

THE KETOGENIC DIET AND PARKINSON'S DISEASE

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ABSTRACT

Parkinson's disease (PD), characterized by degeneration of dopaminergic cells in the substantia nigra, is a devastating neurological condition that impairs quality of life. Currently, there are no known cures for the condition and existing treatments are flawed in long-term applications and are often accompanied by severe motor side effects. The ketogenic diet (KD), a high-fat diet that has been used to manage intractable epilepsy for several decades, might be a potential protective measure in PD. The protection offered in epilepsy might also be offered in PD through elevation of serum levels of ketone bodies, specifically β -hydroxybutyrate. This study tested the effectiveness of the KD in reducing hemispheric preference reversal in a unilateral 6-hydroxydopamine (6-OHDA) lesion rat model of PD. Both male and female animals were fed either the KD or control diet for three weeks prior to receiving a unilateral lesion contralateral to their baseline preferred side, which was determined using the corridor task. The same task was used to determine the extent of preference reversal. Buffered Thionin Blue and Fluoro-Jade C stains were also employed to investigate the cellular effects of the diet. This study found insufficient evidence from either behavioral tests or histological analysis to support the hypothesis that the KD was effective in protecting dopaminergic cells in the substantia nigra from 6-OHDA lesions. Furthermore, this study provides evidence that the ketogenic diet was associated with negative health effects when chronically administered including significant weight loss.

1. INTRODUCTION

1.1 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurological disorder in the United States, preceded only by Alzheimer's disease, and is estimated to affect 1% of the population of the United States over the age of 60 (Abou-Sleiman, Muqit & Wood, 2006). This devastating disorder has been a topic of consideration in research since it was first clinically described in 1817 by James Parkinson. Although it has been the focus of much investigation, PD continues to be an area of interest in neuroscience as neither the etiological mechanism of characteristic cell death or effective curative treatments of the disease have been identified.

Parkinson's disease is a neurological disorder that involves degeneration of dopaminergic neurons of the substantia nigra at a rate of about 5% loss per year after the onset of the disorder (Blum et al., 2001) producing the significant motor deficits evident in patients with PD. Clinical signs of PD are observed in patients when dopaminergic cell death in the substantia nigra *pars compacta* (SNpc) approaches a "dopamine threshold." This threshold is estimated to occur between 50% and 80% loss (Foley & Riederer, 1999; Hamani & Lozano, 2003) and is associated with a deficiency of striatal dopamine (Fahn, 2003). Therefore, the mechanisms of cell death in Parkinson's occur substantially before any clinical manifestations are apparent.

The motor and non-motor symptoms of Parkinson's disease are often severely debilitating and significantly impair quality of life. The motor symptoms of PD appear gradually as the "dopamine threshold" is reached and progress as degeneration of dopaminergic neurons in the SNpc becomes more severe. Initial symptoms often include resting tremor, which typically begins unilaterally and spreads across the body as the disease progresses, and bradykinesia, a slowness of motion. Muscular rigidity is also common early in the progression of PD and is

characterized by stiffening of muscles and joints and increased resistance to both passive and active movement. As PD progresses, patients might develop flexed posture, freezing, and a shuffling, “festinating” gait (Fahn, 2003). Motor symptoms of PD can lead to a reduced quality of life; problems with movement lead to disruption of self-care activities, embarrassment and even social isolation (Damiano et al., 1999).

The classic motor symptoms of Parkinson's disease are most often associated with the disorder, and are even used to define it, but many patients with PD present significant non-motor symptoms as well. Cognitive deficits are very frequently observed in patients with PD, as many as 93% of patients diagnosed with the disease have reported some cognitive deficit (Dubois & Pillon, 1997). These cognitive deficits include visuospatial dysfunction, memory impairment (both short and long-term), and executive dysfunction, especially in processes related to set-shifting and maintenance. Up to 80% of individuals with late-stage PD meet the criteria for dementia, which includes memory dysfunction paired with multiple other cognitive deficits (Chaudhuri & Shapira, 2009). Other non-motor symptoms include depression, apathy, sleep disorders, and erectile dysfunction. Current research estimates that 45% of individuals diagnosed with PD meet diagnostic criteria for both depression and anxiety and a large subset of individuals with PD report sleep dysfunction including insomnia, rapid-eye movement sleep behavior disorder and restless leg syndrome (Chaudhuri & Schapira, 2009).

The wide variety of motor symptoms seen in PD is caused by degeneration of neurons in the basal ganglia, specifically the nigrostriatal pathway, which interferes in the regulatory pathway of motor control. The primary physiological marker of Parkinson's disease post mortem is degeneration of dopaminergic cells of the midbrain, particularly of the substantia nigra *pars compacta*. The interaction of dopaminergic cell death in the substantia nigra and motor

deficits is complex. Research has implicated a variety of dysfunctions that result from striatal dopamine deficiency (Hamani & Lozano, 2003). Among these are decreased activity in the direct pathway of GABAergic striatal neurons to the internal globus pallidus and increased activity in an indirect pathway involving the external segment of the globus pallidus and subthalamic nucleus. Together, these changes result in disruption of activity in the brain's motor areas, particularly the pedunculopontine nucleus and thalamocortical motor system, that is thought to be responsible for the difficulty in initiation of movements and poverty of motion characteristic of PD (Hamani & Lozano, 2003).

In addition to degeneration of dopaminergic nigral cells, the pathophysiology of Parkinson's disease is characterized by formation of cytoplasmic inclusions composed of neurofilaments, called Lewy Bodies, in monoaminergic neurons. Lewy Bodies are useful in a definitive post-mortem diagnosis of Parkinson's disease as these unique cellular inclusions are always found in the substantia nigra of patients with PD (Gibb, 1988). Lewy Bodies are not unique to PD, however, and may represent markers of disease processes or of general cell death as they are occasionally found in the brains of elderly adults and are sometimes, although rarely, found upon postmortem examination of the brains of individuals with other neurological disorders such as Pick's disease and corticobasal degeneration (Gibb, 1988).

Though it has received much attention since it was first clinically described, the cause of PD is still not known. The hypothesis that PD is a clinical manifestation of several varied causes has received a great deal of attention. A small number, fewer than 5%, of Parkinson's disease cases have been associated with genetic dysfunction (Warner & Schapira, 2003). In these cases, genetic abnormalities affect protein production that causes depletion of striatal dopamine levels through a variety of mechanisms (Fahn, 2003).

The vast majority of Parkinson's disease cases, greater than 95%, are not genetic and are therefore considered sporadic. The exact mechanism of dopaminergic cell death in sporadic cases of the disease is not known but research has investigated several hypotheses regarding biochemical changes that suggest oxidative stress and mitochondrial dysfunction play a role in the degenerative processes characteristic of PD. A large subset of research in this area has investigated the role of impaired mitochondrial function, specifically of impaired NADH CoQ reductase (Complex I) activity, in oxidative damage in PD (Schapira et al., 1990; Beal, 2003; Fiskum, Starkov, Polster & Chinopoulos, 2003; Abou-Sleiman, Muqit & Wood, 2006).

1.2 Oxidative Stress and Parkinson's Disease

It is known that all aerobic organisms are continually exposed to reactive oxygen species (ROS), which are a variety of molecules derived from the reduction of molecular oxygen (Halliwell, 1992). An imbalance between production of ROS and the physiological ability to detoxify them with antioxidant defenses or repair the cellular damage they cause is called oxidative stress (Turrens, 2003). Uncontrolled increases in ROS leads to free radical-mediated chain reactions that target proteins, lipids, polysaccharides, and DNA and lead to cellular death or dysfunction.

Mitochondria, particularly the Complex I component, are thought to be the most important cellular source of free radicals that generate superoxide radicals (Turrens, 2003). Superoxides, a group of molecules that contain an O_2^- group, are usually converted by superoxide dismutases to hydroxides (H_2O_2) that are then broken down into metabolic water by glutathione. Although superoxides are normally broken down into harmless water molecules by antioxidants, transition metals and nitric oxide can form free oxygen radicals from these superoxides. These

radicals are considered to be some of the strongest oxidants found in nature and are the primary mediators of cell damage when not balanced by antioxidants (Figure 1).

The first evidence of mitochondrial dysfunction as a potential mechanism for cell death in Parkinson's disease resulted from the accidental exposure of drug abusers to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a toxin that causes irreversible parkinsonian syndrome (Abou-Sleiman, Muqit & Wood, 2006). MPTP has since been shown to metabolize into MPP⁺, an inhibitor of Complex I of the mitochondrial electron transport chain. By inhibiting Complex I, production of ATP in cells via the mitochondrial respiratory chain is decreased and free radical production and oxidative stress are increased. In idiopathic cases of Parkinson's disease, research has shown that there is decreased Complex I activity in the substantia nigra of brain specimens examined post-mortem (Fiskum, Starkov, Polster & Chinopoulos, 2003).

