Pharmacology, Biochemistry and Behavior 124 (2014) 297–304

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Evaluation of the association between blood homocysteine concentration and the degree of behavioral symptoms in the 6-hydroxydopamine-induced Parkinsonism in rat

Hashem Haghdoost-Yazdi [a,*], Mohammad Sarookhani [a], Ayda Faraj [a], Negin Fraidouni [a], Tahereh Dargahi [a], Mohammad Hosein Yaghoubidoust [a], Hassan Azhdari-Zarmehri [b]

[a] Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran
[b] Department of Basic sciences, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran

ARTICLE INFO

Article history:
Received 9 January 2014
Received in revised form 1 June 2014
Accepted 22 June 2014
Available online 30 June 2014

Keywords:
Homocysteine
Parkinson's disease
6-Hydroxydopamine
Apomorphine-induced rotational test
Cell replacement therapy
B vitamin supplementation

ABSTRACT

Growing evidence indicates that homocysteine (Hcy) may be involved in the pathophysiology of several neurological disorders including Parkinson's disease. In the present study, the association between blood Hcy concentration and the degree of behavioral symptoms in the 6-hydroxydopamine (6-OHDA)-induced Parkinsonism in rat was evaluated. Total serum Hcy (tHcy) was measured before and 6 weeks after the intracerebral injection of 6-OHDA. Apomorphine-induced rotational test was performed at second, third and sixth weeks after 6-OHDA injection. Subsequently, cell replacement therapy was performed on rats with good rotation score. No correlation between tHcy in before 6-OHDA injection and severity of the rotations after 6-OHDA injection was observed. On the other hand, 6-OHDA treatment significantly decreased tHcy level. However, this reduction was only observed in animals with low degree of rotations and in rats with high number of rotations; tHcy did not change significantly. Furthermore, 10 weeks after cell transplantation, tHcy was significantly lower than that found before therapy if the rats showed good improvement in the degree of rotations. We also examined the effect of different supplements of B vitamins on tHcy before and after 6-OHDA injection. In healthy rats, all kinds of B vitamins and also supplement B6 or B12 alone reduced tHcy. Following 6-OHDA injection, B vitamin supplementation failed to cause remarkable effect. Considering the direct correlation between the severity of rotational behavior and the degree of lesion in the substantia nigra (SN), our data indicate that higher tHcy values can predict higher SN dopaminergic neurodegeneration.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting about 1–3% of the population over the age 50. The primary pathological feature of PD is the loss of dopaminergic (DA) neurons within the substantia nigra pars compacta of the midbrain. There is no consensus as to mechanism(s) contributing to DA cell loss; however, growing evidence suggests that oxidative stress and mitochondrial dysfunction play important role (Jenner and Olanow, 1996; Tatton, 2000). Although both genetic and environmental factors are involved in the pathogenesis of PD (Lau et al., 2005; Hancock et al., 2007), 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 (Betarbet et al., 2000) and low antioxidant intake (de Rijk et al., 1997) 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 (Kuhn et al., 1998; Diaz-Arrastia, 2000; O'Suilleabhain et al., 2004). 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 (Bertsch et al., 2001; Prins et al., 2002; Seshadri et al., 2002; Tiemeier et al., 2002; Quadri et al., 2005). There is a rising body of evidence that shows Hcy levels increase in the blood and CSF of patients with PD (Allain et al., 1995; Kuhn et al., 1998; Yasui et al., 2000; dos Santos et al., 2009). High levels of Hcy might accelerate DA cell death through oxidative stress and excitotoxicity (Duan et al., 2002; Sachdev et al., 2002; Obeid and Herrman, 2006). Animal studies have demonstrated that focal infusion of Hcy into either substantia nigra (SN) or striatum exacerbates the symptoms of 6-OHDA and MPTP-induced Parkinsonism (Duan et al., 2002; Xing et al., 2008). The pro-oxidant and pro-apoptotic effects of homocysteine have been also confirmed for *in vitro* models of

* Corresponding author at: Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran. Tel.: +98 281 3366001 6; fax: +98 281 3324970 1.
E-mail addresses: hhaghdoost@yahoo.com, hhaghdoost@gmail.com (H. Haghdoost-Yazdi).

http://dx.doi.org/10.1016/j.pbb.2014.06.020
0091-3057/

PD; e.g. homocysteine aggravated the neurotoxic effects of pesticide rotenone in human dopaminergic cells (Todorovic et al., 2006). On the other hand, there is a considerable body of evidence indicating that high level of Hcy in patients with PD is induced by treatment with levodopa (L-DOPA) (Miller et al., 1997; Kuhn et al., 1998; Rogers et al., 2003; Religa et al., 2006). A possible mechanism for the L-DOPA-induced hyper-Hcy is the biotransformation of L-DOPA to dopamine which leads to a depletion of S-adenosylmethionine required for Hcy conversion to methionine (Miller et al., 1997; dos Santos et al., 2009). This transformation needs O-methylation which is catalyzed by COMT (Zoccolella et al., 2005). Several studies have shown that treatment of PD with a combination of L-DOPA and COMT inhibitors decreases Hcy level (Siniscalchi et al., 2006).

