

# Effect of Dietary Restriction on 6-Hydroxydopamine-induced Damage of Dopaminergic Terminals in Mice

Shin-ichi Iwata, MD

### Abstract

There are several reports that dietary restriction (DR) can prevent or lessen the severity of cancer, stroke, coronary heart disease, autoimmune disease, allergy, Parkinson’s disease and Alzheimer’s disease. Therefore, the protective effect of DR on toxin-induced impairment of dopaminergic terminals was examined in mice. Adult 12-week-old ddY mice were bred under DR to maintain a stable weight over a 12-week period. Meanwhile, control mice were allowed food *ad libitum* (AL). Then, dopaminergic terminals were impaired by bilateral intraventricular administration of 6-hydroxydopamine (6-OHDA) at three different doses (5, 10, 20 µg). The content of dopaminergic terminals in the striatum, expressed as tyrosine hydroxylase (TH) level, was measured one week after the administration. There were no significant differences between DR and AL in TH levels, which suggested that DR does not assist in protecting dopaminergic neurons.

**Key words:** Dietary restriction, Dopaminergic neuron, 6-Hydroxydopamine, Parkinson’s disease

Animal studies have shown that DR can prevent or lessen the severity of cancer (Chen et al., 1990), stroke (Yu and Mattson, 1999), coronary heart disease (Ahmet et al., 2005), autoimmune disease (Kubo et al., 1992), allergy (Nakamura et al., 2004), and Parkinson’s disease and Alzheimer’s disease (Mattson et al., 2003). However, the effect of DR in Parkinson’s disease animal models on preservation of dopaminergic neurons (Duan and Mattson, 1999; Maswood et al., 2004) is controversial (Armentero et al., 2008). Therefore, we studied the effect of DR on dopaminergic terminals impaired with 6-hydroxydopamine (6-OHDA).

### METHODS

Male ddY mice (Kyudo Company, Tosu 841-0075, Japan) were purchased at 8 weeks old. The animals were housed with free access to standard food (Clea Japan Inc. Tokyo 153-8533, Japan) in an air-conditioned room with a temperature of 22-24°C and humidity of 60-70% and maintained under a constant 12-hr light-dark cycle (light on 7:00a.m.). After 4 weeks of acclimatization in our animal facility, mice were divided into 6 groups, 3 DR groups

and 3 groups fed *ad libitum* (AL). The average body weight of DR and AL in every groups was the same at the start of the experiment. The amount of food pellets provided to the DR group was controlled in order to maintain the same body weight as at the start of the experiment (i.e., at 12 weeks old). The food pellets were given once a week. We established a Parkinson’s model by administering 6-OHDA into the striatum after 12 weeks of DR (i.e. at 24 weeks old). These mice were sacrificed one weeks after 6-OHDA administration (Table 1).

Table 1. Experimental Protocol

weeks old	8	8 ~ 12	13 ~ 24	24	25
A	purchase	acclimatization	DR	6-OHDA 5ug	sacrificed
	purchase	acclimatization	AL	6-OHDA 5ug	sacrificed
B	purchase	acclimatization	DR	6-OHDA 10ug	sacrificed
	purchase	acclimatization	AL	6-OHDA 10ug	sacrificed
C	purchase	acclimatization	DR	6-OHDA 20ug	sacrificed
	purchase	acclimatization	AL	6-OHDA 20ug	sacrificed

To cause degeneration of dopaminergic terminals, three different doses (5, 10 or 20  $\mu$ g) of 6-OHDA (Sigma, St. Louis, MO) dissolved in 2  $\mu$ l 0.9% saline containing 0.01% ascorbic acid, i.e., the concentration of the solution was 2.5, 5.0 or 10  $\mu$ g/ $\mu$ l, was infused bilaterally into the lateral ventricles using a 1- $\mu$ l Hamilton micro-syringe manually at a rate of 1.0  $\mu$ l/min. 6-OHDA infusions were performed using a stereotaxic apparatus (Model 900; David Kopf Instruments, Pembroke Pines, FL), with the tip of the needle inserted vertically 5.0 mm ventral to the bregma through a small hole in the skull 0 mm rostral and 1.0 mm lateral to the bregma. The needle remained in place for 2 min following the infusion to minimize the spread of the drug through the injection track. Desipramine hydrochloride (Sigma) at 20 mg/kg was dissolved in 40% propylene glycol containing 10.5% ethanol and 0.34 mg/ml NaOH and intraperitoneally administered 20 min before the 6-OHDA treatment to preserve noradrenergic nerve terminals in the striatum. All surgeries were performed under 70 mg/kg pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL, USA) anesthesia.

