Research Report

Ketogenic diet protects dopaminergic neurons against 6-OHDA neurotoxicity via up-regulating glutathione in a rat model of Parkinson’s disease

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ABSTRACT

The high-fat ketogenic diet (KD) leads to an increase of blood ketone bodies (KB) level and has been used to treat refractory childhood seizures for over 80 years. Recent reports show that KD, KB and their components (\(\alpha\)-beta-hydroxybutyrate, acetoacetate and acetone) have neuroprotective for acute and chronic neurological disorders. In our present work, we examined whether KD protected dopaminergic neurons of substantia nigra (SN) against 6-hydroxydopamine (6-OHDA) neurotoxicity in a rat model of Parkinson’s disease (PD) using Nissl staining and tyrosine hydroxylase (TH) immunohistochemistry. At the same time we measured dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum. To elucidate the mechanism, we also measured the level of glutathione (GSH) of striatum. Our data showed that Nissl and TH-positive neurons increased in rats fed with KD compared to rats with normal diet (ND) after intrastriatal 6-OHDA injection, so did DA and its metabolite DOPAC. While HVA had not changed significantly. The change of GSH was significantly similar to DA. We concluded that KD had neuroprotective against 6-OHDA neurotoxicity and in this period GSH played an important role.

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1. Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by motor symptoms including tremor, muscle rigidity, paucity of voluntary movements, and postural instability (Qu et al., 2007). It is characterized by a progressive and selective degeneration of dopaminergic neurons of the substantia nigra (SN) and the exact cause and underlying mechanism responsible for the progressive neurodegenerative condition of sporadic PD remains unknown (Chen et al., 2007). Reduced glutathione (GSH) is a cellular reductant, which protects against oxidative stress (Leret et al., 2002). Post-mortem research shows 40% decrease of GSH in SN of PD patients (Sian et al., 1994). Thus, it has been suggested that low levels of nigrostriatal GSH contents and consequent oxidative stress might contribute to the degeneration of dopaminergic neurons in idiopathic PD (Pinnen et al., 2007). Although GSH is not the only antioxidant molecule reported...
to be altered in PD, the magnitude of GSH depletion appears to parallel the severity of the disease and is the earliest known indicator of nigral degeneration (Chinta and Andersen, 2006).

In a clinical trial, patients with PD remained on a ketogenic diet (KD) for 28 days in an open trial conducted at the Movement Disorders Clinic of the Beth Israel Medical Center and Unified Parkinson’s Disease Rating Scale (UPDRS) scores were determined. The results showed that the changes in the UPDRS scores that occurred in PD patients during the KD. The mean total decrease in UPDRS scores was 43.4%. Among symptoms that improved were resting tremor, freezing, balance, gait, mood, and energy level (Vanitallie et al., 2005). D-beta-hydroxybutyrate, the reduced form of the ketones, confers protection against the structural and functional deleterious effects of the parkinsonian toxin MPTP; these include degeneration of SN dopaminergic neurons and striatal dopaminergic fibers, loss of striatal dopamine, and PD-like motor deficit (Tieu et al., 2003). In mesencephalic neuronal culture, addition of 4 mM of sodium D-beta-hydroxybutyrate significantly protected mesencephalic neurons from MPP+ toxicity and increased the rate of survival (Koustova et al., 2003). Pretreatment of cells with 8 mM D-beta-hydroxybutyrate provided significant protection to SH-SY5Y cells against toxicity induced by rotenone, a PD model in vitro (Imamura et al., 2006).

In this study, we investigated the effects of KD on the rat model of PD induced by 6-hydroxydopamine (6-OHDA). Dopaminergic neurons in the SN and density of fibers in the striatum were observed using Nissl and tyrosine hydroxylase (TH) immunohistochemistry. HPLC was used for measuring dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). We also detected the level of GSH. Our data showed that KD protected dopaminergic neurons of SN against 6-OHDA neurotoxicity via up-regulating GSH in a rat model of PD.

