Research report

High intake of folic acid or complex of B vitamins provides anti-Parkinsonism effect: No role for serum level of homocysteine

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HIGHLIGHTS

High intake of folic acid or B complex ameliorates 6-OHDA-induced Parkinsonism.
Neuroprotective effect of folic acid supplement is dose dependent.
Neuroprotective effect of B vitamins is not mediated by lowering of homocysteine.
High intake of B12 or B6 or folate+B12+B6 has no anti-Parkinsonism effect.
High intake of folic acid increases plasma homocysteine in 6-OHDA treated rats.

ABSTRACT

Several lines of evidence show that homocysteine (Hcy) levels are increased in blood and CSF of patients with Parkinson’s disease. B vitamins are necessary for Hcy metabolism and their deficiencies cause hyperhomocysteinemia and neurodegeneration. In present study, effect of B vitamin supplementation on the severity of 6-hydroxydopamine (6-OHDA)-induced Parkinsonism was investigated. Rats were nourished with B vitamin supplements from 1 month before of stereotaxic injection of 6-OHDA to the end of experiments. Total serum Hcy was measured at the end of experiments to identify its association with Parkinsonism. Both rotational and rotarod tests revealed that supplementation of folic acid, in a dose dependent manner, attenuates severity of Parkinsonism. Supplement of B complex also had beneficial effect and improved motor performance in rotarod test and decreased biased swings in elevated body swing test but had no effect on the apomorphine-induced rotational behavior. Supplement of B6 attenuated rotational behavior but had no effect on the rotarod performance and swinging behavior. Supplement of B12, or combination of folic acid with B6 and B12, had no effect on the behavioral symptoms of Parkinsonism. Except one group, the levels of Hcy in other vitamin B treated groups were near to that in control group. Surprisingly, Hcy in group of rats that received high intake of folic acid was significantly higher than that in control group. Our results indicate that high intake of folic acid or B complex provides anti-Parkinsonism effect but it is not mediated by lowering plasma Hcy.

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting about 1–3% of the population over 50 years of age and characterized by relatively selective nigrostriatal dopaminergic degeneration. There is no consensus as to mechanism(s) contributing to dopaminergic cell loss but growing evidence suggests that oxidative stress and mitochondrial dysfunction play important role. Although both genetic and environmental factors influence in the pathogenesis of PD [1–4] however, specific gene defects have been linked to a very small percentage of the cases and increasing evidence shows that environmental factors such as exposure to toxins [5], and low antioxidant intake [6] are important risk factors for the common sporadic forms of PD.

In the last decades, homocysteine (Hcy) has received special attention because of its association with pathogenesis of atherosclerosis and various cerebrovascular and cardiovascular diseases [7–9]. Also, elevated plasma Hcy level is a risk factor for cognitive decline and dementia in the general population and has been associated with mild cognitive impairment, Alzheimer's disease (AD), vascular dementia and depression [10–14]. In vitro experiments have shown that exposure of cultured cortical and hippocampal neurons to Hcy increases their vulnerability to excitotoxicity [15,16], which describes the possible mechanism by which Hcy promotes cognitive decline and AD.

Several lines of evidence show that Hcy levels are increased in blood and CSF of patients with PD [7,17–19]. High levels of Hcy might accelerate dopaminergic cell death through oxidative stress and excitotoxicity [20–22]. Duan et al. [20] showed that Hcy exacerbates oxidative stress, mitochondrial dysfunction and apoptosis in human dopaminergic cells exposed to the pesticide rotenone and pro-oxidant Fe2+–. Furthermore, several animal studies have demonstrated that focal infusion of Hcy into either substantia nigra (SN) or striatum exacerbates symptoms of 6-OHDA and MPTP-induced Parkinsonism [20,21].