The level of tyrosine hydroxylase (TH) protein was measured to determine the degree of impairment of dopaminergic terminals in the striatum. Mice were decapitated 7 days after the 6-OHDA injection, and the whole bilateral striatum was removed on an ice-cold plate. The brain was then sonicated in 10 volume of 1% SDS. Total protein (2.5 mg wet weight/lane) was separated on a 10% polyacrylamide gel. The proteins were transferred onto a PVDF membrane (Immobilon-P; Millipore Co., Billerica, MA) at 30 V for 16 h. The blots were blocked in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 1% bovine serum albumin (blocking buffer) and incubated with anti-tyrosine hydroxylase (TH) monoclonal antibody (MAB 318, 1:4000; Chemicon International, Inc., Temecula, CA) for 1 h. The blot was then incubated with anti-mouse IgG horseradish peroxidase (A5278, 1:40,000; Sigma) for 30 min. Immunoreactivity on the blot was visualized using an enhanced chemiluminescence (ECL) technique (ECL Plus; Amersham Co., Arlington Heights, IL). Image analysis was performed using Image-J (Abramoff et al., 2004).

The present experiment was carried out after

obtaining permission from the Committee of Animal Experimentation, Kagoshima Junshin University.

## RESULTS

Significant differences in body weight were observed between the DR and AL groups one week after the initiation of DR. The body weight of AL was maximum at 22 to 23 weeks old (Fig. 1). There were no significant differences between DR and AL in TH levels after 6-OHDA injection at all three doses (Table 1). TH levels were proportionally decreased according

Table 2 Effect of dietary restriction on the amount of dopaminergic terminals impaired by 6-OHDA

Dose of 6-OHDA		n	TH
Total dose/mouse			mean $\pm$ S.E.
A : 5 $\mu$ g	DR	7	44 $\pm$ 7
	AL	7	45 $\pm$ 6
B : 10 $\mu$ g	DR	6	37 $\pm$ 1
	AL	7	36 $\pm$ 1
C : 20 $\mu$ g	DR	5	31 $\pm$ 4
	AL	7	31 $\pm$ 1

Content of TH is expressed as densitometry value. Number of mice used was expressed as n.

to the amount of 6-OHDA administered.

## DISCUSSION

DR did not prevent the destructive effects of 6-OHDA on dopaminergic terminals even at the smallest dose of 6-OHDA (5  $\mu$ g).

Duan and Mattson (1999) reported that dopaminergic neurons were more resistant against systemic administration with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) after 3 months alternate-day food restriction in mice. The mice were sacrificed one week after the MPTP administration. This experimental protocol demonstrated that mice under DR were resistant to MPTP when there is a short time period between MPTP administration and sacrifice. The same group (2004) reported that a 30% restriction in calories attenuated dopaminergic impairment by MPTP in rhesus monkeys. These animals were bred for 6 months under DR. MPTP was then injected and the animals were bred under DR for approximately 4 more months. Dopamine levels in the DR group were two-fold greater than in AL animals. Notably, the degree of destruction was

measured 4 months after MPTP injection. Therefore, it is possible that residual dopaminergic neurons restored their nerve terminals during the last 4 months. Thus, DR could assist in regenerating nerve terminals. Armentero et al. (2008) studied the effect of DR (i.e., access to food on alternate days) for 2 or 8 weeks before an 6-OHDA injection and 4 weeks after the injection on the survival rate of dopaminergic neurons in the pars compacta of the substantia nigra and dopaminergic terminals in the striatum after a unilateral striatal injection with 20  $\mu$ g of 6-OHDA in rats. No differences were observed in the survival rate of dopaminergic neurons between DR and AL. There were a couple of experimental differences between the 2 groups, i.e., the neurotoxin used (MPTP or 6-OHDA) and the duration of DR (3 months or 2 months). We used 6-OHDA and the duration of DR was 3 months. Therefore, this may suggest that DR is effective against MPTP-induced impairment but not against 6-OHDA-induced impairment.