![Fig. 1](image_url)

Fig. 1 – Nissl staining of SN (A) ND; (B) KD; (C) ND + 6-OHDA; (D) KD + 6-OHDA; (E) Data of counting cells. Data were representative as mean ± SEM. (n=5) *p<0.01 compared to control rats (ND without 6-OHDA injection); †p<0.05 compared to ND with 6-OHDA injection; ††p>>0.05 compared to control rats (ND without 6-OHDA injection).
2. **Results**

2.1. **KD protected dopaminergic neurons in the SN and their neurites in the striatum against 6-OHDA neurotoxicity**

We examined the effects of intrastriatal 6-OHDA injection on SN dopaminergic neurons in ND or KD rats for 2 weeks using Nissl staining and TH immunohistochemistry (Figs. 1 and 2). After 2 weeks of 20 μg intrastriatal 6-OHDA injection, Nissl-positive and TH-positive neurons of SN were significantly decreased in rats fed with ND compared to the ND without 6-OHDA injection (control) \((p<0.01)\). While Nissl-positive and TH-positive neurons of SN in rats fed with KD increased more significantly than in rats fed with ND after 2 weeks of 6-OHDA injection \((p<0.05)\). There were no differences between ND and KD rats without intrastriatal 6-OHDA injection \((p>0.05)\). The density of TH-positive fibers had the similar changes in the striata (Fig. 3).

2.2. **KD inhibited the decrease of striatal DA and its metabolite DOPAC but not HVA induced by 6-OHDA**

We used HPLC analysis to measure the levels of striatal dopamine and its metabolites DOPAC and HVA. After 2 weeks of 20 μg 6-OHDA intrastriatal injection, the levels of striatal DA and its metabolites DOPAC and HVA were significantly decreased in rats fed with ND compared to the ND rats without 6-OHDA injection (control) \((p<0.01)\). While the levels of striatal DA and its metabolite DOPAC in rats fed with KD increased more significantly than in rats fed with ND after 2 weeks of 6-OHDA injection \((p<0.05)\). The metabolite HVA of DA in rats fed with KD did not increase more significantly than in rats fed with ND after 2 weeks of 6-OHDA injection \((p>0.05)\). There were no differences of DA and its metabolites DOPAC and HVA between ND and KD rats without 6-OHDA injection \((p>0.05)\) (Fig. 4).

![Fig. 2 – TH immunohistochemistry of SN (A) ND; (B) KD; (C) ND + 6-OHDA; (D) KD + 6-OHDA; (E) Data of counting cells. Data were representative as mean ± SEM. \((n = 5)\) #\(p<0.01\) compared to control rats (ND without 6-OHDA injection); \(*p<0.05\) compared to ND with 6-OHDA injection; \(*p>0.05\) compared to control rats (ND without 6-OHDA injection).](image-url)
2.3. KD inhibited the decrease of SN and striatal GSH induced by 6-OHDA

After 2 weeks of 20 μg intrastrial 6-OHDA injection, the levels of GSH in the SN and striatum were significantly decreased in rats fed with ND compared to the ND without 6-OHDA injection (control) (p < 0.01). While the levels of SN and striatal GSH in rats fed with KD increased more significantly than in rats fed with ND after 2 weeks of 6-OHDA injection (p < 0.05). There were no difference of GSH levels in the SN and striatum

<table>
<thead>
<tr>
<th></th>
<th>DA (ng/g wet tissue)</th>
<th>DOPAC (ng/g wet tissue)</th>
<th>HVA (ng/g wet tissue)</th>
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</thead>
<tbody>
<tr>
<td>ND</td>
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<td>1016.04±68.38</td>
<td>1088.88±81.88</td>
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<tr>
<td>KD</td>
<td>6239.3±256.65*</td>
<td>1028.68±61.92*</td>
<td>1040.32±72.30*</td>
</tr>
<tr>
<td>ND+6-OHDA</td>
<td>2948.32±263.56*</td>
<td>339.88±47.45*</td>
<td>332.38±36.91*</td>
</tr>
<tr>
<td>KD+6-OHDA</td>
<td>4162.1±426.31*</td>
<td>465.68±45.78*</td>
<td>390.56±582.69*</td>
</tr>
</tbody>
</table>

