen with nickel enhancement.

Cell Counting

The number of TH positive substantia nigra (SN) and ventral tegmental area (VTA) neurons were counted from the brain tissue of all experimental animals. The counts occurred in the SN and VTA of every sixth section between levels -5.3mm and -5.5mm with respect to Bregma. The criterion for delineating the SN from the VTA was the localization of the oculomoter nerve root. The VTA is medial to the root whereas the SN is laterally located. The degree of dopamine neuron depletion was determined by the loss of TH-IR SN neurons on the lesioned side with respect to the control side of the brain. Images of the substantia nigra were captured using an Olympus DP12 Microscope Digital Camera System.

Statistical Analysis

The experimental data were statistically analyzed by means of analysis of variance (ANOVA) for the first part of the study and unpaired t-test for the second part. Results are provided as the mean +/- S.E.M.

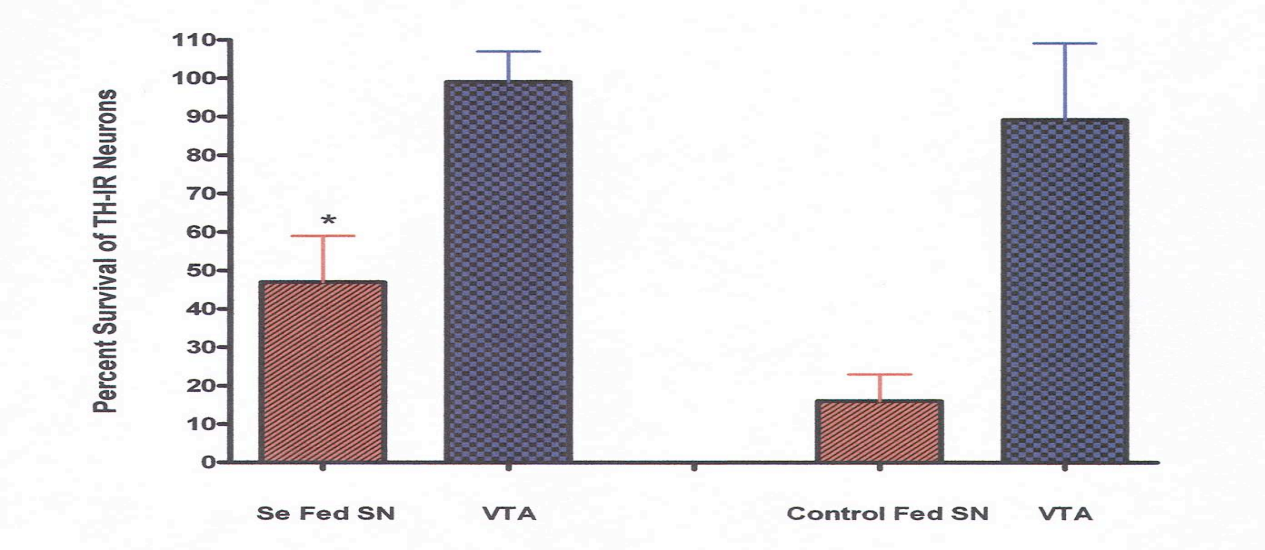

Figure 1. Percent neuronal survival in the substantia nigra and ventral tegmental area for selenium fed and control fed groups. Values are represented as the means+/- S.E.M.. * $P<0.05$, significantly different from the control group.

Results

In the first part of this study, when substantia nigra neurons of the selenium fed animals were compared to those of the control fed group, results indicated that the selenium treatment provided some protection against 6-OHDA neurotoxicity (p value <0.05). Average dopamine neuron survival in the substantia nigra for the selenium fed group was 49.7% compared to an average of 19.0% survival for the control fed group (Figure 1).

Comparison of substantia nigra neuron survival between animals administered selenium through intraperitoneal injection and animals treated with intraperitoneal injections of distilled water, also showed some protection (p value <0.05). Average percent survival of substantia nigra dopamine neurons for the selenium injected group was 56.0% compared to an average of 21.3% survival for the control injected group (Figure 2).

When comparing the methods of selenium administration, results were not significantly different between the two groups. Selenium delivery through diet yielded a protective value of 49.7% for substantia nigra dopamine neurons while selenium delivered through intraperitoneal injection yielded a protective value of 56.0%.