There are several DR regimes employed in experimental models (Kouda and Iki, 2010). Both Duan and Mattson (1999), and Armentero et al. (2008) adopted alternate-day food restriction in all

experiments. Animals devour food pellets after one-day starvation. Therefore, intermittent fasting is a more suitable name than DR for these experimental protocols. In humans, however, the amount of food is reduced at every meal during dieting. Therefore, the experimental protocol per se is not similar to the human diet.

Duan and Mattson (1999), and Armentero et al. (2008) considered DR to be mildly stressful (hermetic effect) and not a healthy eating habit. Hormesis is the term for generally favorable biological responses to low exposures to toxins and other stressors. The biochemical mechanisms of hormesis are thought to involve the activation of the repair mechanisms of the body by low-dose toxins or other stressors (Stranahan and Mattson, 2012). Very mild experimental manipulation such as DR is not thought to block the effect of neurotoxins.

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FIGURE LEGENDS

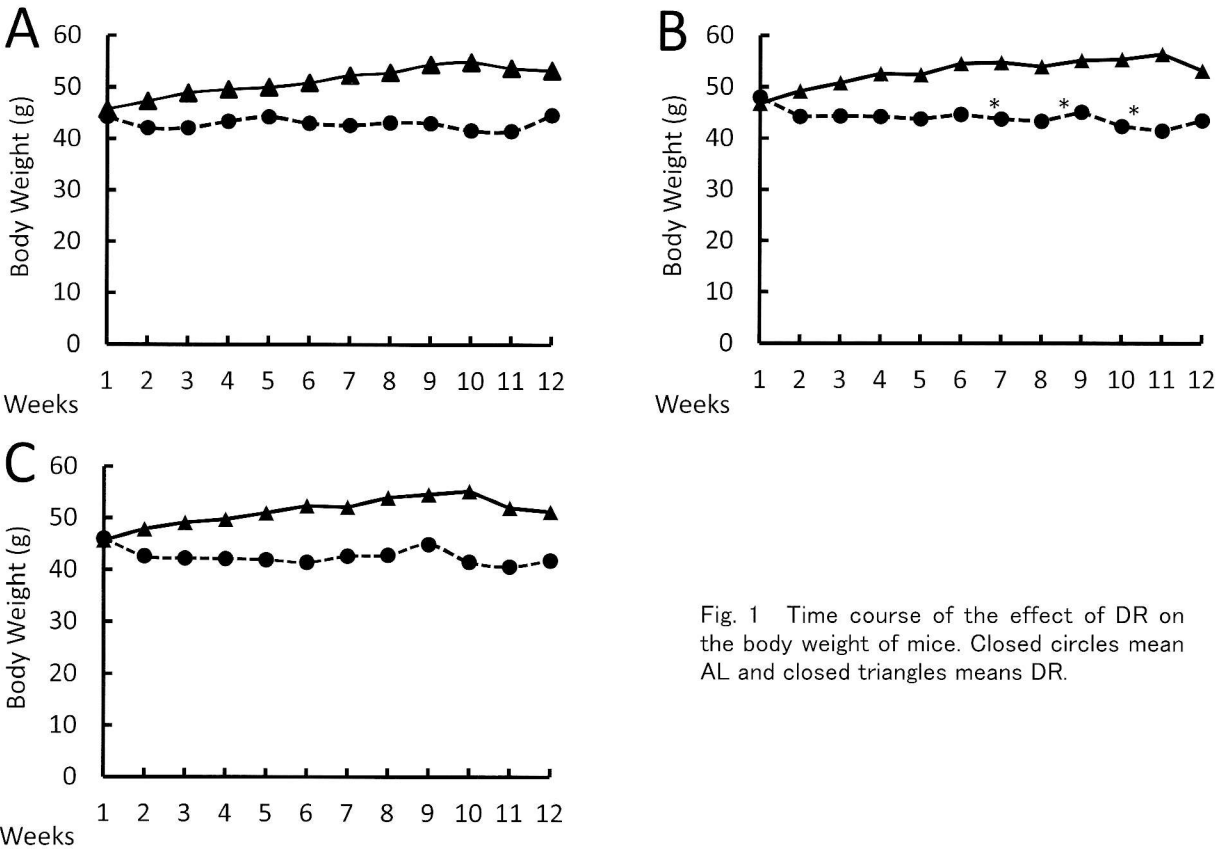


Fig. 1 Time course of the effect of DR on the body weight of mice. Closed circles mean AL and closed triangles means DR.

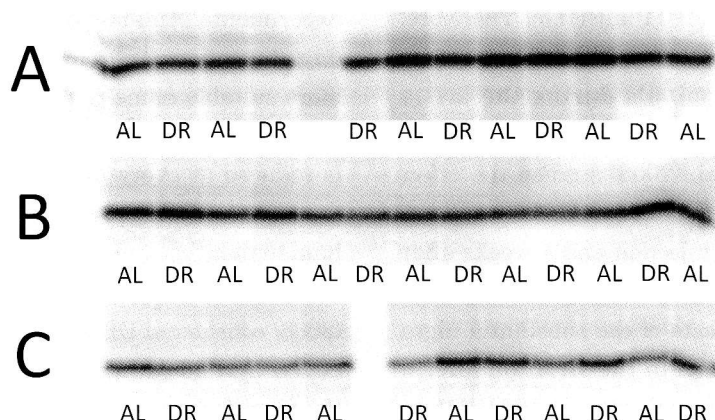


Fig.2 An immunoblot of TH treated with 6-OHDA either in DR or AL.

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## 食事制限をするとパーキンソン病になりにくくなるのか(マウスによる実験)

岩田 真一

鹿児島純心女子大学看護栄養学部健康栄養学科

### 要 旨

巷では食事制限をすると長生きできるとのことが流布されている。げっ歯類を用いた実験では自由摂取群に比べて食餌制限をおこなった群のほうが長生きなのは認められている。しかし、げっ歯類は狭い飼育ケージに入れられて運動も十分にできず、自由に餌が食べられる状態であり、すべてのげっ歯類は肥満になっている。つまり、食事制限群は適正量食餌群である可能性が高いので、食餌制限群は長生きであるとの結論が招きだされたと思われる。ヒトでは食事制限ではなく、肥満度により間接的に過食の影響をみており、肥満があれば、肥満関連の疾患が多いのは当然である。ヒトの徒な食事制限は必要栄養素の摂取を減少させ、筋肉量や骨量を低下させ、結果的に不健康に陥らせる。

本研究における食餌制限とは健康的な量の餌を動物に与えるという意味ではない。繰り返される絶食ストレスにより生体の抵抗性が高まり疾患になりにくくなるという理論を土台として行われている (hormesis またはホメオパシー理論)。食餌制限とパーキンソン病の動物実験は2つのグループから報告されている。効果があるとの報告は hormesis の唱導者 Mattson のグループから出させたもので、残りの一つは否定的な報告である。そこでどちらが正しいか、行ってみたのが研究の動機である。本研究では食餌制限は何の影響も与えなかった。6-hydroxydopamine という神経毒を脳内に入れるという激しい傷害方法であるので、餌の制限などということでのこの神経毒の効果が弱くなるなどと考えるほうが不自然である。つまり、Mattson の結果は疑わしい。

日常生活の適度のストレスや適度の運動は健康増進を起こすが、絶食ストレスは良いストレスとは言えないのではないと思われる。