6-Hydroxydopamine (6-OHDA)-induced Parkinsonism is one of the most common animal models of PD. 6-OHDA is a hydroxylated analogue of natural dopamine that selectively destroys catecholamine neurons. In addition to production of reactive oxygen species (ROS) which damage proteins, lipids and DNA, 6-OHDA through inhibition of mitochondrial complexes I and IV leads to mitochondrial impairment and ATP deficiency (Kumar et al., 1995; Soto-Otero et al., 2000; Blum et al., 2001; Rodriquez et al., 2002; Dauer and Przedborski, 2003). In the present study, we investigated the association between blood Hcy and the degree of behavioral symptoms of 6-OHDA-induced Parkinsonism. We also evaluated the effect of cell replacement therapy and B vitamin supplementation on the serum level of Hcy in 6-OHDA-treated rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Razi Institute, Karaj, Iran), weighing 220–250 g at the beginning of study were housed in large cages (38 × 59 × 20 cm, 10–12 rats in each) at a temperature-controlled colony room maintained at 21 ± 3 °C under 12:12 h light/dark cycle with lights on at 6:00 a.m. They were given free access to tap water and standard rat chow. All procedures carried out throughout this study were according to the guidelines for animal experiments approved by the Research Council of Qazvin University of Medical Sciences.

2.2. Surgical procedures

Rats were anesthetized with intraperitoneal injection of a solution containing ketamine (100 mg/kg) and xylazine (10 mg/kg). 6-OHDA (10–15 μg, dissolved in saline containing 0.2% ascorbic acid) was injected unilaterally into 2 sites in the right medial forebrain bundle (MFB) with coordinates of 1: AP: −4.4, L: −1.2, and DV: −7.8 with the tooth bar (TB) positioned below the interaural line: −2.3 and 2: AP: −4, L: −0.8, and DV: −8 with TB: +3.4 using stereotaxic apparatus (Stoelting, USA) and through a 10-μl Hamilton syringe. For the experiments evaluating B vitamin supplementation, 6-OHDA was unilaterally injected into the right striatum with coordinates of AP: 0.2 and L: −3.5 and also DV: −8 with TB: −3.3. AP and L were measured from bregma and DV was measured from the surface of skull. All coordinates were calculated according to the atlas of Paxinos and Watson (2007). 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.

2.3. Apomorphine-induced rotational test

Apomorphine-induced rotational test was performed according to the method previously described by Fujita et al. (1996). 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 38 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 was defined as the positive scores minus the negative ones. All tests were carried out between 01.00 and 04.00 p.m.

2.4. Cell replacement therapy

2.4.1. Cell preparation

Embryonic day 14 (E14) ventral mesencephalic (VM) cells obtained from pregnant female Wistar rats were used for cell replacement therapy. E14 VM tissues were dissected and processed as previously described (Dunnett and Björklund, 1997; Nikkhah et al., 2009). Briefly, fetuses from both uterine horns were removed from timed-pregnant female and collected into saline-glucose (4 and 0.6%, respectively) solution (GS) at room temperature. Then, using sterile dissection instruments, the VM tissue pieces were divided into 4 segments and pooled in GS. To dissociate the tissue pieces into a single cell suspension, the VM segments were incubated in 0.1% trypsin (Worthington), 0.05% DNase (Sigma DN 25) and DMEM (Dulbecco's modified Eagle's medium, Gibco) at 37 °C for 30 min followed by four rinses with 0.05% DNase/DMEM. Later, trituration process was performed using 1-ml and 200-μl Eppendorf pipette tips (about 15 strokes each) in a solution with 50% cell culture medium and 50% trituration solution. Cell culture medium contained 77% DMEM, 20% fetal calf serum, 2% B-27 and 1% Pen-Strep and fungizone (sigma). Trituration solution contained 0.001% DNase and 1% bovine serum albumin (sigma) in Hank's balanced salt solution (sigma). Cell suspensions were pelleted by centrifugation at 600 rpm for 5 min and the remaining was resuspended with 0.05% DNase/DMEM at a final volume of 5 μl per VM. The final cell suspension contained 45,000 cells/μl and the viability was more than 85%, as determined by trypan blue exclusion assay on a hemocytometer. Before transplantation, the cell suspensions were incubated overnight at 0 °C in a hibernation medium containing KCl, glucose, MgCl2, NaH_2PO_4, Na_2HPO_4 and 30% lactic acid at pH = 7.2.

2.4.2. Cell transplantation

The cell suspension was transplanted into the right striatum of 6-OHDA-treated rats with good rotational score using stereotaxic surgery and 2-μl Hamilton syringe. A volume of 2 μl of suspension at the rate of 0.5 μl/min was implanted in each rat at 4 sites with coordinates of (in relation to bregma) AP: +0.5, L: +2.3, and DV: −5 or −4 mm with TB: −3.3 and also AP: +0.5, L: +3.3, and DV: −5 or −4 mm with TB: −3.3. There was 1 min stop after injection into each site and 3 min stop after the last injection. Then, the needle was slowly (1 mm/min) withdrawn.