The glutathione redox cycle is a major protective system in living creatures (Dringen, 2000). Glutathione (GSH) is an endogenous tripeptide that acts as a substrate in the destruction of the hyperperoxides implicated in oxidative stress induced cell death (Figure 1). Therefore, GSH is an antioxidant that prevents damage to important cellular components caused by ROS. GSH protects against oxidative stress by removing of peroxides, thereby preventing the formation of more damaging oxygen radical species such as the hydroxyl radical. Sian and colleagues (1994) found a 40% decrease of GSH levels in the substantia nigra selectively in patients with PD compared to healthy controls that was not seen in patients with Huntington's disease, multiple-system atrophy, or progressive supranuclear palsy. This study also reported that the ratio of GSH and its oxidized form, GSSG, was altered with an increased proportion of the oxidized form. In another study, the ketogenic diet was found to protect dopaminergic

neurons against 6-OHDA neurotoxicity by upregulating GSH (Cheng et al., 2009). These findings are consistent with the hypothesis that oxidative stress plays a role in nigral cell loss in PD and suggest that the GSH alterations selective to PD may be significant in the underlying neuropathology.

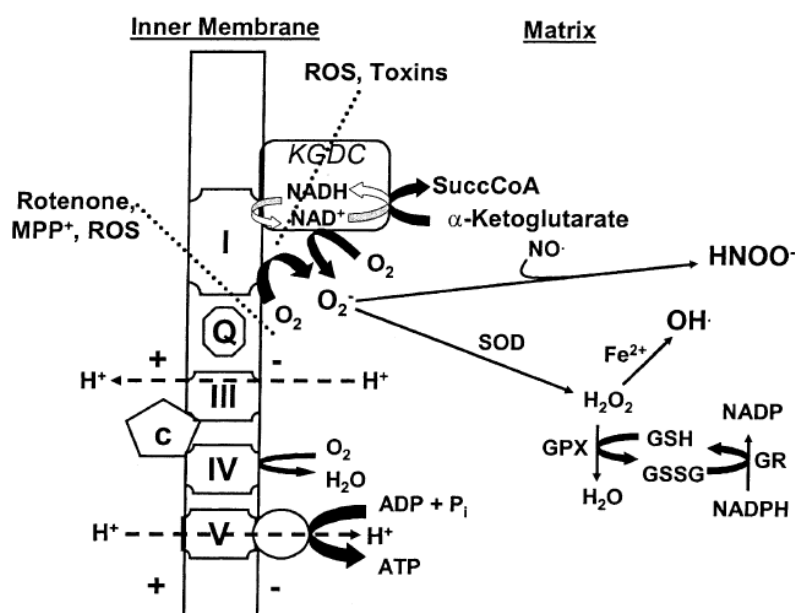


Figure 1. *Oxidative stress and mitochondrial Complex I.* The Complex I component of mitochondria has been suggested to be the most prominent producer of oxidant species that lead to oxidative stress induced apoptosis in Parkinson's disease. The glutathione (GSH) cycle has been shown to reduce hydroxides to benign water molecules and prevent the formation of other oxygen radicals (from Fiskum et al, 2003)

The interaction of the various mechanisms of oxidative stress as they relate to dopaminergic cell death in PD is complex and not yet fully understood. This does not mean, however, that treatments targeting these mechanisms are unworthy of study. The KD offers a potential protective treatment approach to prevent oxidative stress implicated in the cell death characteristic of PD. The KD has been an established method for treating seizure disorders for over 80 years but has only recently been investigated as a potential neuroprotective (Gasior,

Rogawski & Hartman, 2006). This diet mimics the biochemical effects of caloric restriction by reducing carbohydrate intake and causing the body to metabolize fat as a means for cellular energy, increasing levels of ketone bodies in the blood. Although the KD has only started to receive evidence as a potential treatment for PD in the last five years (Gasior et al., 2006), both caloric restriction and ketone bodies, which are elevated in the blood when a subject is sustained on this diet, have been investigated for some time.

1.3 Calorie Restriction, Ketone Bodies, and the Ketogenic Diet

Caloric restriction has been repeatedly studied as a potential method of neuroprotection in cases of neurodegenerative disorders (Maalouf, Rho & Mattson, 2009). In an MPTP mouse model of PD, caloric restriction was shown to improve motor function and enhance cell survival in the substantia nigra (Duan & Mattson, 1999). MPP⁺, the toxic cation product of MPTP, increases reactive oxygen species formation at Complex I of the mitochondria respiratory chain in the SN. The neuroprotective effects of calorie restriction in this study suggest that this diet somehow ameliorates the formation of reactive oxygen species, uptake into the brain, or their effect on Complex I. By extension, the KD, which mimics the effects of calorie restriction, might have similar effects. In fact, research using the MPTP model of Parkinson's disease has demonstrated that the KD has neuroprotective effects in mice and inhibits behavioral dysfunction and cell death caused by the toxin (Yang & Cheng, 2010).

It has been suggested that the KD has neuroprotective effects through similar mechanisms as those seen in calorie restriction. Both the KD and calorie restriction have been found to increase blood levels of the three ketone bodies, acetoacetate, acetone, and β -hydroxybutyrate, up to four times basal levels (Gasior et al., 2006). These ketone bodies can readily cross the blood brain barrier either by simple diffusion or with the help of transporters.

Investigations have shown that β -hydroxybutyrate may be protective in the MPTP model of Parkinson's disease. Tieu and colleagues (2003) reported that rats that received subcutaneous administration of β -hydroxybutyrate for seven days before an MPTP-induced degeneration of dopaminergic neurons showed significantly less neuronal loss and fewer parkinsonian motor deficits. β -hydroxybutyrate may act to improve mitochondrial ATP production in this model, conferring neuroprotection by protecting this vital cellular process from the damaging effects of oxidative stress.

The important protective effects of GSH against ROS may be enhanced in a ketogenic diet which has also been shown to increase mitochondrial levels of GSH. In a previous study, the effects of the KD on mitochondria were studied in rats. Researchers found that animals fed the KD had twice the amount of GSH in hippocampal cells than did control animals fed a standard diet (Jarrett, Milder, Liang & Patel, 2008). These results provide evidence for increases in GSH which may play a role in the neuroprotective effect of the KD.

1.4 The Unilateral 6-hydroxydopamine Lesion Model of Parkinson's Disease

The unilateral 6-hydroxydopamine (6-OHDA) lesion is a common technique utilized to create a model of Parkinson's disease. This model has been shown to produce pathological and physiological deficits similar to PD. The chemical structure of 6-OHDA is analogous to that of catecholaminergic neurotransmitters. Therefore, when 6-OHDA is microinfused selectively into the substantia nigra *pars compacta* or medial forebrain bundle, it can be transported into these cells through catecholaminergic-selective membrane uptake systems (Fulceri et al., 2006).

6-OHDA, much like MPTP, has been shown to affect mitochondrial functioning in dopamine neurons and lead to oxidative stress, ultimately causing cell death. When 6-OHDA is

taken into dopaminergic cells of the nigrostriatal pathway, it is thought to be rapidly oxidized to produce free radicals and hydrogen peroxidase (Fulceri et al., 2006).

1.5 The Current Study

The implication of oxidative stress in PD and the potential neuroprotective effects of the ketogenic diet on oxidative stress in PD are of particular interest. The KD has been established as a treatment method for epilepsy (Freedman, Kossoff & Hartman, 2007) and research has suggested that effects associated with the KD reduce oxidative stress via up-regulation of glutathione and increased levels of β -hydroxybutyrate (Jarret et al., 2008; Tieu et al., 2003).

Considering that the KD increases β -hydroxybutyrate and glutathione in other models of PD and that these both seem to be neuroprotective (Cheng et al., 2009, Gasior et al., 2006, Jarret et al., 2008, Tieu et al., 2003), it may be that the ketogenic diet will have a protective effect in a 6-OHDA lesion rat model of Parkinson's disease. The ketogenic diet may protect nigrostriatal cells from the toxic effects of 6-OHDA, which is thought to act through oxidative stress induced mitochondrial dysfunction, through the protective action of β -hydroxybutyrate and glutathione.

The goal of this study was to investigate both the cellular and behavioral effects of the ketogenic diet on the 6-OHDA model of Parkinson's disease. Little research has investigated the link between the ketogenic diet and behavioral and pathological effects of PD, especially using a 6-OHDA model of cellular degeneration. There is a lack of preventative or curative treatments for PD and currently available treatments are associated with significant motor side effects and lose effectiveness in long-term applications. Levodopa, the most potent drug available for treating symptoms of Parkinson's disease, is associated with an annual 10% cumulative risk of severe motor complications (Schapira et al., 2006). Given this shortage of curative or

preventative measures and effective treatments, combined with the projection that the incidence of PD will double by 2030 (Dorsey et al., 2006), research in this area is exceptionally important.

The implications of this study are numerous. First, the main animal model used in research regarding the ketogenic diet in Parkinson's has been the MPTP lesion model. In this study, the 6-OHDA model will be used to determine if the ketogenic diet produces similar cellular and behavioral effects as studies using the MPTP model. In addition, this study will help advance understanding about the effect of increased ketone bodies and their potential neuroprotective role in Parkinson's disease. This line of research has the potential to eventually produce a preemptive treatment for Parkinson's disease.