Hcy is a metabolite of methionine, an amino acid critical for the generation of methyl groups required for synthesis of DNA. Hcy levels are normally maintained low either through remethylation to methionine by enzymes that require folate or cobalamin (vitamin B12) or through catabolism to cysteine by the pyridoxine (vitamin B6)-dependent enzyme of cystathionine b-synthase. Several epidemiological studies have shown that deficiencies of folate, vitamin B12 and vitamin B6 are the major causes of hyperhomocysteinemia in the general population [24–27]. Folic acid, folate plays an important role and it has been reported that there is generally an inverse correlation between plasma folate and Hcy levels [28]. In addition, B vitamins are the precursors of mitochondrial enzyme cofactors and it has been proposed that high levels of cofactors and substrates of mitochondrial enzymes may reverse some of the aspects of aging through binding to enzymes and protection of the enzyme activity sites from oxidant attack. B vitamins are nutrients without toxicity even at doses several folds of dietary reference intakes and can be administrated for a long time in human [29].

In present study, we hypothesized that Hcy sensitizes SN dopaminergic neurons to neurodegenerative stress and lowering of plasma Hcy may prevent or attenuate development of PD. We also supposed that high intake of B vitamins involving in the metabolism of Hcy (i.e. folate, B6, and B12) can reduce plasma Hcy and increase surveillance of dopaminergic neurons against neurotoxic agents. To test our hypothesis, we examined effect of supplementation of folate, B6, B12 and combination of them on the development and severity of 6-OHDA-induced Parkinsonism. Regarding to inverse association between dietary folate intake and plasma level of Hcy, special attention was occurred on this vitamin and three different doses of folate supplement were used. In addition, we investi- gated effect of high intake of B complex because it has been recently reported that high dose of B vitamins has synergistic anti-Parkinsonism activity [30]. Serum Hcy was measured at the end of experiments to identify possible association between the Hcy level and severity of Parkinsonism in rat.

2. Materials and methods

2.1. Animals and experimental groups

Adult male Wistar rats (Razi Institute, Karaj, Iran), weighing 190–220 g at the beginning of study were housed in large cages (38 cm × 59 cm × 20 cm) at a temperature-controlled colony room under light/dark cycle with free access to tap water and standard food. All procedures carried out throughout this study were according to the guidelines of animal experiments of Research Council at Qazvin University of Medical Sciences.

Animals were divided into eight experimental groups as follows: control which received B vitamins equal to in normal MEM (minimum essential medium); complex which received combination of all of B vitamins (Table 1); 5-folds of that in normal MEM; FA 2X, FA 5X and FA 10X which received folic acid 2, 5 and 10-folds of that in normal MEM, respectively; FA + B6 + B12 which received combination of folic acid, vitamin B6 and vitamin B12; 5-folds of that in normal MEM; B6 and B12 which received vitamin B6 and B12, respectively 5-folds of that in normal MEM, n was 12 for each group. In addition, the data of another group of rats (n = 8) marked as healthy rats, was also used in this study. This extra group was consisted of intact rats subjected to no intervention and did not received 6-OHDA. All of B vitamins were purchased from Sigma–Aldrich Company.

Considering normal dietary regime contains B vitamins equal to normal MEM additional B vitamins to provide required supplements were added to drinking water. Table 1 displays the normal MEM of B vitamins, drinking water, and food and also amounts of added B vitamins to drinking water for preparation of the supplements. Drinking water was replaced in each two or three days to avoid possible disturbances due to evaporation or chemical destruction of B vitamins.

2.2. Experimental design

All animals (except healthy group) were subjected to stereotaxic surgery and received 6-OHDA into their striatum. Feeding with B vitamin supplements was begun one month before the surgery and continued to the end of experiments (Fig. 1). Apomorphine-induced rotational test and elevated body swing test (EBST) were performed at three time-points: before the surgery, within the second, and fourth weeks post-surgery (Fig. 1). Rotard test was performed during the fifth week post-surgery. After rotard test blood was sampled from heart of the rats and serum Hcy was measured.

2.3. Surgical procedures

Rats were anesthetized with intraperitoneal injection of a solution containing ketamine (100 mg/kg) and xylazine (5 mg/kg). Then 4 μl of 6-OHDA was unilaterally injected into the right striatum using stereotaxic apparatus (Stoelting, USA) and through a 10-μl Hamilton syringe. Coordinates were AP: 0.2 and L: −3.3 measured from bregma and also V: −8 measured from the surface of skull according to the atlas of Paxinos and Watson [32]. 6-OHDA was dissolved in isotonic NaCl solution containing 0.2 mg/ml of ascorbic acid and was injected at a single dose of 4 μl (4 μg of 6-OHDA in 1 μl). At the end of injection, the needle was left in place for an additional 5 min and then withdrawn at a rate of 1 mm/min.