Although the area of interest in this study is the substantia nigra, dopamine neurons in the ventral tegmental area were also examined primarily to determine the extent of the 6-OHDA lesion. Neurons in the ventral tegmental area were not affected by the 6-OHDA administered into the substantia nigra suggesting that the toxin was well localized within the target area (Figures 1 and 2).

Based on the results of this earlier experiment, selenium administration through a supplemented rat chow was employed for the second part of this study to minimize the discomfort of the selenium delivery process in the experimental animals. A longer period of dietary selenium exposure significantly ($p < 0.01$) protected dopamine neurons in the substantia nigra against 6-OHDA neurotoxicity (Figure 3). In the control treated animals, loss of nigral dopamine neurons appeared to be extensive (Figure 3B). Macrophages were evident in the needle tracts leading to the injection site. Damage to the substantia nigra in the selenium treated animals appeared to be less severe than observed in the control group (Figure 3D).

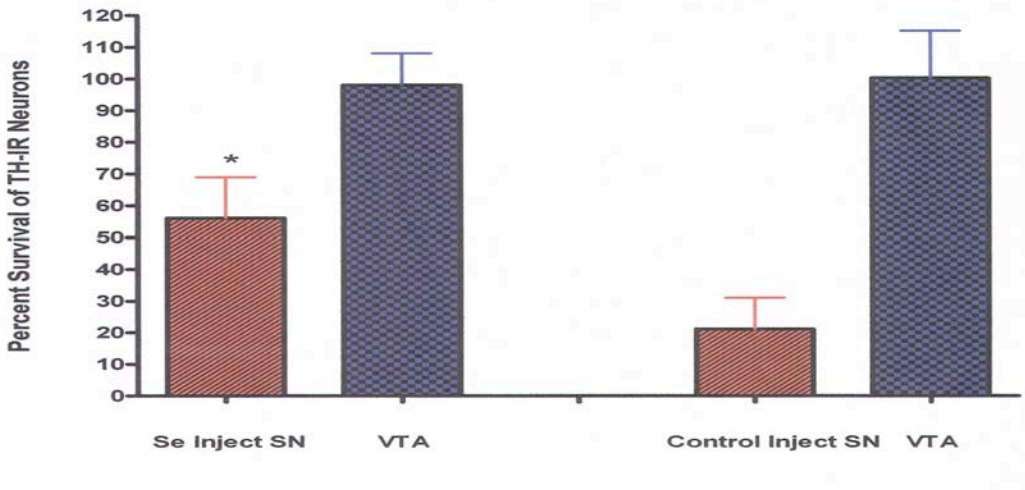

Figure 2. Percent neuronal survival in the substantia nigra and ventral tegmental area for selenium injected and control injected groups. Values are represented as the means +/- S.E.M. * P <0.05, significantly different from the control group.