In this study, it was hypothesized that the ketogenic diet will reduce the impact of 6-hydroxydopamine injection as evidenced by several dependent variables. First, reversal of hemispheric preference when the initially preferred side is lesioned is expected to be reduced, as this reversal should be correlated with the extent of the lesion. Additionally, it is expected that animals exposed to the ketogenic diet should exhibit a smaller difference in cell survival in the substantia nigra in the lesioned hemisphere when compared to the unlesioned hemisphere through Nissl staining. Finally, it is expected that indication of cell death using Fluoro-Jade C will be less prominent in 6-OHDA exposed substantia nigra in animals treated with the ketogenic diet when compared to control animals.

2. METHODS

2.1 Subjects

A total of twenty Sprague-Dawley rats (*Rattus norvegicus*) were used in this experiment, of which ten were males that initially weighed 320 – 430g and ten females that initially weighed 180 – 230 g. All animals were bred between 12 and 13 weeks before the beginning of the study and were housed individually in Allegheny College's colony for the duration of the study. Both male and female animals were divided randomly and received either the ketogenic diet or a control diet for the duration of the study, creating four groups: I) Females fed the KD, II) Females fed a control diet, III) Males receiving the KD, and IV) Males fed a control diet. Animals were allowed free access to water throughout the duration of the study. All procedures were approved by the Allegheny College Animal Research Committee in accordance with NIH guidelines.

2.2 Diet

Groups II (n=5) and IV (n=5) were fed a standard diet (Prolab® RMH 3000) throughout the experiment from which 26% of calories were provided by protein, 14% from fat, and 60% from carbohydrates. The test diet groups, I (n=5) and III (n=5), were fed the ketogenic diet (Bio-Serv #F3666, Frenchtown, NJ) for approximately 53 days, beginning immediately after a baseline assessment of hemispheric preference was completed until each animal was sacrificed for histology. The ketogenic diet has a 6:1 ratio of fat to carbohydrates 78% of calories in the ketogenic diet are derived from its fat content (Table 1). All animals were fed their respective diets *ad libitum* throughout the duration of the study except for habituation and post-lesion testing when it was necessary to restrict animal weight.

Table 1. The Ketogenic Diet*

Component	Weight (%)
Lard	47.50
Butter	19.95
Corn Oil	11.40
Protein	9.50
Fiber	5.00
Ash	3.80
Vitamin Mix	2.09
Dextrose	0.76

**BioServ diet #F3666 (Formula Rat Diet AIN-76, Modified High-fat Paste)*

2.3 6-hydroxydopamine Lesion Procedure

All surgical procedures were performed in the Physiology Lab at Allegheny College using a David Kopf stereotaxic with a mandible bar set at -3.7mm horizontally. Lesion procedures occurred 23 days after animals began receiving either the ketogenic or control diets.

Rats were anesthetized with a 70mg/kg ketamine and 6mg/kg xylazine mixture injected intraperitoneally at a volume of 0.1cc per 100g body weight. The dorsal surface of each rat's head was shaved and the animal was placed in a David Kopf stereotaxic. A midline incision was made in the dorsal surface of the head with a scalpel. After the periosteum was scraped away to expose bregma, coordinates of the location of each injection site were marked and a burr hole was drilled to allow access to the brain. Once dorsal/ventral coordinates were marked from the surface of the brain, the injection needle was lowered to the correct depth using a back and forth motion to minimize trauma to the brain. The 6-OHDA neurotoxin was prepared by mixing 10 mL of 0.9% saline with 5mg of ascorbic acid (preserved with nitrogen gas). This solution was added to 6-hydroxydopamine (Sigma) at a concentration of 5 mg in 2.15 mL and stored in a freezer between uses.

The intended target of the lesion was the substantia nigra *pars compacta*, an area of dense dopaminergic cell bodies that project to the striatum. The hemisphere in which the lesion would be placed was determined after analysis of data from a pre-lesion behavioral test using a corridor task. Initial side preference was defined based upon a greater than 50% retrieval on the to side of the body averaged across three trials. Animals were lesioned on the side contralateral to their initial side preference in order to reverse their side preference so that reversal of side preference could be used as an indicator of the ketogenic diet's efficacy. The 6-OHDA solution was injected into the brain at the coordinates -5 mm A/P, ± 2 mm M/L, and -7.4 mm D/V from bregma to create a unilateral lesion. Two microliters of neurotoxin were injected at a rate of 0.25 μ L per minute for a total of eight minutes to allow it to be absorbed into brain tissue. Slower injection rates produce more effective lesions and produce less mechanical damage caused by pressure from the volume of injected fluid (Fulceri et al., 2006). After the toxin was injected, it was left to absorb for several minutes before removing the microsyringe.

Once the toxin was injected, the injection needle was gently removed and the incision sutured in standard square knots using 4-0 silk thread. After surgery, rats were removed from the stereotaxic and placed in cages with food, water and antibiotics for several days. Animals assigned to receive the ketogenic diet continued to receive the diet during recovery, with oxytetracycline HCl (Terramycin ®), an antibiotic, mixed directly with the KD for three days after the procedure. Control animals were fed the control diet throughout recovery and received antibiotics mixed with $\frac{1}{2}$ teaspoon peanut butter for three days after the procedure.

2.4 The Corridor Task

Animals were assessed behaviorally both prior to assignment to diet groups beginning the ketogenic diet and again after a two week long recovery following the unilateral 6-

hydroxydopamine lesion procedure. The corridor task is a behavioral test first described by Dowd and colleagues (2005) that has since been repeatedly reported to shown lateralized behavior in the unilateral 6-OHDA model without the use of dopamine agonists. Evidence shows that rats with unilateral lesions in the nigrostriatal pathway preferentially retrieve food from the side of their body ipsilateral to the lesion (Fitzsimmons, Moloney & Dowd, 2006).

In this study, animals were placed in a modified version of the corridor task first described by Dowd and colleagues that measured 8cm wide x 220cm long x 19cm deep. Sugar pellets were arranged in adjacent pairs of wells sunk $\frac{3}{4}$ cm into the base along the length of the corridor at 13 cm intervals (Figure 2). Rats were maintained at 90% of feeding weight for the duration of testing to encourage retrieval behavior without the use of drug induced hyperactivity common to other tests of hemispheric preference. Animals were habituated to the box with sugar pellets scattered randomly across the floor for ten minutes each for two day prior to trials. Data were collected over three trials in the following three days in which rats were allowed to explore the corridor and make retrievals at will. "Retrieval" was operationally defined in this study as a nose poke in a well, with or without the animal taking a sugar pellet and was recorded based on the side of the animals midline on which it occurred. Trials were completed when five minutes elapsed from the time the partition between the start block and the corridor was removed or when 20 retrievals were recorded.

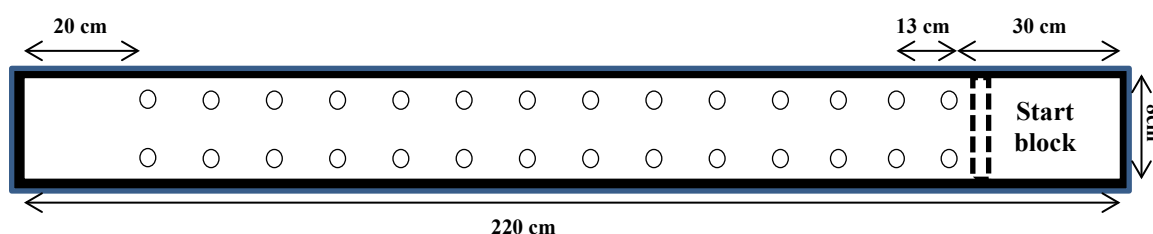


Figure 2. The *corridor task apparatus*. A 220 cm x 8 cm x 19 cm deep box lined with adjacent pairs of pellet $\frac{3}{4}$ cm deep wells spaced 30 cm from the start block wall continuing in 13 cm increments up to 20 cm from the end of the corridor. A start block was separated from the corridor with a removable partition that, when removed, signaled the start of a trial.

2.5 Histology

Both buffered Thionin Blue Nissl and Fluoro-Jade C stains were used to analyze brain tissue from three animals from each group in this experiment. Animals were sacrificed with a lethal dose of sodium pentobarbital (Euthasol®, Virbac) injected intraperitoneally and perfused intracardially with 10% formalin in 0.9% NaCl for several minutes. The brain was allowed to fix in 10% formalin for at least 48 hours before it was sectioned in 50 μ m sections using a freezing microtome. Alternating sections were mounted on slides prepared with albumin glycerine (Sargent-Welch) and were designated for either Nissl or Fluoro-Jade C stains.

Nissl Staining. In order to obtain a general view of the substantia nigra and make a qualitative analysis of the area, a buffered Thionin Blue stain was used. For this procedure, slides were submersed in a series of solutions to remove lipids, dehydrate tissue, and stain cell bodies (Appendix Table A). Slides were coverslipped with Permount® mounting medium and viewed under a compound microscope.