2.4. Behavioral testing

2.4.1. Apomorphine-induced rotational test

Administration of dopamine agonists such as apomorphine after unilateral degeneration of SN neurons overactivates striatum neurons in the lesion side which is revealed by rotations contralateral to lesion side. 6-OHDA treated rats were tested by apomorphine-induced rotational test according to method as described previously by Fujita et al. [33]. Briefly, Animals were initially given a 5-min habituation time followed by injection of apomorphine hydrochloride (0.5 mg/kg, i.p., dissolved in saline, Sigma). A minute later, the number of full rotations was counted at 10-min intervals for 1 h in a cylindrical container (at a diameter and height of 28 and 36 cm, respectively). Contralateral and ipsilateral rotations (far away and toward the lesion side, respectively) were counted as positive and negative scores and the net number of rotations defined as the positive scores minus the negative ones.

2.4.2. Elevated body swing test

6-OHDA treated rats show swinging behavior biased ipsilateral or contralateral to the lesion side. The more biased swings indicate the more intensive lesion in the SN dopaminergic neurons. The EBST was performed before the apomorphine-induced rotational test according to a slightly modified method as described before [34]. Briefly, the animal was placed in a cylindrical container, allowed to habituate for 10 min to attain a neutral position, defined as having all four paws on the floor. Subsequently, the animal was held at a position approximately 2 cm from the base...
of its tail. It was then lifted up 2 cm above the surface on which it was resting. The animal was held in the vertical axis, defined as no deviation of more than 10° to either side. A swing was recorded whenever the animal moved its head out of the vertical axis to either side. Before attempting another swing, the animal should have return to the vertical position for the next swing to be counted. Swings were counted for a period of 1 min. One observer was responsible for timing the test session, determining and recording the direction and the frequency of swings, while another observer held the rat. All tests were conducted to the groups. Biased swing behavior was calculated as follows: L/(L+R) (%) for left-biased swings and R/(R+L) (%) for right-biased swings (L = amount of left-biased swings, R = amount of right-biased swings).

### 2.4.3. Rotarod test

A rotating rod apparatus (M.T6800, Bonj Sanat, Iran) was used to assess the motor performance and measure the ability of rats to improve motor skill with training. Rotarod test was performed at three consecutive days and two sessions per day. Each session lasted a maximum of 200 s, during which the rotating rod underwent a linear acceleration from 5 to 40 rpm over the first 120 s of the trial and remained at maximum speed for the remaining 200 s. Animals were scored for their latency (in seconds) to fall (height 30 cm) for each trial. Rats were given a minimum rest of 30 min between trials to avoid fatigue. Rotarod data are expressed as the area under the curve (AUC), which was computed according to the following formula:

\[ AUC = \text{time on the rod}(s) \times \left[ \frac{\text{time on the rod}(s)}{1044} \right] \]

where 0.44 is the acceleration speed per second.

### 2.5. Blood sampling and Hcy measurement

Under deep anesthesia, blood was collected from the heart of rats in order of first from control group, then from complex, FA2X, FA5X, FA10X, FA + B6, FA + B12, and B12 groups. Then, bloods were allowed to clot and sera were separated using centrifugation at 5000 rpm for 5 min and stored at −80°C until use. Total serum Hcy (Hcyx) was measured using ELISA kit (Axis-Shield Co. UK). The principle of this assay is based on the reduction of protein-bound Hcy to free Hcy followed by enzymatic conversion of Hcy to S-adenosyl-L-homocysteine (SAH) in a separate procedure. SAH is finally determined by Enzyme Immuno Assay (EIA).

Six calibrators were used for preparing the calibration curve and calculation of unknown samples. All 3 control samples were within their ranges. The performance characteristics of the assay were: within run precision: 8%, limit of quantification: 1 μmol/L, and linearity: up to 50 μmol/L.