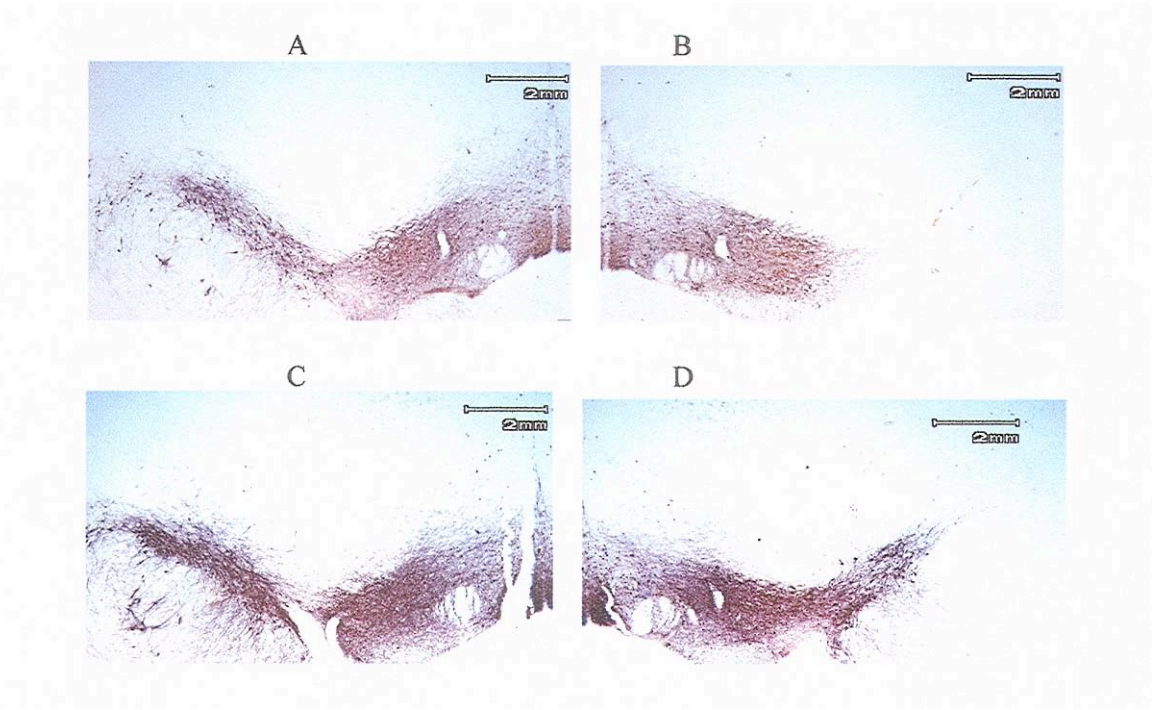

Figure 3. Representative sections are shown through the midbrain of animals in both the control and selenium treated groups from the first part of the study. Figures 3A and B correspond to the unlesioned side (A) and 6-OHDA lesioned side (B) of the control group while figures 3C and 3D represent unlesioned side and 6-OHDA lesioned side of the selenium treated animals, respectively. There is significant sparing of substantia nigra neurons on the lesioned side of the brain in the selenium treated animals.

The average percent survival of substantia nigra dopamine neurons in the selenium fed group was 58.8%, while the average percent survival of nigral dopamine neurons in those animals exposed to control rat chow was 29.6% (Figure 4). These findings are consistent with the preceding experiment, which also demonstrated the protective properties of selenium supplementation. However, there was no significant improvement in the protective effect elicited by long-term selenium treatment (58.8% survival) when compared to the short-term selenium exposure (49.7% survival) in the first part of the study.

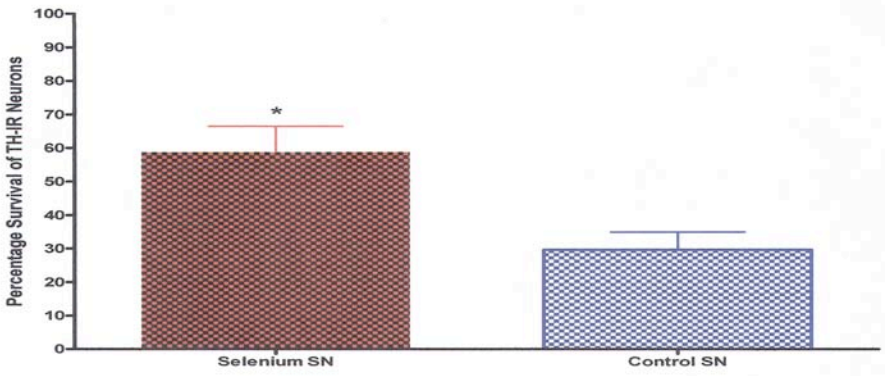

Figure 4. Percent survival of dopamine neurons in the substantia nigra of selenium and control treated animals. Values are represented as the means +/- S.E.M. *P value<0.01.