Fluoro-Jade C Staining. The extent of damage to neurons in the substantia nigra was analyzed with a fluorescent stain called Fluoro-Jade C. Fluoro-Jade C has been previously reported to fluoresce degenerating neurons (Schmued et al., 2005). Slides with adjacent sections to those used in Nissl staining were stained in a series of solutions in order to maximize the ability to compare sections between stains (Appendix Table B). Slides were coverslipped with DPX and viewed with a compound fluorescence microscope using a Nikon FITC HYQ filter set with an excitation wavelength range of 460 to 500 nanometers.

3. RESULTS

3.1 Behavioral Testing

All animals in the study were tested in the corridor task to assess their initial hemispheric preference and again after receiving a unilateral 6-hydroxydopamine lesion contralateral to their preferred side. Left and right retrievals were counted in each of three trials with a maximum of 20 retrievals per trial. Contralateral side biases were calculated for each animal by dividing the number of retrievals on the side contralateral to the lesion by the total number of retrievals across all three trials and were expressed as percentages (Figure 3).

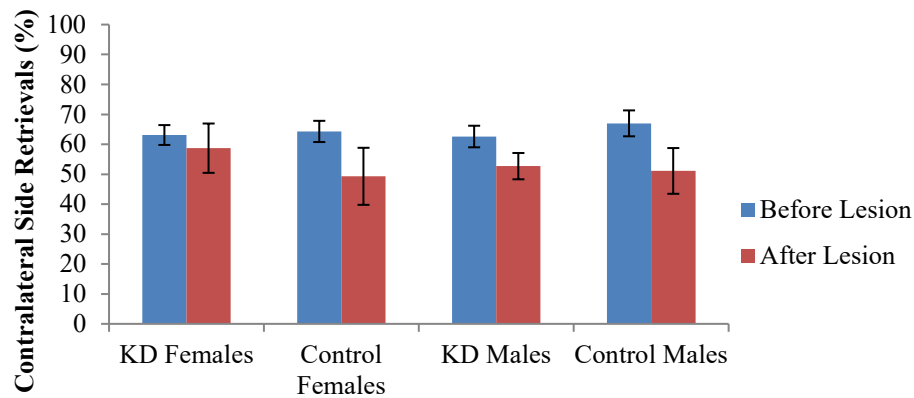


Figure 3. *Percent contralateral turns before and after a unilateral 6-OHDA lesion.* The number of retrievals made on the left and right sides of each animal's body were recorded over three trials. Percent contralateral turns were calculated by dividing retrievals made on the side opposite the lesion by the total number of retrievals made across each of three trials. Standard error of the mean for each group was used to add error bars.

Analysis of behavioral data was performed using three way mixed ANOVA. Tests of between-subjects effects revealed no significant effect of sex on hemispheric preference (female animals, $M=0.588$, $SD=0.033$, male animals, $M=0.583$, $SD = 0.033$ [$F(1,16)=0.012$, $p=0.915$]). There was also no significant diet effect between females animals ($M=0.593$, $SD=0.033$) compared to male animals ($M=0.579$, $SD=0.033$), $F(1,16)=0.089$, $p=0.769$. The sex by diet

interaction was not significant [female animals that received the ketogenic diet ($M=0.609$, $SD=0.046$), female animals that received the control diet ($M=0.568$, $SD=0.046$), male animals that received the ketogenic diet ($M=0.576$, $SD=.046$) and male animals that received a control diet ($M=.590$, $SD=0.046$), $F(1,16)=0.355$, $p=0.560$] (Table 2).

Table 2. Mixed ANOVA: Between Subjects Contralateral Side Preference Effects

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Significance
Sex	0.000	1	0.000	0.012	0.915
Diet	0.002	1	0.002	0.089	0.769
Sex x diet	0.008	1	0.008	0.355	0.560
Error	0.342	16	0.021		

Tests of within-subjects effects showed that the effect of lesion on the proportion of contralateral retrievals was significant, $F= (1,16)$, $p<0.0001$ [before lesions ($M=0.642$, $SD=0.0782$) and after lesions ($M=0.529$, $SD=0.1619$)] (Table 3). Other within-subjects effects, including lesion by sex, lesion by diet, and lesion by sex by diet, were not significant (Table 3).

Table 3. Mixed ANOVA: Within Subjects Contralateral Side Preference Effects

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Significance
Lesion	0.128	1	0.128	8.494	0.010
Lesion x Sex	0.003	1	0.003	0.171	0.685
Lesion x Diet	0.017	1	0.017	1.121	0.305
Lesion x Diet x Sex	0.001	1	0.001	0.087	0.772
Error	0.242	16	0.015		

Weights of all animals were recorded before animals began receiving their assigned diets and again at the conclusion of the study. The sex by lesion interaction on weight was not significant, $F=0.443$, $p=0.515$ [female animals before lesion ($M=273.900$, $SD=71.120$), female

animals after lesion ($M=269.700$, $SD=55.666$), males before lesion ($M=295.050$, $SD=99.404$), and males after lesion (347.000 , $SD=112.575$) (Table 4).

There was a significant interaction between lesion and diet on weight, $F(1,16)=117.303$, $p<.0001$. There were significant differences between all group means (Figure 4).

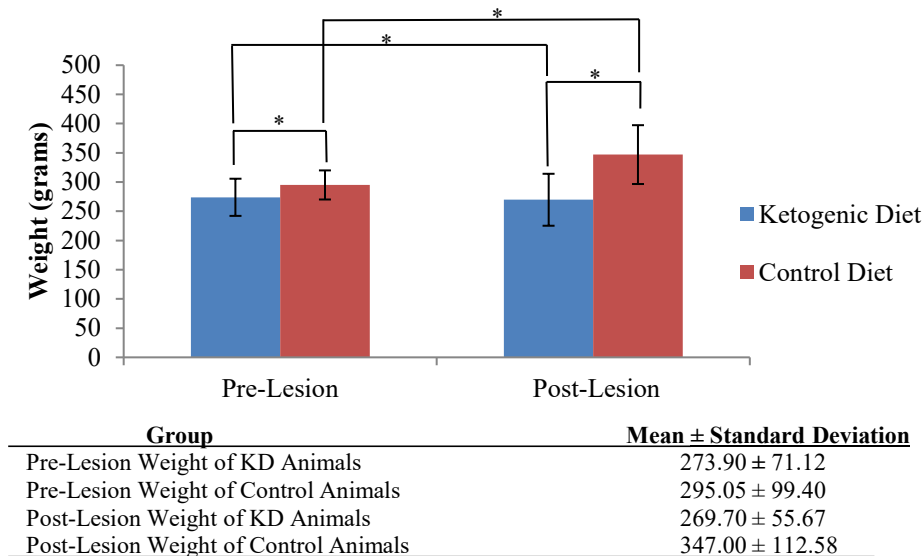


Figure 4. *Lesion by diet interaction on animal weight.* Animals were weighed before and after being fed either a control diet or the ketogenic diet for 53 days. Lesion by diet interaction had a significant effect on animal weight. Tukey's HSD calculations revealed that there were significant lesion x diet interactions between all groups at $\alpha = 0.05$ (indicated by *). Error bars representing the standard error of the mean for each group were added.

The lesion by diet by sex interaction was also significant on weight, $F=84.832$, $p<.0001$ (Table 5). There were several significant differences in this interaction (Figure 5). There was not a significant difference between KD female weight pre- and post-lesion but there was a significant increase in weight of control females pre- to post-lesion. Additionally, control males gained a significant amount of weight pre- to post-lesion while KD males showed a significant decrease in weight.