Fig. 3 – TH immunohistochemistry of striatum (A) ND; (B) KD; (C) ND + 6-OHDA; (D) KD + 6-OHDA; (E) Data of striatal TH+ fibers density. Data were representative as mean±SEM. (n=5) *p<0.01 compared to control rats (ND without 6-OHDA injection); 'p<0.05 compared to ND with 6-OHDA injection; #p>0.05 compared to control rats (ND without 6-OHDA injection).

Fig. 4 – The levels of striatal DA and its metabolites DOPAC and HVA. Data were representative as mean±SEM. (n=5) *p<0.01 compared to control rats (ND without 6-OHDA injection); 'p<0.05 compared to ND with 6-OHDA injection; **p>0.05 compared to control rats (ND without 6-OHDA injection); ^p>0.05 compared to ND with 6-OHDA injection.
between ND and KD rats without intrastriatal 6-OHDA injection \((p > 0.05)\) (Fig. 5).

### 3. Discussion

In this work, we demonstrated that KD protected dopaminergic neurons of SN against degeneration induced by intrastriatal injection of 6-OHDA. And the decrease of DA and its metabolite DOPAC induced by 6-OHDA in the striatum were reversed under condition of KD.

Oxidative stress is the loss of the balance between reactive oxidative species (ROS) and the antioxidants, and it has been implicated in the mechanism of PD (Zhou et al., 2008). Post-mortem shows that oxidative stress appear in the SN of the brain in PD patients and it also is observed in animal models of PD (Greenamyre and Hastings, 2004; Maguire-Zeiss et al., 2005). Sullivan et al. show that KD can decrease ROS level and exert neuroprotection (Sullivan et al., 2004). GSH system plays a major role in controlling cellular redox states and is the primary defense mechanism for peroxide removal from the brain (Jida et al., 1999). The intracellular GSH level is controlled by a balance between the rate of production or salvage and the rate of consumption or loss (Tanaka et al., 2002). GSH is capable of reacting both in vitro and in vivo with 6-OHDA through a nucleophilic attack on the 6-OHDA quinone to form 2-S-(glutathionyl)-6-OHDA, and sulfhydryl antioxidants such as GSH and NAC produce a significant reduction in the autoxidation of 6-OHDA (Shimizu et al., 2002). On the other hand, 6-OHDA neurotoxicity has also been shown to involve mitochondria (Kulich et al., 2007). KD can increase GSH biosynthesis, enhances mitochondrial antioxidant status, and protects mtDNA from oxidant-induced damage (Jarrett et al., 2008). In our present study, 6-OHDA induced GSH level to decrease in rats with ND but the turnover appeared in rats with KD. So GSH played an important role in the therapeutics of KD.

Since KD, \(\beta\)-hydroxybutyrate, acetoacetate and acetone have the effect of neuroprotection, this becomes a new potent therapeutics for PD. KTX 0101 is the sodium salt of the physiological KB (\(\beta\)-hydroxybutyrate) and has been used trial and proved neuroprotective, while some side effects come out and the underlying mechanisms still are not clear (Smith et al., 2005). So further studies need to elucidate the mechanisms and eliminate the side effects.

### 4. Experimental procedures

#### 4.1. Animals

Male Wistar rats (weighing 250–300 g, Medical Animal Center of Shandong University, Jinan, Shandong Province, China) were used in this work and housed under control of temperature (23 ± 2 °C) and light (12h/12 h cycles of day and night). All experiments were carried out under the guide of the Lab Animal Rules of Shandong University.