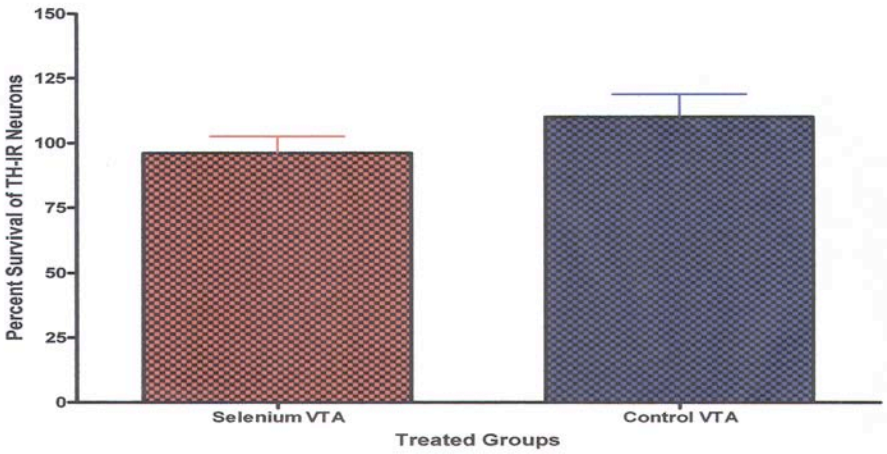

Figure 5. Percent survival of dopamine neurons in the ventral tegmental area of selenium and control treated animals. Values are represented as the means +/- S.E.M.

Once again, the ventral tegmental area in all selenium treated and control animals remained unaffected, suggesting that the toxin was well localized within the substantia nigra (Figure 5).

Discussion

Once free radicals were hypothesized to contribute to the pathogenesis of several neurological diseases, an increasing body of research has focused on attempting to understand the mechanisms behind the protection offered by antioxidants. Selenium, an essential dietary element, has recently been shown to have antioxidant properties due to its localization within the active center of glutathione peroxidase (Schweizer et al., 2004). As mentioned earlier, the neuroprotectant properties of selenium have been demonstrated against MPP+, quinolinic acid, methamphetamine and intrastriatal 6-OHDA (Vizuete et al., 1994, Santamaria et al., 2003, Imam et al., 1999, Kim et al., 1999 and Zafar et al., 2003). The results of our study provide further evidence that the antioxidant, selenium is capable of protecting substantia nigra dopamine neurons against the free radical damage imposed by 6-OHDA when administered directly into the substantia nigra.

The selective effects of 6-OHDA in the rat midbrain are a result of its structural similarity to dopamine and its ability to efficiently bind to the high affinity transport system present on dopamine cell bodies and axon terminals. Since it is highly electroactive, 6-OHDA undergoes a rapid auto-oxidative process to form hydrogen peroxide, the superoxide radical and the hydroxyl radical (Sachs and Jonnson, 1975). When 6-OHDA is administered directly into the substantia nigra, the result is immediate and almost complete destruction of dopamine neurons (Ungerstedt and Arbuthnott, 1970). Extensive loss of substantia nigra dopamine neurons is evident within one week of the lesion. In contrast to the nigral lesion which produces a model for end-stage Parkinson's disease, the striatal lesion creates a neuronal degeneration similar to the progressive type of cell death observed throughout the course of this disease. The chronic loss of dopamine neurons in the substantia nigra due to 6-OHDA exposure is first reported at two weeks post lesion and continues to sixteen weeks (Sauer and Oertel, 1994). For the purpose of this study, the nigral lesion model was adopted to investigate the effectiveness of selenium in preventing the development of the end-stage model of Parkinson's disease.

The first part of our study was primarily designed to determine an optimal method for

administering selenium to our experimental animals challenged with a 6-OHDA nigral lesion. In our short term study, selenium was significantly effective in protecting dopamine neurons of the substantia nigra when administered either through diet (49.7% survival) or intraperitoneal injection (56% survival) prior to an intranigral 6-OHDA lesion. In fact, the percent survival of nigral dopamine neurons following both types of treatment did not display any statistical difference.

Based on these results, a subsequent long term study was designed to examine whether prolonged exposure to dietary selenium could offer a more robust protective effect. However, the timeline of selenium exposure in our experimental animals needed to be carefully considered. Selenium first appeared as a poisonous element in stock animals in the early 1930's. Those animals exposed to large amounts of selenium developed two diseases from selenosis now known as alkali disease and blind staggers (Himeno and Imura, 2002). Rats fed a diet containing over 6ppm selenium (as sodium selenite) for six weeks developed cirrhosis of the liver, enlargement of the spleen and a depression in growth rate. It has also been shown that rats fed a diet containing 4ppm selenium died months earlier than their control fed counterparts (Himeno and Imura, 2002). At this time, the mechanism of selenium toxicity remains to be completely understood. It has been proposed that selenite, the more toxic form of selenium, is capable of inhibiting both DNA and RNA synthesis as well as stimulating apoptosis in several cell lines including premalignant and malignant cells (Himeno and Imura, 2002). Therefore, depending on dosage, there appears to be a dual role for selenium as a neuroprotectant and toxic agent.