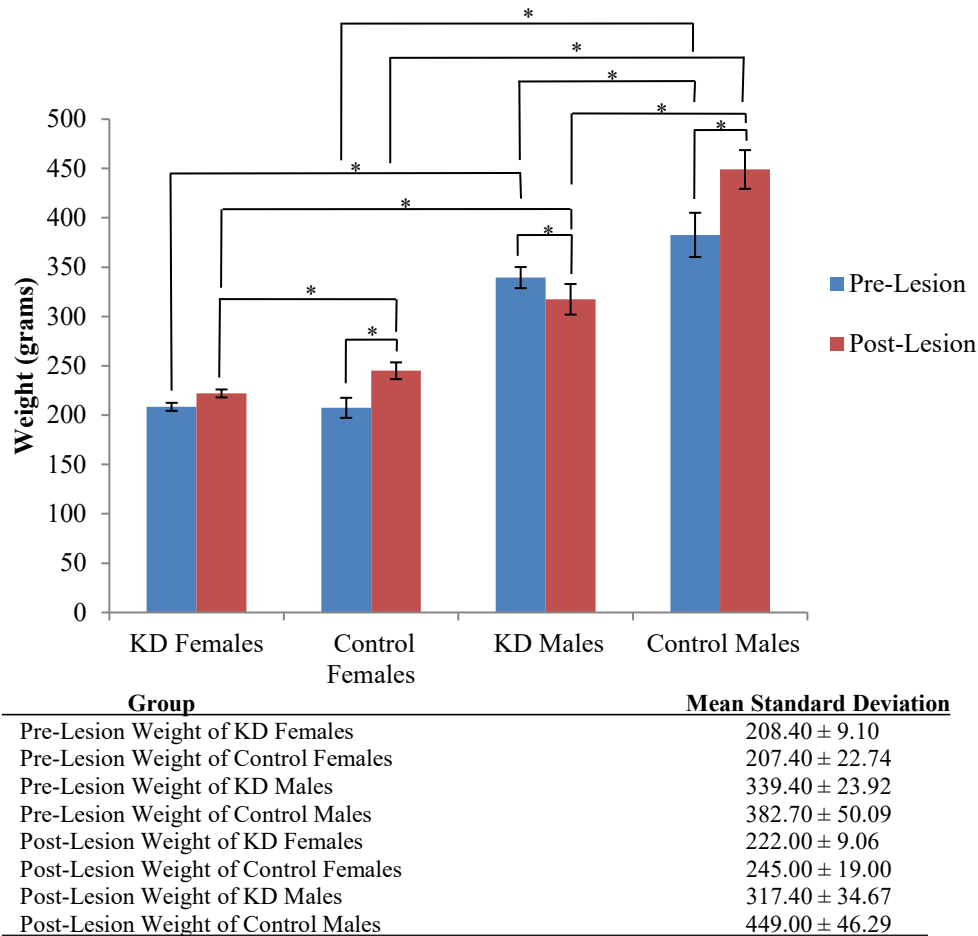


Figure 5. Lesion by diet by sex interaction on weight. Animals were weighed before and after being fed a control or ketogenic diet for 53 days. Mixed ANOVA revealed a significant interaction between lesion, diet, and sex. Tukey's HSD calculations showed that several interactions were significant at $\alpha=0.05$ (indicated by *). Error bars representing the standard error of the mean for each group were added.

Table 4. Mixed ANOVA: Within Subjects Weight Effects

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Significance
Lesion	5700.156	1	5700.156	84.832	0.000
Lesion x Sex	29.756	1	29.756	.443	0.515
Lesion x Diet	7882.056	1	7882.056	117.303	0.000
Lesion x Sex x Diet	2584.056	1	2584.056	38.457	0.000
Error(Lesion)	1075.100	16	67.194		

3.2 Histology

At the conclusion of behavioral testing (53 days after animals were randomly assigned to begin receiving either a control diet or the ketogenic diet), animals were sacrificed and their brains were sectioned into 50 μm sections and mounted on slides coated with albumin glycerine. Alternate sections were mounted on slides to be stained with a buffered Thionin Blue stain. After slides were stained, they were coverslipped and allowed to dry before they were qualitatively analyzed.

Specific attention was paid to the substantia nigra *pars compacta*, which is located between approximately 4.50 mm and 6.60 mm posterior to bregma, 2.00 mm from either side of the midline, and about 7.80 mm from the dorsal surface of the brain. Lesions to this area caused a decreased density of cells apparent in buffered Thionin Blue stains when the SNpc of the lesioned hemisphere was compared to the non-lesioned SNpc (Figures 6 – 9). Lesion caused a break in the continuity of the SNpc by destroying areas of dopaminergic neurons in the area (Figures 6B, 7B, 8B, and 9B). In all animals, the accuracy of lesion placement was noted when observing tissue sections (lesion tracks were apparent in both the buffered Thionin Blue stains and Fluoro-Jade C stains and were used as an indicator of lesion placement). Successful lesions were determined to be those with needle tracks ending in the substantia nigra *pars compacta* or in the *pars reticulata*, which contains dopaminergic dendrites capable of 6-ODHA uptake (Wassef and Berod, 1981). Lesions appeared to be successfully placed in all animals.

There was a noticeable difference in all animals between the lesioned and non-lesioned sides of all animals that is consistent with the statistically significant decrease found in the proportion of contralateral turns pre- and post-lesion. The non-lesioned hemisphere was used as an intra-animal control to compare cellular density and degeneration in sectioned and stained

tissue. . Cellular effects of the KD diet were not apparent from qualitative analysis of cell density in these sections. Animals fed the ketogenic diet had damage to the SNpc similar to animals that were fed a control diet (Figures 6 – 9).

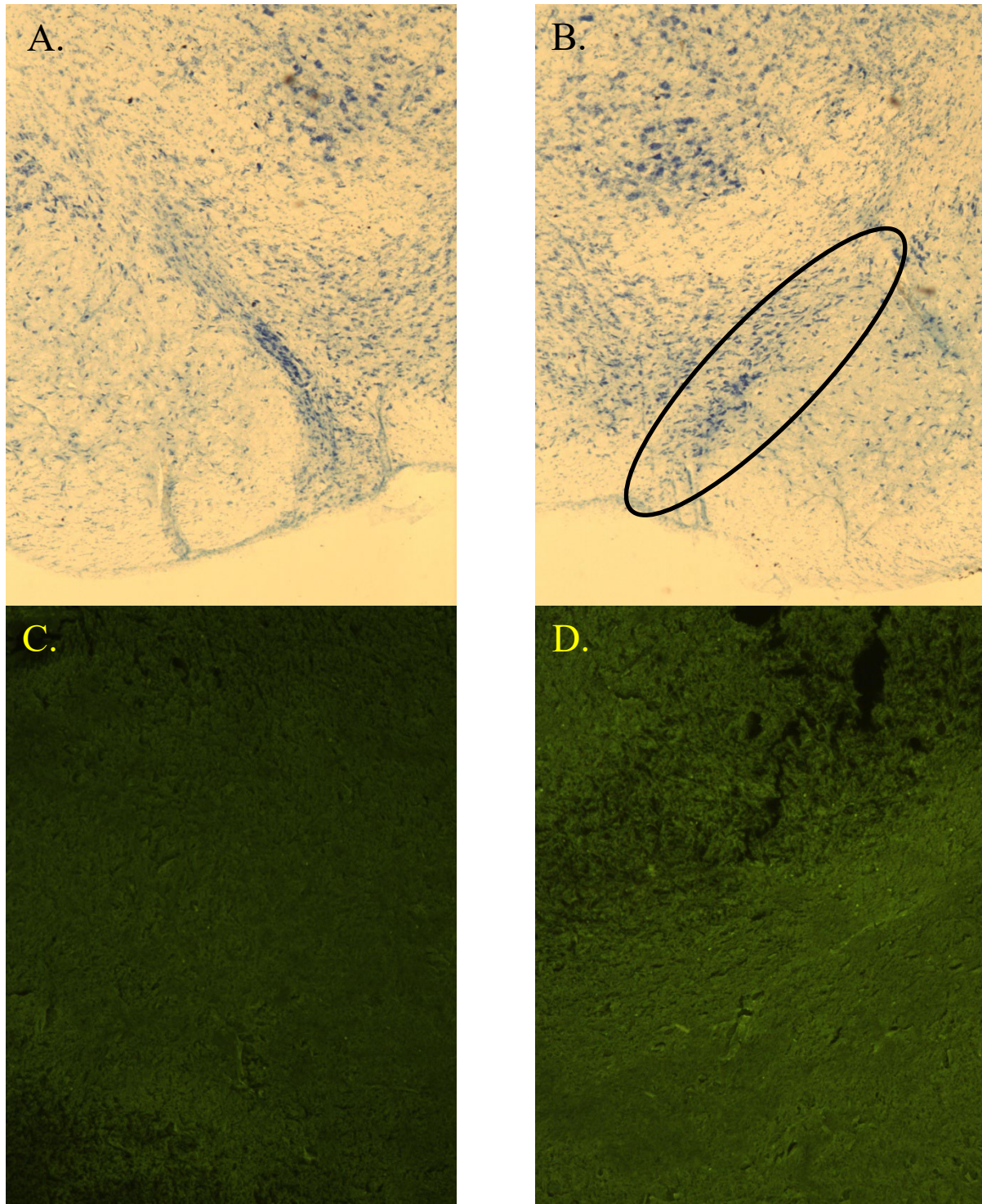


Figure 6. *Nissl and Fluoro-Jade C sections of a female animal fed the ketogenic diet.* Alternate sections were mounted on slides to be stained with either buffered Thionin Blue or Fluoro-Jade C. The substantia nigra of each hemisphere was photographed. The SNpc stained with buffered Thionin Blue (left, A, and right, B, at 5x magnification) and with Fluoro-Jade C (left, C, and right, D, at 10x magnification). Lesions are apparent in both stains (circled in C, brighter green fluorescence in D compared to C).