### 2.6. Statistical analysis

Data are expressed as the mean ± SD, in spite of the probable non-normality of the distribution of scores. Data of behavioral tests were initially analyzed by Kolmogorov–Smirnov test to find the normality of the data. Since these data lacked normal distribution, they were later subjected to Kruskal–Wallis nonparametric ANOVA followed by a two-tailed Mann–Whitney U test. Data of Hcy measurement was analyzed using One-Way ANOVA. A P value ≤ 0.05 was considered as significant, statistically.

### 3. Results

#### 3.1. Apomorphine-induced rotational test

Significant apomorphine-induced contralateral (to the left side) rotations were observed in all groups indicating vitamin B supplements could not prevent development of 6-OHDA-induced Parkinsonism. However, enrichment of drinking water with some of B vitamins affected significantly rotational behavior (Fig. 2). The most significant effect was observed in rats belonging to FA 10X group. In this group, number of rotations in the both first and second post-surgery tests was more than 60% lower than in control group. Also, all FA 10X treated rats showed less than 100 rotations/h, while in other groups number of rotations was between 110 and 746. Furthermore, in comparison to the first test, number of rotations was prominently increased in the second test in all experimental groups but this increment in the FA 10X group was remarkably less than that in other groups.

Animals treated with moderate dose of folic acid (FA 5X group) also showed remarkably less rotations (~30%) in compared to control group. However, this difference was only seen in the first test (Fig. 2). Also, rotational test revealed no marked difference in severity of Parkinsonism between control and FA 2X groups indicating effect of folic acid supplementations was dose dependent.

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**Table 1**

Displays minimum essential medium (MEM) of different B vitamins for maintenance in rats and also the amounts of added vitamins to drinking water for preparation of the required supplements. Amount of required food in the pellets form and required drinking water for maintenance was considered as 15 g/day and 12 ml/day, respectively. **Note:** 2-fold supplementation means that 1-fold was provided from the pellets and another 1-fold from adding of the required vitamin to drinking water.

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>MEM (mg/kg diet)</th>
<th>Requirements for 2-fold Supplementation (mg/1L)</th>
<th>Requirements for 5-fold Supplementation (mg/1L)</th>
<th>Requirements for 10-fold Supplementation (mg/1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin (d-biotin)</td>
<td>0.2</td>
<td>1.25</td>
<td>5</td>
<td>11.25</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1</td>
<td>125</td>
<td>25</td>
<td>1125</td>
</tr>
<tr>
<td>Niacin (nicotinic acid)</td>
<td>15</td>
<td>25</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>Pantothenate (Ca-pantothenate)</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>4</td>
<td>20</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>Thiamin (thiamin-HCl)</td>
<td>4</td>
<td>20</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>B6 (pyridoxine)</td>
<td>6</td>
<td>30</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>B12</td>
<td>0.05</td>
<td>0.25</td>
<td>0.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The information has been taken from Committee on Animal Nutrition of National Research Council [31].

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**Fig. 1.** Time schedule used for animal experiments: except of control and healthy groups, all of other animals received drinking water enriched with B vitamins from 1 month before of the stereotaxic injection of 6-OHDA to the end of experiments. Animals were tested by apomorphine-induced rotational test and elevated body swing test (EBST) at three times: before the stereotaxic surgery and in the second and fourth weeks after that. Rotational test were performed at least 1 h after termination of the EBST. Walking on the rotating rod was tested in the fifth week post-surgery. Blood sampling and homocysteine (Hcy) measurement were performed after rotarod test.
Rotational behavior of FA + B6 + B12–treated rats was similar to that in FA 5X group.

Another important data obtained from B6 treated rats. In this group, number of rotations in the first and second tests was respectively 36 and 57%, lower than that in control group. Also, similar to FA 10X treated rats, increment in number of rotations from first test to the second test was small.