2.4.3. B vitamin supplementation experiments

All kinds of B vitamins were purchased from the Sigma-Aldrich Company. Feeding with B vitamin supplements was begun 1 month before the injection of 6-OHDA and continued for 6 weeks afterwards. Animals were divided into eight experimental groups as follows: (1) control, which received B vitamins equal to that in normal MEM (minimum essential medium which obtained from the Committee on Animal Nutrition of National Research Council (National Academy Press, 1995); (2) complex, which received a combination of all kinds of B vitamins (Table 1) 5-folds of that in normal MEM; (3–5) FA 2X, FA 5X and FA 10X which received folic acid 2, 5 and 10-folds of that in normal MEM, respectively; (6) FA + B6 + B12 which received a combination of folic acid, vitamin B6 and vitamin B12, 5-folds of that in normal MEM; (7 and 8) B6 and B12 which received vitamin B6 and B12, respectively 5-folds of that in normal MEM. The number of animals (n) was 12 for each group. Considering normal dietary regime contains B vitamins equal to normal MEM, additional B vitamins to provide required supplements were added to drinking water. Two 500 ml bottles of water were

Table 1
Displays the minimum essential medium (MEM) of different B vitamins for maintenance in rats and also the amounts of added vitamins to drinking water to prepare required supplements. The amount of required food in the form of pellets and the required volume of drinking water for maintenance was considered as 15 g/day and 12 ml/day, respectively. Note, 2-folds supplementation means that 1-fold was provided from the pellets and another 1-fold was provided by addition required vitamin to drinking water. The information was extracted from the Committee on Animal Nutrition of National Research Council (National Academy Press, 1995).

Vitamins	MEM (mg/kg diet)	Requirements for 2-fold Supplementation (mg/1 L)	Requirements for 5-fold Supplementation (mg/1 L)	Requirements for 10-fold Supplementation (mg/1 L)
Biotin (d-biotin)	**0.2**		**1**	
Folic acid	**1**	**1.25**	**5**	11.25
Niacin (nicotinic acid)	**15**		**75**	
Pantothenate (Ca-d-pantothenate)	**10**		**50**	
Riboflavin	**4**		**20**	
Thiamin (thiamin-HCl)	**4**		**20**	
B_6 (pyridoxine)	**6**		**30**	
B_{12}	**0.05**		**0.25**	

placed in each cage to ensure that each rat receives enough amounts of water and B vitamins. Drinking water was replaced every 2 days.

2.4.4. Blood sampling and Hcy measurement

Before and after 6-OHDA injection, venous blood samples were collected from the animal's tail while the animals restricted within a restrainer. After cell transplantation, blood samples were collected from the heart while the animals were anesthetized with intraperitoneal injection of ketamine and xylazine. Blood specimens were allowed to clot and the sera were separated using centrifugation at 5000 rpm for 5 min and stored at −80 ºC until use. Total serum Hcy (tHcy) 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 as follows: within run precision: 8%, limit of quantification: 1 μmol/L and linearity: up to 50 μmol/L.

2.5. Statistical analysis

Data are expressed as 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. Student's paired t-test and one-way ANOVA were used for analyzing apomorphine-induced rotations and tHcy level. Correlation analysis between tHcy level and number of rotations was performed by Spearman's test using SPSS software. A P value ≤ 0.05 was considered as significant, statistically.

3. Results

3.1. Effect of 6-OHDA treatment on the total serum level of Hcy

6-OHDA treatment significantly ($P < 0.001$, paired t-test, $n = 32$) reduced the total serum level of Hcy (tHcy). It was 13.3 ± 0.47 μmol/L prior to 6-OHDA injection and 10.65 ± 0.63 μmol/L at sixth week post-injection ($n = 32$). However, the severity of apomorphine-induced rotations among the 6-OHDA-treated rats was not similar. Based on the number of rotations, the animals were divided into three groups marked as: 1 – severe parkinsonian rats ($n = 12$) with more than 6 rotations/min, 2 – moderate parkinsonian rats ($n = 12$) with 2–6 rotations/min, and 3 – weak parkinsonian rats ($n = 8$) with less than 2 rotations/min. While tHcy level in the weak group significantly decreased following 6-OHDA treatment, there was no significant reduction in the severe group (Fig. 1). Statistical analysis indicated significant correlation between number of rotations and tHcy level after 6-OHDA injection (Spearman's correlation coefficient = 0.471 and $P < 0.01$, $n = 32$). However, there was no correlation between tHcy level prior to the injection and number of rotations (correlation coefficient = −0.04, $n = 32$). Separate analysis of different parkinsonian rats showed that there was positive correlation between number of rotations and tHcy level post-injection only in severe parkinsonian rats (correlation coefficient = 0.342 and $P < 0.05$, $n = 12$). These findings indicate that (1) there was no correlation between tHcy prior to 6-OHDA injection and the degree of behavioral symptom of Parkinsonism and (2) at least in severe parkinsonian rats there is an obvious and direct correlation between the severity of behavioral symptom and tHcy level, i.e. rats with more severe symptoms had higher tHcy level.