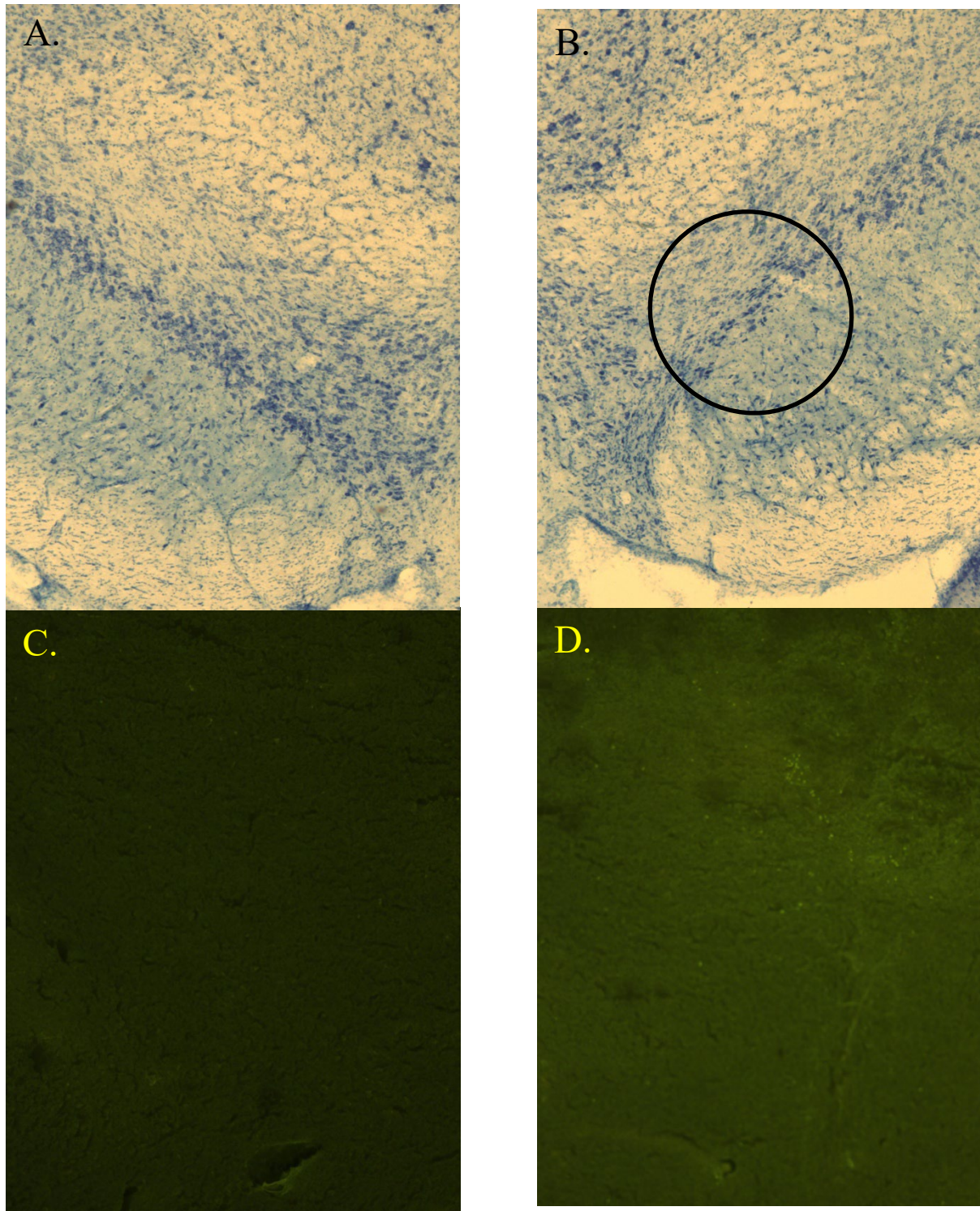


Figure 7. *Nissl and Fluoro-Jade C sections of a female animal fed a control diet.* Alternate sections were mounted on slides to be stained with either buffered Thionin Blue or Fluoro-Jade C. The substantia nigra of each hemisphere was photographed. The SNpc stained with buffered Thionin Blue (left, A, and right, B, at 5x magnification) and with Fluoro-Jade C (left, C, and right, D, at 10x magnification). Lesions are apparent in both stains (circled in C, brighter green fluorescence in D compared to C).

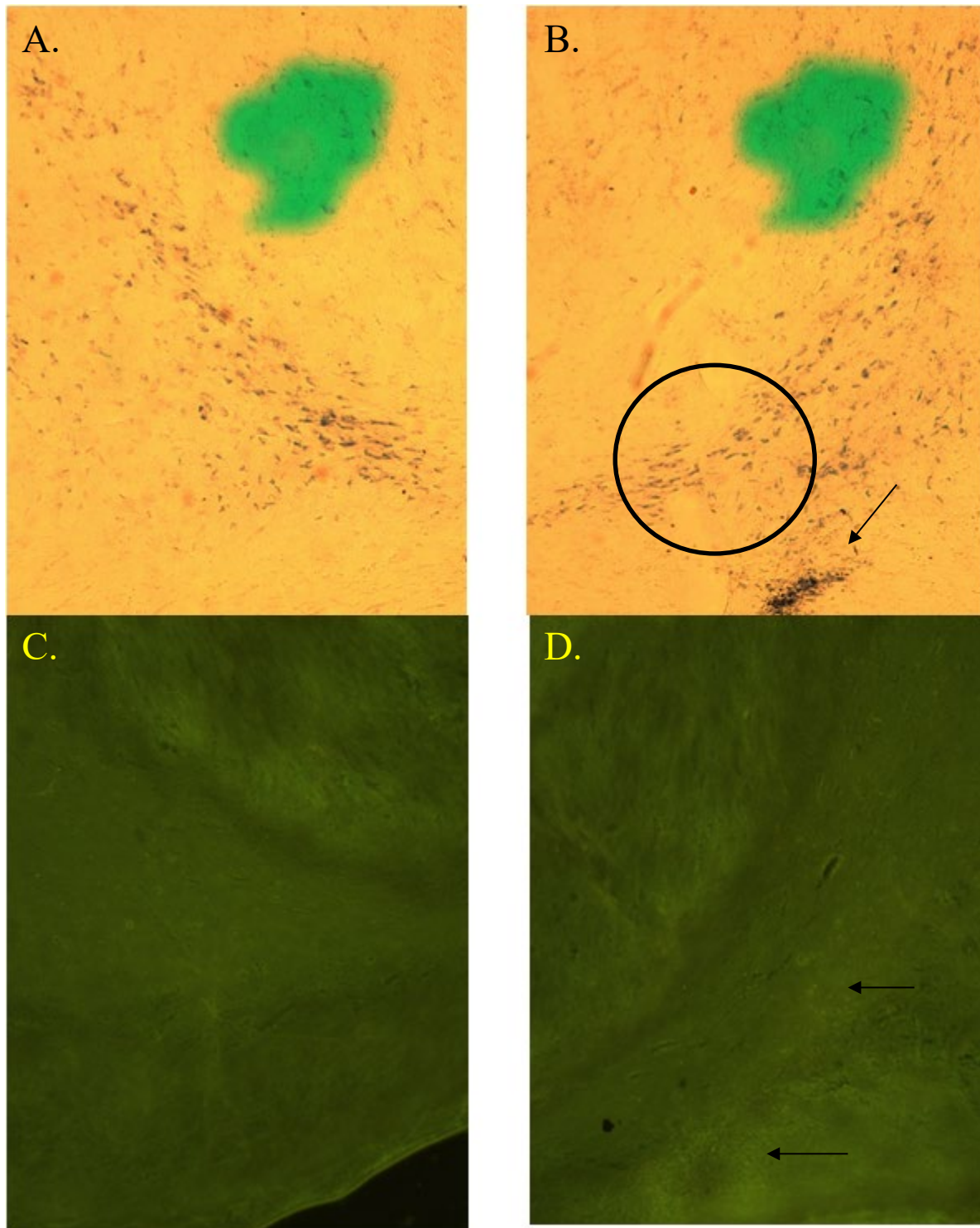


Figure 8. *Nissl and Fluoro-Jade C sections of a male animal fed the ketogenic diet.* Alternate sections were mounted on slides to be stained with either buffered thionin blue or Fluoro-Jade C. The substantia nigra of each hemisphere was photographed. The SNpc stained with buffered Thionin Blue (left, A, and right, B, at 10x magnification) and with Fluoro-Jade C (left, C, and right, D, at 10x magnification). Lesions are apparent in both stains (circled in C, brighter green fluorescence in D compared to C). Needle tracks caused gliosis that appears as dark blue when stained in buffered thionin blue (arrow in B).