3.2. Elevated body swing test

Fig. 3 displays swinging behavior of different experimental groups in the EBST. Rats belonging to control group showed swinging behavior mainly (about 75% of all swings) toward the lesion side. Bias in the swings in second post-surgery test was slightly more than that in the first post-surgery test. Similar results were observed in FA 2X, 5X, 10X and FA + B6 + B12 groups except that bias in swings in the second test was more obvious. Rats in the B complex group showed 13% less biased swings in comparison to control group but it was statistically insignificant. On the other hand, based on the mean of the data, rats treated with B6 or B12 supplements showed swings biased slightly contralateral to lesion side. However, statistical analysis revealed no significant difference between these groups and control group probably because of large variations in the biased swings in rats treated with these supplements.

3.3. Rotarod test

Rotarod test was conducted at three consecutive days, two sessions in each. As shown in Fig. 4, there was marked difference in learning pattern of walking on the rotating rod between healthy
and parkinsonian (control group) rats. Healthy rats showed fast learning and reached to maximum performance at session 4 (R4) but parkinsonian rats showed very weak learning and did not reach to maximum performance even in the last session (R6). Treatment with B complex had marked effect so that motor performance of this group of rats was very close to healthy rats and significantly better than that in control group. FA 5X and FA 10X groups also showed learning pattern similar to healthy and B complex-treated rats but reached to maximum performance at last session (R6) instead of R4. On the other hand, learning pattern of FA 2X, FA + B6 + B12 and B12 groups was more close to parkinsonian rats although B12 group showed remarkably better performance in the last session. Rats belonging to B6 group showed very weak motor performance in all sessions of the test indicating B6 supplementation has no protective effect which is in contrast to the our findings from rotational test.

3.4. Homocysteine level

In order to investigate whether plasma concentration of Hcy involve in possible neuroprotective effect of B vitamin supplementations, we measured tHcy at the end of experiments after rotaion test. In control group, tHcy level was $5.7 \pm 1.1 \mu M/L$. Except of B complex-treated group, tHcy levels in all other experimental groups were higher than that in control group (Fig. 5). Statistically, only significant difference was observed between FA 10X and control groups. The tHcy level in this FA group was 86% higher than that in control group. In other FA-treated groups, tHcy level was 26% and 16% (for FA 2X and FA 5X groups, respectively) higher than that in control group. The tHcy levels in FA + B6 + B12, B6, B12 and B complex groups were $7.4 \pm 2$, $6.8 \pm 1.2$, $7.9 \pm 1.5$ and $4.8 \pm 0.9 \mu M/L$, respectively.

4. Discussion

In present study, we tested the effect of prolonged B vitamin supplementations on the development and severity of 6-OHDA-induced Parkinsonism, with possible association with serum level of Hcy. 6-OHDA was injected into striatum in order to produce slow progressing Parkinsonism similar to development of PD in human [35]. Severity of Parkinsonism was assessed by three behavioral tests of the rotational, rotaion and EBST. Findings of both rotational and rotaion tests show that 10-fold supplementation of folic acid provides significant anti-Parkinsonism effect. 5-fold supplement of folic acid also had some beneficial effect but 2-fold supplement of folic acid, 5-fold supplement of B12 and 5-fold supplement of combination of folic acid with B12 and B6 had no effect. Rotational test revealed some beneficial effect of 5-fold supplementation of B6 but it was not confirmed by rotaion test and EBST. We also examined the effect of 5-fold supplementation of B complex because it has been recently shown that high dose of B vitamins has synergistic anti-Parkinsonism activity [30]. B complex had no effect on the rotational behavior but increased significantly time on the rod in rotaion test and decreased biased swing in EBST.

Discrepancies observed in the findings of these behavioral tests have been probably aroused from sensitivity of each test in prediction of nigral cell death. Rotational test which is the most valid test in the assessment of 6-OHDA-induced Parkinsonism [34–37] can distinguish between partial and near complete lesions of the SN but has rough estimation of motor impairment when nigral lesion size is between 50 and 80% [37]. On the other hand, time spent on the rotating rod correlate inversely with the cell loss in the SN. lancu et al. [38] reported that rotaion test predicts best nigral cell death in comparison to amphetamine- and apomorphine-induced rotation and EBST. Although several authors have confirmed that EBST is a valid behavioral test providing accurate measure of a dopamine- mediated motor function [34,39–41] but it is not sensitive enough in the low-grade lesion [37]. Based on these reports and findings of our experiments, 10-fold supplement of folic acid has certainly neuroprotective effect against 6-OHDA neurotoxicity. 5-fold supplement of folic acid or B complex produces a lower grade of neuroprotection. Effect of B6 is suspicious and our data do not confirm neuroprotective effect of this vitamin.