3.2. Effect of cell replacement therapy on tHcy level

Cell replacement therapy was performed on the severe and moderate parkinsonian rats. The cell suspension was prepared from the VM of E14 rat embryos and was transplanted into the striatum of parkinsonian rats following overnight hibernation. The therapy was effective and remarkably attenuated the behavioral symptoms. The number of rotations decreased from 433 ± 78 prior to transplantation to 245 ± 124 in the sixth week post-transplantation ($n = 24$). There was no significant difference in tHcy prior to transplantation and that observed prior to 6-OHDA injection; however, tHcy after transplantation was significantly lower than that found before 6-OHDA injection (Fig. 2). Again, the effect of cell replacement therapy was not the same in these rats. Some parkinsonian rats responded well and showed remarkable improvement in the degree of rotations but some failed to do so. In the rats with good improvement ($n = 9$), tHcy after transplantation was significantly lower than that measured before 6-OHDA injection. However, in the rats with no significant improvement ($n = 14$), the reduction in tHcy after transplantation was minor and insignificant. Again significant correlation was recognized between number of rotations and tHcy level after transplantation (correlation coefficient = 0.371 and $P < 0.05$, $n = 9$ for rats with good improvement and correlation coefficient = 0.441 and $P < 0.01$, $n = 14$ for rats with no significant improvement). These data confirm the presence of a direct correlation between the severity of behavioral symptoms and tHcy level in 6-OHDA-treated rats.

3.3. Effect of B vitamin supplements on tHcy level

B vitamins are involved in Hcy metabolism and several epidemiological studies have shown that the deficiencies of these vitamins are the major causes of hyper-Hcy in the general population (Jacques et al., 2001; de Bree et al., 2002; Castro et al., 2006; Selhub, 2006). In another series of experiments, we studied the effect of B vitamin supplementation on the tHcy before and after 6-OHDA treatments (Fig. 3). The supplementation began 1 month before 6-OHDA injection and continued for 6 weeks post-injection. Primary measurements showed that each cage of rats consumes about 300 ml water in 2 days that indicate our

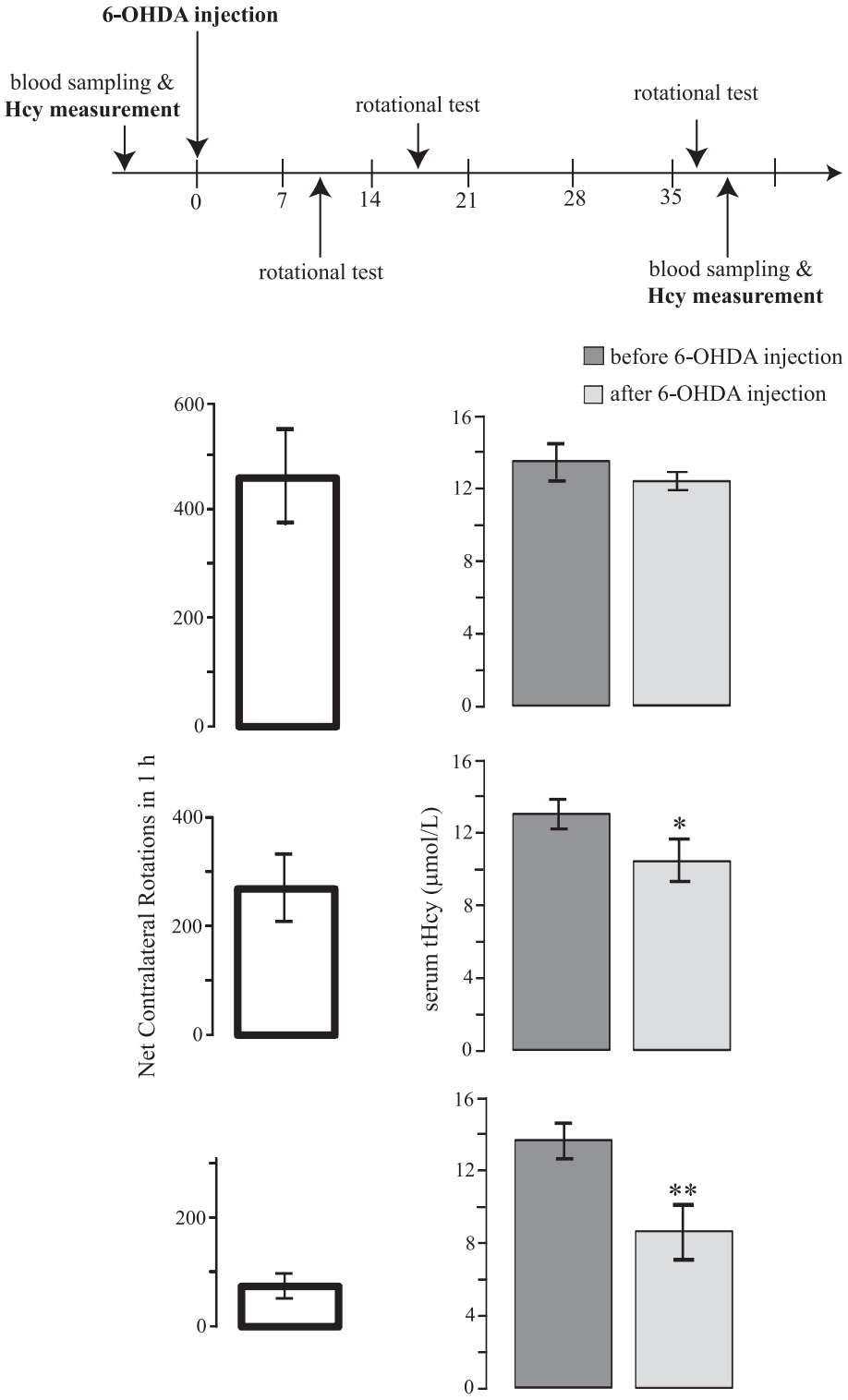

Fig. 1. Association between the severity of apomorphine-induced rotations and tHcy in the 6-OHDA-treated rats. Diagram illustrates the time schedule used for these series of experiments. Histograms on the left side show the number of rotations 6 weeks after 6-OHDA injection. Histograms on the right side show the tHcy before and 6 weeks after 6-OHDA injection in three groups of rats with severe (upper panel, $n = 12$), moderate (mid panel, $n = 12$) and weak (lower panel, $n = 8$) degree of rotations. *: $P < 0.05$; **: $P < 0.01$, paired t-test.