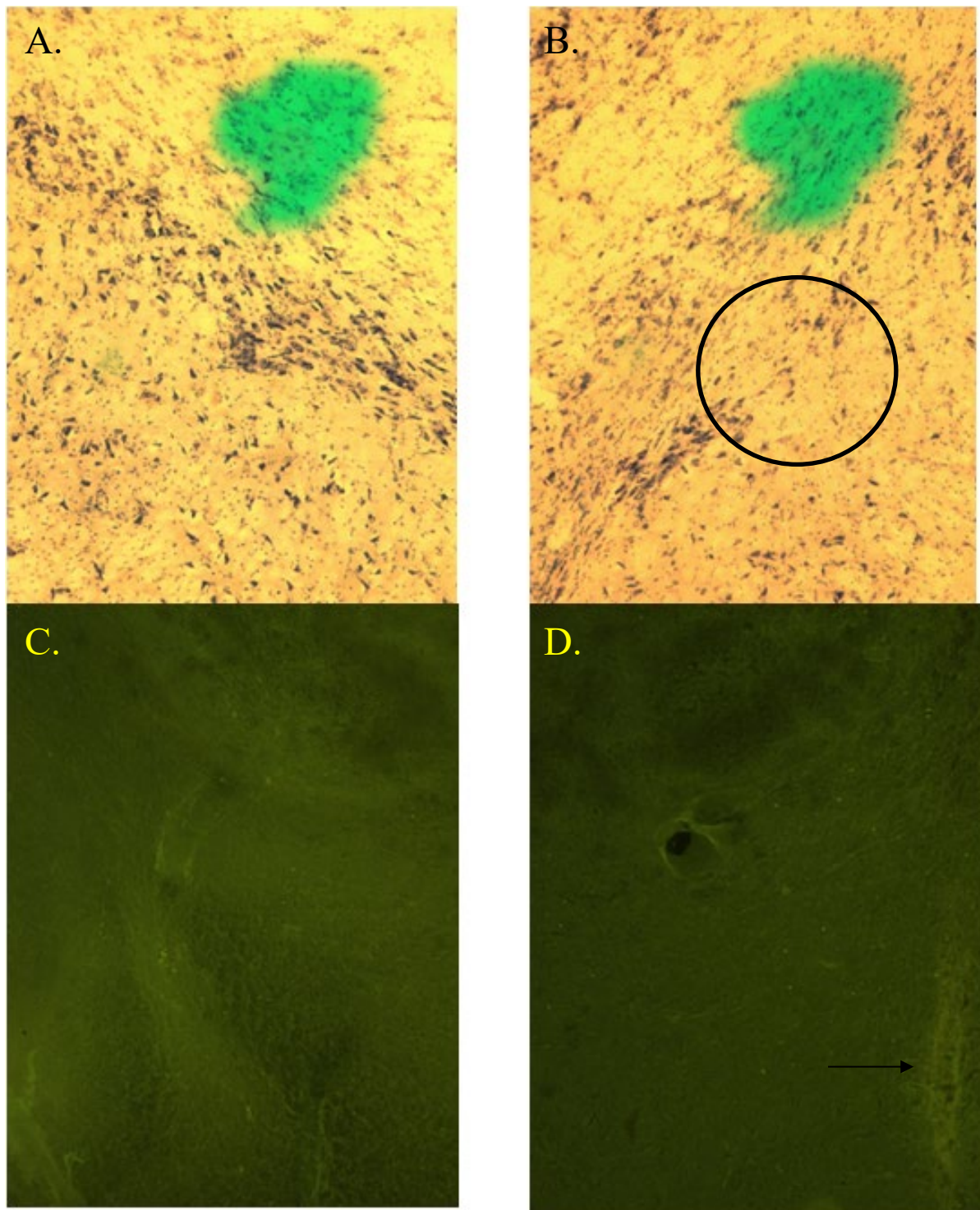


Figure 9. *Nissl and Fluoro-Jade C sections of a male animal fed a control diet.* Alternate sections were mounted on slides to be stained with either buffered thionin blue or Fluoro-Jade C. The substantia nigra of each hemisphere was photographed. The SNpc stained with buffered thionin blue (left, A, and right, B, at 10x magnification) and with Fluoro-Jade C (left, C, and right, D, at 10x magnification). Lesions are apparent in both stains (circled in C, brighter green fluorescence in D compared to C). Needle tracks fluoresce brightly in FJ-C (arrow in D)

4. DISCUSSION

4.1 Overview

The primary goal of this investigation was to study the potential effectiveness of the ketogenic diet as a method of protection in a unilateral 6-hydroxydopamine lesion model of PD. For many years, the ketogenic diet has been clinically reported as an effective treatment option for patients with uncontrolled epilepsy (Gasior et al., 2006). More recently, the neuroprotective effects of the ketone body β -hydroxybutyrate, which has been repeatedly shown to be increased in serum concentration when subjects are fed the KD, have been studied (Tieu et al., 2003). Some research has investigated these effects in conjunction with dopaminergic cell death in the nigrostriatal pathway that characterizes PD. This connection relies on evidence that cell death in PD is related to mitochondrial dysfunction caused by increased reactive oxidative stress (Abou-Sleiman et al., 2006; Cheng et al., 2009; Sian et al., 1994). It might be, then, that the KD is neuroprotective in PD via mechanisms based in its power to promote concentrations of antioxidant molecules in neural tissue.

While this line of investigation has already achieved some success, few studies have investigated cellular and behavioral effects of the KD in a unilateral 6-hydroxydopamine model of PD. Previous research has focused mostly on the methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) model, which creates a model of PD by exposing cells to the oxidant form of the toxin, MPP⁺. A concern of this study was to determine whether future research with the ketogenic diet is possible using 6-hydroxydopamine models of PD. This model more accurately mimics the chronic degenerative human condition of PD by causing cell death over several weeks whereas the MPTP model causes very rapid cell death and is less useful in studying long-term cell death (Sauer & Oertel, 1994).

4.2 Behavioral Effects

This study provided further evidence that the corridor task is a behavioral measure sensitive to unilateral lesion models of PD. The lesion effect on proportion of contralateral turns was significant (Table 3). These results are supported by previous research that found the corridor task detected changes in hemispheric preference in the 6-OHDA model of PD (Dowd et al., 2005, Fitzsimmons et al, 2006).

In this study, it was hypothesized that animals treated with the KD would be protected from 6-hydroxydopamine lesions and would therefore show a decreased hemispheric preference reversal from a pre-lesion baseline. Considering that paw preference in rats is controlled by the contralateral hemisphere of the brain (Gao & Zhang, 2008), a lesion to the side contralateral to the preferred side, as determined by retrieval behavior in the corridor task, would abolish that side preference and cause it to reverse. Behavioral data obtained in this study, when analyzed using mixed ANOVA, did not support the hypothesis that the KD protects animals from a reversal of hemispheric preference caused by a unilateral 6-hydroxydopamine lesion (Table 2). It is notable that a comparison of means shows that both male and female animals fed control diets demonstrated a larger side bias reversal, which may indicate a protective effect of the diet albeit not a statistically significant one (Figure 3). These data suggest that the KD might have a small protective effect against 6-OHDA lesions that the corridor task was not sensitive to in this study.

Contrary to previously reported results using similar corridor tasks, results of this study found that there was large variability over trials (Figure 3). Variability found in this study suggests data collected from trials over several days is not as reliable as previously reported (Dowd et al., 2005). For three days, rats were tested in the corridor task in order to maximize its

sensitivity to hemispheric preference and retrieval behavior varied widely between trials.

Contralateral bias scores were derived from averages across these three trials, resulting in a large statistical variability in mean scores. Dowd and colleagues (2005) used only two trials on two consecutive days and found that trials were significantly correlated, whereas trials in this study were not. In a modified version of the task, Fitzsimmons and colleagues (2006) tested rats across 15 days and also found that the proportion of contralateral retrievals in each animal was consistent over this time.

It should be noted that the operational definition of “retrieval” was modified slightly in this study from that used by Dowd and colleagues to include any time a rat nose poked into or took a sugar pellet from a well, whether or not it was a consecutive retrieval from the same well. In previous studies using the task, consecutive retrievals from the same well were not counted. This modified definition might have contributed to the large variability as rats tended to retrieve several pellets from the same well before moving down the corridor. This effectively increased the number of retrievals on one side without requiring the rat to re-choose between the left and right side of its body, artificially inflating one side preference without most accurately reflecting contralateral side bias.

4.4 Histology

Histological analysis of brain tissue in this study was utilized for three reasons, to confirm the accuracy of lesions, to determine if lesions were effective within individual animals, and to compare the extent of lesions across animals in different treatment groups. Qualitative analysis found that lesions were accurately placed and that damage to cells of the SNpc correlated with the significant lesion effect on the proportion of contralateral turns found in behavioral testing (Figures 6 – 9, Table 3).

Results of this study concerning the third application of histological analysis in this study were less revealing. Previous research has found that neurons in this area are protected by both direct application of the ketone body β -hydroxybutyrate (Tieu et al, 2003) and application of blood ketosis indirectly using the ketogenic diet (Cheng et al., 2009), but results of histological methods in this study fail to support the hypothesis that the ketogenic diet is protective against 6-hydroxydopamine injection. Sections of brain tissue from both animals fed a control diet and the ketogenic diet show similar qualitative evidence of damage to the SNpc of the lesioned hemisphere.

It must be noted that the SNpc contains neurons that are not catecholaminergic, therefore 6-hydroxydopamine would have little, if any, effect in causing death to these cells (Wassef and Berod, 1981). This is potentially problematic when using Nissl staining histology to quantify the effectiveness of a protective treatment using cell survival as a dependent variable, as these neurons will not be affected by the lesion and are indistinguishable from dopamine neurons in the area using the methods employed in this study.