In considering the protective studies already published, as well as the physiological role of selenium, the experimental animals in the long term study were administered selenium as 2ppm sodium selenite in rat chow for two months prior to 6-OHDA exposure. So far, there are no studies examining the effects of in utero selenium exposure on development. However, all animals appeared healthy at the time of the lesion. When the animals were euthanized two weeks post surgery, all tissues appeared normal. The long term exposure from in utero through young adulthood conveyed similar protection (58.8% survival) against 6-OHDA neurotoxicity as observed in the previous short term study. There was no significant increase in neuroprotection by extending the treatment period.

It is hypothesized that this increased exposure to selenium enhances the activity of the free radical scavenging enzyme, glutathione peroxidase thereby preventing the conversion of hydrogen peroxide into the hydroxyl radical (Vizuete et al., 1994, Santamaria et al., 2003, Imam et al., 1999, Kim et al., 1999 and Zafar et al., 2003). It has also been reported that following ingestion, selenium is incorporated into selenoproteins as selenocysteine (Brauer and Savaskan, 2004). In fact, all selenoproteins with enzymatic functions contain selenocysteine at their catalytic center (Schweizer et al., 2004). In addition to glutathione peroxidase, glutathione peroxidase isoenzymes and thioredoxin reductases containing selenocysteine are involved in redox defense mechanisms or cellular regulation through their activation of specific transcription factors (Mustacich and Powis, 2000). Based on this rationale, the greater percent survival of dopamine neurons in the substantia nigra observed in our selenium supplemented animals leads us to conclude that selenium exposure may have led to an increase in antioxidant levels in those specially treated animals. Therefore, this increase in antioxidant levels may have offered the neurons in the substantia nigra significantly more protection against 6-OHDA neurotoxicity than those neurons of the control-fed animals.

Dopamine itself is known to contribute to neurotoxicity in the brain. Its ability to spontaneously auto-oxidize to generate harmful quinones and hydrogen peroxide has added to the vulnerability of the substantia nigra. The addition of hydrogen peroxide to the high levels of iron already present within this area of the midbrain stimulates the development of hydroxyl radicals via the Fenton reaction (Romeros-Ramos et al., 2000). It has been hypothesized that the neuroprotection offered by selenium supplementation may be due to its ability to reduce the auto-oxidation of dopamine by enhancing the activity of free radical

scavenging enzymes such as glutathione peroxidase (Zafar et al., 2003). In the presence of 6-OHDA, the subsequent lipid peroxidation, DNA damage and impairment of calcium homeostasis may be the result of weakened antioxidant enzymatic activity. However, in a study published by An et al. (1992) selenium was also shown to serve as a calcium channel antagonist in vitro thereby preventing the unregulated influx of calcium known to contribute to neuronal degeneration.

The short term study revealed a protective response of dopamine neurons to selenium when animals were fed a selenium enhanced diet for three weeks at a daily dose of 2ppm selenium. However, no significant increase in protection was observed following a selenium exposure period beginning in utero and continuing through young adulthood utilizing the same daily dose of selenium. Zafar et al. reported a dose dependent increase in the antioxidant status and behavioral recovery of intrastriatal 6-OHDA lesioned animals exposed to increasing doses of selenium. Perhaps in our study, the amount of selenium administered in the rat chow was sufficient to initiate a protective response by partially up regulating the activity of glutathione peroxidase, but a higher dose could have generated a more robust effect.

Although the use of selenium as a neuroprotectant is relatively new, there is compelling evidence that it may serve as a preventive and therapeutic agent within the brain. Further research targeting the underlying mechanisms contributing to the neuroprotective properties of this element as well as the behavioral improvements observed is needed to clarify its role. Future studies from our lab will continue to investigate a safe and appropriate dosage of selenium to administer to our experimental animals as a neuroprotective agent against 6-OHDA neurotoxicity. Since functional recovery is an essential outcome of this research, rotation behavior experiments will be conducted to determine whether selenium exposure has the ability to improve the motor function of animals exposed to an intranigral 6-OHDA lesion.

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