assumption that each rat drinks 12 ml water per day is more or less correct. The Hcy measurement prior to 6-OHDA injection showed that the supplements containing all kinds of B vitamins altogether (complex) or B6 or B12 alone at 5-folds of that in normal MEM (minimum essential medium) significantly decreased tHcy. The supplements of folic acid at 2, 5 or 10-folds of MEM (FA 2X, FA 5X and FA 10X, respectively) or a combination of folic acid, B6 and B12 at 5-folds of MEM had no effect. On the other hand, the tHcy measurement at the sixth week after 6-OHDA injection showed that nourishing the 6-OHDA-treated rats with FA 10X causes a significant increase in tHcy while the supplements of other B vitamins and even FA 2X and FA 5X have no effect. Interestingly, tHcy concentration after 6-OHDA injection was

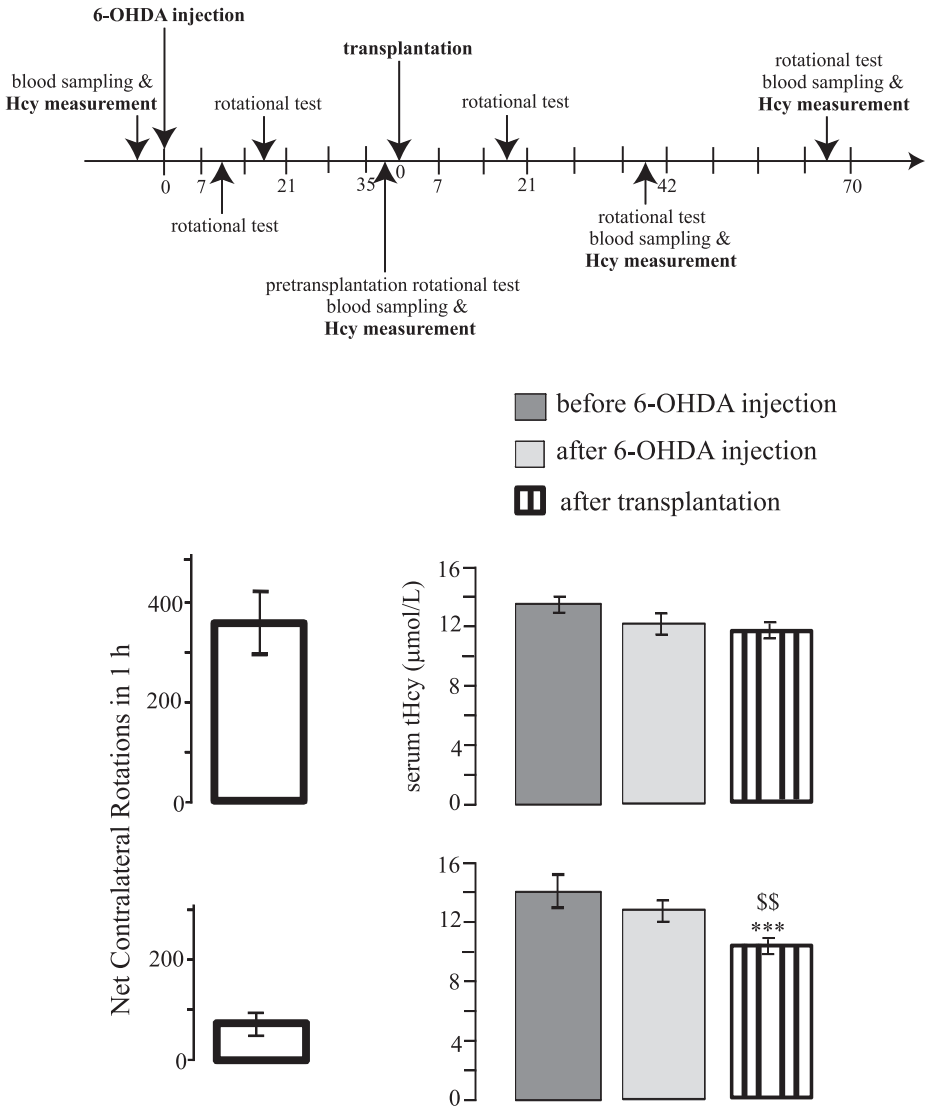

Fig. 2. Effect of cell replacement therapy on the tHcy level. Diagram illustrates the time schedule used for these series of experiments. Cell suspension was prepared from embryonic ventral mesencephalic cells and was transplanted into the striatum of parkinsonian rats 6 weeks after 6-OHDA injection. The histograms on the left side show the number of rotations in the sixth week after transplantation. Histograms on the right side show the tHcy prior to 6-OHDA injection, 6 weeks post-injection and 10 weeks post-transplantation. Some rats which receive cell suspension showed good improvement in the number of rotations (lower panel, $n = 9$) but others failed to show remarkable improvements (upper panel, $n = 14$). ***: $P < 0.001$ paired t-test, compared to tHcy level prior to injection. $$: $P < 0.01$ paired t-test, compared to tHcy level in sixth week post-injection.

significantly lower than that observed before 6-OHDA injection for the complex, FA 2X, FA 5X and control (receiving no supplement) groups.