In consistence with the results of this study, Chen et al. [42] reported that folic acid supplementation protects various brain regions against lipid peroxidation, mitochondrial genotoxicity and decreases remarkably neuronal death associated with folate deprivation in rats treated with β-amyloid peptide, a pathogenic hallmark of Alzheimer’s disease. On the other hand, the findings of the present study are both in agreement and disagreement with several human studies which have investigated association of higher intake of B vitamins with risk of PD. A case-control study in Germany found that a higher intake of folate, vitamin B6 and vitamin B12, but not of riboflavin, associate with a lower risk of PD [43]. A prospective cohort study in the Netherlands showed that a higher intake of vitamin B6, but not of folate or vitamin B12, associate with a decrease in risk of PD [3]. Conversely, a large US-based prospective study in health professionals found no association between intake of folate, vitamin B6 or vitamin B12 and PD risk [44].

To examine the mechanism of neuroprotective effect of B vitamin supplementations, we measured the serum level of Hcy at the end of experiments. A few studies have demonstrated that Hcy levels are increased in blood of PD patients [7,17–19]. Hcy exacerbates oxidative stress, mitochondrial dysfunction and apoptosis in human dopaminergic cells exposed to the pesticide rotenone or the pro-oxidant Fe$^{2+}$ [20]. In consistence with this, Xing et al. [23] reported that focal infusion of Hcy into SN of 6-OHDA-treated rats increases the severity of rotational behavior and decreases numbers of tyrosine hydroxylase (TH)-stained neurons in comparison to control group treated just by 6-OHDA. Also Duan et al. [20] showed that direct infusion of Hcy into either the SN or striatum exacerbates MPTP-induced dopamine depletion, neuronal degeneration and motor dysfunction. Our results are not in agreement with these reports. Except for FA10X group, there was no significant difference in the tHcy levels between all vitamin B-treated groups with control group. In this group, amount of serum Hcy was $5.7 \pm 1.1 \mu M/L$ which is near to concentration obtained by Martins et al. [45] ($6.42 \pm 1.65 \mu M/L$) for 6 months old rats. Our findings are in consistent with some human studies describing no association between PD and Hcy [46]. The Rotterdam Study in Netherlands (a prospective, population-based cohort study of people aged 55 years and older) evaluated the association between dietary intake of folate, vitamin B12, and vitamin B6 and the risk of incident PD among 5289 participants and concluded that dietary...
vitamin B₆ may decrease PD risk, probably through antioxidant effects unrelated to Hcy metabolism [3]. In fact, elevated level of Hcy in the patients with PD has been attributed to the treatment with levodopa (L-DOPA) [7,47–49]. A possible mechanism for this increase is the biotransformation of L-DOPA to dopamine that leads to a depletion of S-adenosylmethionine which is needed for Hcy conversion to methionine [19,47]. Actually, most of the studies associated Hcy levels with B vitamins have examined the effect of deficiency of B vitamins on the plasma Hcy level [19,24–27].