#### 4.2. Diet regime

Rats were fed with normal diet (ND) and KD as described by Hae Sook Noh et al. (2006). The ND is composed of fat (5%), protein (20%), carbohydrate (66%), and inert matter (9%). The KD is composed of fat (90.7%), protein (9%), and carbohydrate (0.3%). The goal of a KD is to produce and maintain ketonemia.
4.3. 6-OHDA lesion

After 2 weeks fed with ND or KD, rats were anesthetized by sodium pentobarbital (Pengyuan Co. China) and injected unilaterally stereotaxically with 20 μg 6-OHDA hydrobromide (Sigma) (dissolved in 4 μL saline with 0.02% ascorbic acid) or the corresponding volume of saline (containing 0.02% ascorbic acid) into the left striatum as described by Carmen Henze et al. (2005). Coordinates (tooth bar: ±0.0 mm; anterior/posterior: +1.0 mm; medial/lateral: +3.0 mm; ventral/dorsal: −4.5 mm) were determined from bregma, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982). After surgery, rats were maintained to be fed by KD or ND for 2 weeks.

4.4. Tissue preparation

2 weeks after 6-OHDA injection, some rats of every group were anesthetized and perfused with 4% paraformaldehyde. Then the brains were taken out and kept in 4% paraformaldehyde. After 2 days they were transferred into phosphate-buffered saline (PBS) containing 30% sucrose at 4 °C. The tissue was sunk for sectioning in order to Nissl staining in the SN and TH immunohistochemistry in the striatum and SN. The left striata of some brains were dissected out and kept at −80 °C for measuring DA, DOPAC and HVA using HPLC and the levels of GSH.

4.5. Nissl staining

One section was selected every six sections in the SN area of each brain and eight sections were selected every brain. All the sections from each brain were matched as closely as possible for Nissl staining. The sections were mounted on the slides, dried and kept in xylene overnight. The slides were then dehydrated in alcohol and washed with dH₂O. Following the slides were stained with Cresyl Violet (0.5% cresyl violet acetate). The slides were washed with dH₂O and dehydrated in alcohol again. In the end, the slides were coverslipped. Using microscope the dopaminergic neurons of SN were counted all the eight sections manually of each brain. The data were expressed as percent of control rats (ND without 6-OHDA injection).

4.6. TH immunohistochemistry

The sections of SN were picked up as described as Nissl staining. The same method was used for picking up sections of striatum. The sections were collected and washed with phosphate buffer (PBS). Following treatment with 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase, the sections were treated by 0.04% normal goat serum with 0.4% Triton X-100 in PBS including 0.2 g bovine serum albumin (BSA) for 1 h at 37 °C, followed by incubation with TH primary antibody (Dingguo Co. China) overnight at 4 °C. Slides were then washed three times with PBS and incubated with biotinylated secondary antibody (Boshide Co. China) 37 °C for 1 h. After washing three times with PBS, slides were incubated with ABC (Boshide Co. China) kit for 1 h. Slides were developed in diaminobenzidine (DAB) (Pengyuan Co. China) for 2–20 min. Using microscope the dopaminergic neurons of SN were counted all the six sections manually of each brain. The data were expressed as percent of control rats (ND without 6-OHDA injection). For TH-immunoreactivity fibers in the striatum, the optical density was measured using a computerized image analysis system (Olympus) and the corpus callosum were measured as nonspecific background densities. The optical density values of the striatum in the lesioned sides were expressed as a percentage loss of the same side of control rats (ND without 6-OHDA injection).

4.7. HPLC analysis

Tissue levels of DA and its primary metabolites (DOPAC and HVA) were measured using HPLC. Data were expressed as ng/g wet weight of tissue.

4.8. Measurement of GSH

The SN and striata were homogenized and homogenate was precipitated with 0.3 mol sodium phosphate buffer (pH 8.0) and 5 mL of 0.04% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Then it was incubated for 5 min at room temperature. The absorbance value of the sample was read against the blank at 412 nm. GSH concentration was calculated from the standard curve. The data were expressed as percent of control (ND without 6-OHDA injection).

4.9. Statistics analysis

Data were shown as means±SE. Comparisons between two groups (n=5) were analyzed using t-test. Differences were considered significant when p≤0.05.

REFERENCES