4. Discussion

Growing evidence indicates that Hcy may be involved in the pathophysiology of several neurological disorders including PD. In this study, we investigated the association between tHcy level and the degree of apomorphine-induced rotations in 6-OHDA-treated rats. The most important finding was a significant decrease in tHcy following 6-OHDA treatment. However, this reduction was observed in the animals with low degree of rotations and in the rats with high number of rotations; tHcy did not change significantly. Furthermore, 10 weeks after the cell replacement therapy of 6-OHDA-treated rats with high number of rotations, tHcy was significantly lower than that seen prior to cell therapy. However, this effect was only observed in the rats which well responded to the therapy and showed significant decrease in the number of rotations. Also statistical analysis showed that there is a positive correlation between tHcy after 6-OHDA injection with the number of apomorphine-induced rotations.

Why did tHcy decrease after 6-OHDA injection? Martins et al. (2005) reported that in rats, plasma Hcy concentration increases from 2.94 ± 0.47 µmol/L in newborns to 8.29 ± 0.67 µmol/L at 3 months old but after that values decrease with aging so that at 6 months it is 6.42 ± 1.65 µmol/L and at 28 months is 4.87 ± 0.81 µmol/L. Although these concentrations are considerably different from our data (in our experiments, prior to 6-OHDA injection, rats were about 3 months old and had tHcy level about 13 µmol/L), but Martins' data indicate that aging decreases plasma Hcy concentration. Nevertheless, we cannot attribute the reduction in tHcy level following 6-OHDA treatment to aging because tHcy did not decrease in all experimental groups (for example: the severe parkinsonian group (Fig. 2), FA 10X, B6, and B12 groups (Fig. 3). Also, a number of severe parkinsonian rats which were treated with cell replacement therapy did not show marked improvement in the degree of rotations. In these rats, following cell transplantation, i.e. 16 weeks after 6-OHDA injection, tHcy was not significantly lower than that observed prior to 6-OHDA injection. Another possible mechanism is the interaction of 6-OHDA with catechol-O-methyltransferase (COMT), an enzyme that degrades catecholamines. Several human studies have demonstrated that Hcy levels increase in the blood and