However, tHcy level in FA 10X group which showed the lowest grade of Parkinsonism among experimental groups was significantly higher than that in control group. There is no evidence to associate the increase in level of Hcy by folic acid supplement with its neuroprotective effect. Indirect evidence comes from reports associated smoking with high levels of Hcy [24,50,51] and epidemiological studies suggest that smoking reduces the risk of PD [52,53]. On the other hand a large body of evidences shows that Hcy has neurotoxic effect [20,23,42]. It has been reported that total homocysteine in whole blood increases at room temperature because of a continuous production and release of homocysteine from blood cells [54]. This may be aroused a concern that the time left between blood sampling and sera separation was significantly longer in the FA10X group than control or other groups. However, we extrude this concern because the blood collection was made first from control group and the last collection was made from B₁₂ group. So if this time left be so effective, Hcy level in control group must be significantly higher than B₁₂ or FA groups. Another concern may be aroused through interaction of folate with B₁₂. It has been reported that in vitamin B₁₂ deficiency, higher serum folate is associated with increased total homocysteine and methylnalonic acid concentrations [55,56]. However, the food of the rats in the form of pellets contains necessary amounts of all vitamins and minerals. Also, if our experimental groups had vitamin B₁₂ deficiency, rats belonging to FAX and FAXR groups must have higher Hcy levels too. On the other hand, increase in the level of Hcy might be aroused through interaction of 6-OHDA with folate because 6-OHDA affects noradrenergic neurons and noradrenal in act as acceptor of methyl group [57]. Production of Hcy needs methylation and folate is also involved in the methylation reactions. Also, hyperhomocysteinemia can arise from the effect of several polymorphisms on genes involving in Hcy metabolism [58].

Therefore, if serum level of Hcy do not involve in neuroprotective effect of B vitamins, which mechanism does mediate this effect? Oxidative stress and mitochondrial dysfunction have been proposed as the main mechanisms involving in the neuropathology of PD. High levels of several mitochondrial cofactors and substrates of mitochondrial enzymes may reverse some aspects of aging because high levels of substrates/cofactors could bind to enzymes and protect enzyme activity sites from oxidant attack. High levels of substrates/cofactors could also stimulate activity of partially oxidized damaged enzymes [29]. Recently Jia et al. [30] examined neuroprotective effect of B vitamins on mitochondrial dysfunction, oxidative stress, and Parkinsonism in a 4-week long rotenone treatment-induced cellular model of PD. Their results showed that pretreatment with B vitamins at 2.5 and 5-fold and not 10-fold of that in normal MEM for 4 weeks prevents rotenone-induced: (1) mitochondrial dysfunction, including reduced mitochondrial membrane potential and activities of complex I; (2) oxidative stress, including increase in reactive oxygen species, oxidative DNA damage and protein oxidation, and (3) Parkinsonism parameters, including accumulation of α-synuclein and poly-ubiquitin. Their experiments also showed that these effects are only seen when B vitamins work synergistically and individual B vitamins at the same doses do not have similar effect. These data provide supportive evidences for the proposal considering protection of mitochondrial enzyme activity as neuroprotective mechanism of B vitamin suppletations. However and based on the results of present study, the synergistic effect of B vitamins is not necessary for their neuroprotective effect and individual B vitamins can also induce neuroprotection. Furthermore and in contrast to Jia et al. report, our results indicate that the effect of folate supplement is dose dependent with no effect at 2-fold, moderate effect at 5-fold and the best effect at 10-fold suppletations. In agreement with this, Anderson et al. [59] reported that nicotinamide administration resulted in a dose-dependent sparing of striatal DA levels and SN neurons in acute MPTP-treated animals. Only the highest dose of nicotinamide had similar effects in sub-acute MPTP-treated animals. However, in our study, only 5-fold supplement of B complex was used and higher doses of B complex might be more effective even more than that of folic acid supplementation.

5. Conclusion

We have demonstrated that supplement of folic acid alone or complex of B vitamins could provide neuroprotective effect and attenuates severity of 6-OHDA-induced Parkinsonism. The effect of folic acid was dose dependent and its 10-fold supplement was more effective than 5-fold supplement. Also, higher intake of B vitamins did not decrease the sera level of Hcy in the 6-OHDA treated rats. This finding indicate that neuroprotective effect of B vitamins is not mediated by lowering plasma Hcy and might be mediated rather by protection of mitochondrial enzyme activity sites from oxidant attack and stimulation of activity of partially oxidized damaged enzymes. Considering that B vitamins are not toxic even at large doses and can be safely used for long time, further studies should be done to find exact neuroprotective mechanism of B vitamins and optimize their best effective dose.

Conflict of interest

All authors declare no actual or potential conflicts of interest that could inappropriately influence this study.

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