Despite the potential problem of non-catecholaminergic cells confounding analysis of cell density in the substantia nigra, previous research has reported significant differences in the number of cells in this area between animals fed a ketogenic diet versus those fed a control diet. Cheng and colleagues used a similar Nissl staining protocol to determine that cell bodies in the SNpc were significantly protected from 6-OHDA in animals fed the ketogenic diet for 14 days compared to those fed a control diet. In this study, researchers counted cell bodies in eight comparable sections of tissue from each of a total of 20 rats. They found that if they calculated the percent of surviving SNpc cells compared to control animals fed a normal diet that were not

lesioned, animals lesioned with 6-OHDA that were fed the KD had about 10% more cell bodies in the SNpc than did lesioned animals fed a control diet (Cheng et al., 2009).

The current study did not utilize a randomization method for counting cell bodies in the SNpc as it was hard to find comparable sections from the same A/P coordinates because two staining protocol used adjacent sections. It might be that, had this study conducted such a precise quantitative analysis, that a similar cellular effect would be evident.

4.3 Health Effects

Although the main focus of this study was not to analyze the effect of the ketogenic diet on weight, mixed ANOVA on weight data, which were originally intended only to keep track of animal weight for behavioral testing, revealed significant relationships worth discussion. The lesion by sex by diet interaction on weight was significant in this study and proved to represent a multitude of significant interactions that might represent a potential weakness of the ketogenic diet as a protective treatment for Parkinson's disease.

In both control males and control females, there was a significant increase in weight pre- to post-lesion (Figure 5). This increase in weight is not abnormal, as the animals that were used in this study were only 12-13 weeks old when they were first weighed. Interestingly, females fed the ketogenic diet did not gain a significant amount of weight pre- to post-lesion while their counterpart control females did. Males fed the KD actually lost a significant amount of weight pre- to post-lesion, while their counterpart males gained weight.

Considering these data, it can be concluded that chronic implementation of the ketogenic diet affects normal growth. These differences are not unexpected when the composition of the ketogenic diet is analyzed and compared to research. The KD, which is designed to provide calories mostly from fat, is low in both carbohydrates and proteins. This composition is intended

to mimic the bodily reaction to caloric restriction, thereby inducing blood ketosis. Low carbohydrate diets have been associated with significant decreases in weight in humans (Westman et al., 2002).

Literature focusing on the protective effect of the ketogenic diet in individuals with epilepsy points out other negative affects on health in humans. Vomiting, hypoglycemia, and dehydration have all been reported in clinical applications of the diet leading to diet refusal in humans (Vining, 1999). Considering this effect in humans, it might be that rats exposed to the diet experienced similar discomfort, potentially consumed fewer calories, and suffered from chronic exposure to the diet. It is important, therefore, to consider that the ketogenic diet might have overall negative health consequences and that these might limit its clinical efficacy in protective applications for Parkinson's disease.

4.5 Limitations

Analysis of data in this study highlighted a few limitations that must be noted. Primarily, statistical power in this study was reduced by a number of factors including a small sample size and large variability between behavioral trials. Sample sizes of just five were too small to provide enough statistical power to observe significant relationships in group mean differences of contralateral retrieval data. It might be that with larger sample sizes, a significant relationship might be seen.

A potential weakness of this study lies in the fact that only one behavioral test was utilized. In an attempt to avoid the application of drug-induced rotation behavioral tasks such as the rotometer, which uses d-amphetamine or apomorphine, a behavioral test was chosen that did not rely on amphetamines to induce hyperactivity. This study might have benefitted from the addition of behavioral tasks that do not rely on hemispheric preference or drug-induced

hyperactivity. In future studies, application of a variety of behavioral tests including reaching tasks or forelimb asymmetry tasks might help determine the potential effectiveness of protective treatments in Parkinson's disease.

4.6 Future Studies

There is potential for future research using β -hydroxybutyrate in a 6-hydroxydopamine lesion model of Parkinson's disease. The mechanism by which the 6-OHDA lesion causes dopaminergic cell death prolonged over several weeks makes for an ideal situation to study the chronic effects of Parkinson's and potential preventative and therapeutic effects (Sauer & Oertel, 1994).

In future studies, increasing the concentration of β -hydroxybutyrate might be an interesting and informative modification of the current study. Inducing elevated blood ketosis by feeding animals a ketogenic diet has been proven to be successful (Cheng et al., 2009) but might not be the most effective treatment route considering the inability to accurately control dosage or limit nutrition-based health effects associated with a food based β -hydroxybutyrate application. Previous studies have used subcutaneous β -hydroxybutyrate infusion with a miniosmotic pump and reported partial protection of dopaminergic cells against MPTP-induced lesions (Tieu et al., 2003). Studying the effect of this method in the 6-OHDA lesion model of Parkinson's disease might be particularly informative considering ability to control dosage over long term cellular degenerative processes produced in the model.

In this study, animals were tested 14 days after receiving 6-OHDA lesions. Previous studies have found that 6-OHDA lesions continue to cause cell death in the nigrostriatal pathway for as many as eight weeks (Sauer & Oertel, 1994). Extending the time of behavioral testing will shed light on the effect of this diet in the long-term. A study with this intent could explore

the long-term benefits of either the ketogenic diet or β -hydroxybutyrate as a treatment for Parkinson's disease.

Although the 6-hydroxydopamine lesion has been used for several years to create rodent models of Parkinson's disease (Sauer & Oertel, 1994), modification of the lesion procedure is a potential avenue for improvement in future studies. The human condition of Parkinson's disease is characterized by a chronic cellular degeneration of the substantia nigra (Blum et al., 2001), therefore, studying minimally effective doses of 6-OHDA that better represent the early stages of this chronic degeneration may provide a more accurate representation of the disease. In this study, histological analysis of lesion effects showed that in most cases, lesions completely destroyed portions of the substantia nigra *pars compacta* or greatly reduced cell density (Figures 6 – 9), which mimics an advanced Parkinsonian state in which clinical manifestations of the disease are apparent (Hamani and Lozano, 2003). Future investigations of neuroprotective effects might benefit from less complete lesions of the substantia nigra. This line of investigation would benefit from a comparison of behavioral and cellular effects across a variety of doses.

In considering future studies regarding the potential effectiveness of β -hydroxybutyrate as a neuroprotective, an essential concern must be the method used to apply the chemical into the brain. The lack of clear behavioral improvement or cellular benefit from the ketogenic diet must be considered given the ketogenic diet's negative systemic effect on weight seen in this study compared to its potentially beneficial neuroprotective effects.

4.7 Concluding Remarks

The potential effectiveness of the ketone body β -hydroxybutyrate as a neuroprotective in Parkinson's disease remains to be a concern in neurodegenerative disease research. The

ketogenic diet has offered protection against epileptic activity for many decades and despite the lack of statistical significance in this study, might offer the same significant effect in Parkinson's disease. This study failed to provide evidence for the hypothesis that the ketogenic diet would protect animals against a unilateral 6-hydroxydopamine lesion. It can be concluded, then, that the ketogenic diet might not be as appropriate for clinical application as it is for epilepsy. This does not mean that the ketone body β -hydroxybutyrate, which has been repeatedly reported to have neuroprotective effects, does not have neuroprotective effects that are worth studying in the 6-hydroxydopamine lesion rat model of Parkinson's disease. Use of this chemical in previous studies using the MPTP model of Parkinson's disease has been met with some success (Tieu et al., 2003). Due to the advantage of the 6-hydroxydopamine lesion in mimicking the long-term cellular degeneration characteristic of the human condition of Parkinson's disease, it makes sense that β -hydroxybutyrate continue to be investigated in this model. Although evidence in this study suggests that the ketogenic diet might not be powerful enough to influence cell survival in a 6-OHDA lesion, a more deliberate investigation of the cellular and behavioral effects of β -hydroxybutyrate is essential to a more comprehensive understanding of the molecule's potential therapeutic effects in Parkinson's disease.

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APPENDIX

Table A. Nissl Staining Protocol

Bath	Time (Minutes)
50% ETOH	10
75% ETOH	10
Chloroform Solution ¹	10
95% ETOH	2
100% ETOH	2
Xylene	5
95% ETOH	2
95% ETOH	2
Distilled Water	2
Buffered Thionin Stain Solution ²	3
Distilled Water (3 changes)	1
Acetic-formalin ³	2-3
Distilled Water (3 changes)	1
50% ETOH	2
100% ETOH	2
95% ETOH	2
Xylene (2 changes)	2

¹ 480ml chloroform in 60ml ether and 60ml 95% ETOH

² 25 ml of Stock Dye (1g thionin Cl 52000 in 100ml boiling distilled water) in 475ml buffer (7g sodium acetate in 2ml glacial acetic acid and 1000ml distilled water)

³ 4ml 40% formaldehyde in 0.2ml glacial acetic acid and 100ml distilled water.

B. Fluoro-Jade C Protocol

Bath	Time (Minutes)
Basic Alcohol Solution ¹	5
70% ETOH	2
Distilled Water	2
0.06% Potassium Permanganate	10
0.0001% Fluoro-Jade C Solution ²	10
Distilled water (3 changes)	1

¹ 1.0% sodium hydroxide in 80% ETOH

² 1:100 ratio of .01% Fluoro-Jade C Stock to 0.1% acetic acid vehicle.