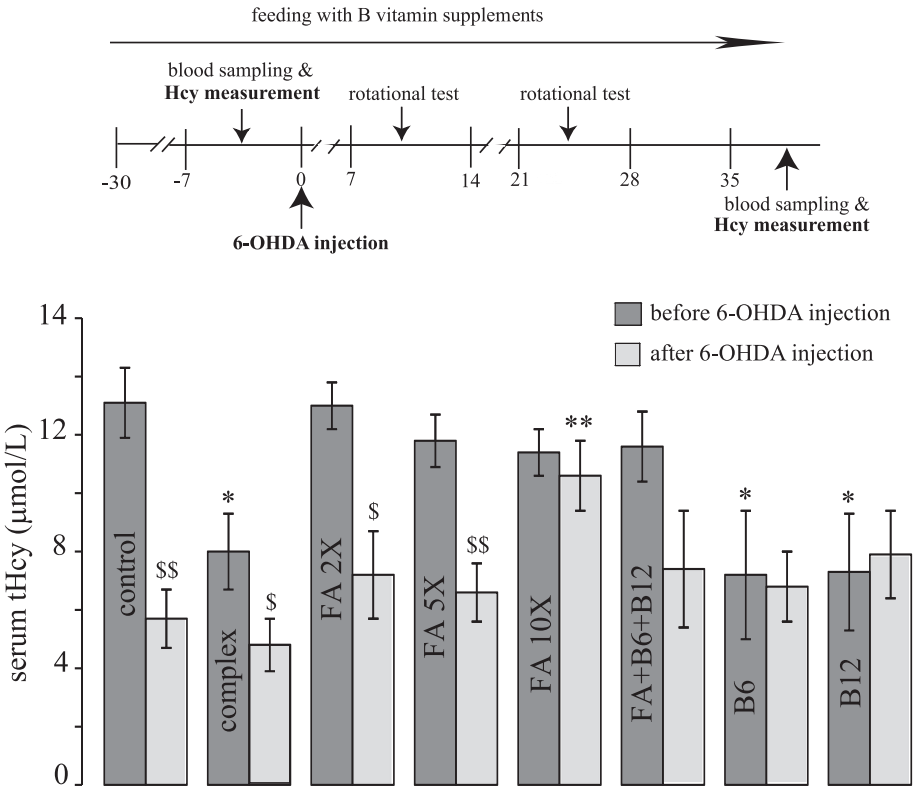

Fig. 3. Effect of B vitamin supplementation on the tHcy level. Diagram illustrates the time schedule used for these series of experiments. Except for the control group, all animals received drinking water enriched with B vitamins 1 month before 6-OHDA injection to 6 weeks post-injection. Blood sampling and tHcy measurements were performed before and 6 weeks after 6-OHDA injection. The histograms show tHcy in different experimental groups. *: $P < 0.05$; ***: $P < 0.001$ One-Way ANOVA, compared to control group. $: $P < 0.05$, $$: $P < 0.01$ paired t-test, in compared to tHcy prior to injection. control: a group of rats which did not receive any supplements, complex: a group of rats nourished with a combination of all kinds of B vitamins, 5-folds of MEM (minimum essential medium), FA 2X, 5X and 10X: groups of rats nourished with folic acid at 2, 5 and 10-folds of MEM, respectively, FA + B6 + B12: a group of rats nourished with a combination of folic acid, B6 and B12 at 5-folds of MEM, B6 and B12: groups of rats nourished with B6 or B12 at 5-folds of MEM. $n = 12$ for all groups.

CSF of patients with PD (Allain et al., 1995; Kuhn et al., 1998; Yasui et al., 2000; dos Santos et al., 2009). Some authors have attributed this elevated level of Hcy to the treatment with levodopa (L-DOPA) (Miller et al., 1997; Kuhn et al., 1998; Rogers et al., 2003; Religa et al., 2006). A possible mechanism for this increment is the biotransformation of L-DOPA to dopamine that leads to a depletion of S-adenosylmethionine which is needed for Hcy conversion to methionine (Kuhn et al., 1998; dos Santos et al., 2009). Transformation of L-DOPA to dopamine needs O-methylation which is catalyzed by COMT. It is reported that a combination of L-DOPA with COMT inhibitors peripherally increases the bioavailability of L-DOPA and decreases Hcy levels (Lamberti et al., 2005; Siniscalchi et al., 2005; Zoccolella et al., 2005). Chemical experiments indicate that COMT is inactivated by 6-OHDA (Borchardt et al., 1976). Therefore, it is possible that in our experiment, 6-OHDA acted as a COMT inhibitor, leading to a decrease in tHcy. Although this mechanism can explain how 6-OHDA treatment decreased tHcy but some of our data failed to confirm this possibility. We measured tHcy 6 weeks after 6-OHDA injection which provided enough time for terminating the chemical effects of 6-OHDA. Also, if the decrease in tHcy level was due to the inhibition of COMT by 6-OHDA, tHcy must have been decreased in all rats including the severe parkinsonian rats.

On the other hand, our data show a direct correlation between tHcy and the degree of rotational behavior. The rotational test is a standard test for confirming the efficacy of a lesion in the SN dopaminergic neurons. It has been shown that the magnitude of rotations depends on the degree of lesion in the SN neurons (Ungerstedt and Arbuthnott, 1970; Hefti et al., 1980; Przedborski et al., 1995) and also the degree of striatal dopamine depletion (Hefti et al., 1980; Schwarting and Huston, 1996). Therefore, the higher level of tHcy in the severe parkinsonian rats might indicate the presence of more severe lesion in the SN dopaminergic neurons. But how does the loss of DA neurons elevate tHcy? It has been shown that besides the DA neurons, 6-OHDA could also damage noradrenergic (NA) or serotonergic neurons (Sadakierska-Chudy et al., 2010; Szot et al., 2012). Noradrenalin acts as an acceptor of methyl group and the production of Hcy is dependent on methylation (Selhub, 1999). Also, both DA and NA systems are important in PD and the loss of either NA or DA neurons can affect the function of each other's neurons. For example, it has been suggested that the loss of locus coeruleus (LC) NA neurons can enhance the susceptibility of DA neurons to damage. Also, human studies show that the NA neurons in the LC are significantly reduced in Parkinson's disease (PD) and the LC exhibits neuropathological changes early in the disease process (Szot et al., 2012).

Therefore, neurological disorders by themselves or their treatments induce hyper-Hcy and Hcy itself may not be a potential neurotoxic agent. However, several studies have confirmed that Hcy acts as a neurotoxin and potentiates or exacerbates other neurotoxins with neurodegenerative effect on the SN dopaminergic neurons. Hcy exacerbates oxidative stress, mitochondrial dysfunction and apoptosis in human DA cells exposed to the pesticide rotenone or the pro-oxidant Fe^{2+}. Also, direct infusion of Hcy into either SN or striatum exacerbates MPTP-induced dopamine depletion, neuronal degeneration and motor dysfunction (Duan et al., 2002). Moreover, Xing et al. (2008) reported that the focal infusion of Hcy into the SN of 6-OHDA-treated rats increases the severity of rotational behavior and decreases the numbers of tyrosine hydroxylase (TH)-stained neurons in comparison to rats treated just by 6-OHDA. Our data did not show any correlation between tHcy level prior to 6-OHDA injection and the degree of rotational behavior after 6-OHDA injection. However, we cannot rule out the neurotoxic effect of Hcy because no assessment on the direct effect of Hcy on

Parkinsonism was conducted. Also, we did not administer any systemic or focal infusion of Hcy. It is possible that Hcy acutely exerts its toxic effect at very high concentrations or chronically at the highest level of normal range as it has been shown for atherosclerosis in human (Todorovic et al., 2006).

Anyway, even if high level of Hcy does not act as a risk factor for PD and neurodegeneration or medications result in high tHcy concentration, it is very important to decrease Hcy level because of its association with cerebrovascular disease and neuropsychiatric symptoms (like dementia, cognitive slowing, and depression) (Giles et al., 1995). High level of Hcy could also exacerbate the clinical manifestations of PD (Yasui et al., 2000). It has been reported that folate, vitamin B6 and vitamin B12 effectively lower the plasma concentration of Hcy (Quadri et al., 2005; Castro et al., 2006; dos Santos et al., 2009). We examined the effect of different supplements of B vitamins on tHcy level before and after 6-OHDA injection. Feeding of intact and 6-OHDA-untreated rats with supplements of B complex, B6 or B12 for 1 month decreased the level of tHcy. Folate supplements failed to produce significant effect. However, following 6-OHDA injection, B vitamins supplements were not effective. In fact, high dose of folate (10-folds of MEM) significantly increased tHcy. This increase may have been aroused through the interaction of 6-OHDA with folate as the later is involved in the methylation reactions. However, as we reported previously (Haghdoost-Yazdi et al., 2012), some B vitamins supplements could significantly decrease the behavioral symptoms of 6-OHDA-induced Parkinsonism which is independent of serum Hcy level. The useful effect of B vitamin supplementation is probably mediated by protecting mitochondrial enzyme activity sites from oxidant attack and the stimulation of activity of partially oxidized damaged enzymes (Jia et al., 2010).

5. Conclusion

Our data show that in 6-OHDA-induced Parkinsonism, there is a direct correlation between total serum Hcy level and the degree of behavioral symptoms. As the severity of the behavioral symptoms reflects the degree of lesion in the SN dopaminergic neurons, a higher Hcy level suggests higher SN dopaminergic neurodegeneration. Therefore, high level of Hcy in PD patients or even other neurological disorders may actually be a side effect of disease and not a risk factor of such clinical condition. If this is true, it is important to decrease the plasma concentration of Hcy because it has been confirmed that high level of Hcy is a risk factor for various cerebrovascular and cardiovascular diseases. Here, we showed that the effect of B vitamin supplements on lowering serum Hcy level depends on the presence or absence of SN lesion. While in healthy rats, the supplements containing B complex, B6 or B12 can reduce the serum level of Hcy, with SN lesion, B vitamin supplementation is ineffective in lowering tHcy.

Acknowledgement

This work was supported by a grant-in aid for scientific research from the Iran National Science Foundation (INSF). The authors thank Dr. M. Alipour-heidari for his help in the statistical analysis of data.

References

Allain P, Le Bouil A, Cordillet E, Le Quay L, Bagheri H, Montastruc JL. Sulfate and cysteine levels in the plasma of patients with Parkinson's disease. Neurotoxicology 1995;16:527–9.

Bertsch T, Mielke O, Höly S, Zimmer W, Casarin W, Aufenanger J, et al. Homocysteine in cerebrovascular disease: an independent risk factor for subcortical vascular encephalopathy. Clin Chem Lab Med 2001;39:721–4.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3(12):1301–6.

Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, et al. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol 2001;65:135–72.

Borchardt RT, Reid JR, Thakker DR. Catechol O-methyltransferase 9. Mechanism of inactivation by 6-hydroxydopamine. J Med Chem 1976;19(10):1201–9.

Castro R, Rivera I, Blom HJ, Jakobs C, Tavares de Almeida I. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. J Inherit Metab Dis 2006;29:3–20.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003;39(6):889–909. [Review].

de Bree A, Verscuren WM, Kromhout D, Kluijtmans LA, Blom HJ. Homocysteine determinants and the evidence to what extent homocysteine determinants the risk of coronary heart disease. Pharmacol Rev 2002;54:599–618.

de Rijk MC, Breteler MM, den Breeijen JH, Launer LJ, Grobbee DE, van der Meché FG, et al. Dietary antioxidants and Parkinson disease. The Rotterdam Study. Arch Neurol 1997;54(6):762–5.

Diaz-Arrastia R. Homocysteine and neurologic disease. Arch Neurol 2000;57:1422–7.

dos Santos EF, Busanello EN, Miglioranza A, Zanatta A, Barchak AG, Vargas CR, et al. Evidence that folic acid deficiency is a major determinant of hyperhomocysteinemia in Parkinson's disease. Metab Brain Dis 2009;24(2):257–69.

Duan W, Ladenheim B, Cutler RG, Kruman II, Cadet JL, Mattson MP. Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease. J Neurochem 2002;80:101–10.

Dunnett SB, Björklund A. Basic neural transplantation techniques. I. Dissociated cell suspension grafts of embryonic ventral mesencephalon in the adult rat brain. Brain Res Brain Res Protoc 1997;1:91–9.

Fujita M, Nishino H, Kumazaki M, Shimada S, Tohyama M, Nishimura T. Expression of dopamine transporter mRNA and its binding site in fetal nigral cells transplanted into the striatum of 6-OHDA lesioned rat. Mol Brain Res 1996;39:127–36.

Giles WH, Kittner SJ, Anda RF, Croft JB, Casper ML. Serum folate and risk for ischemic stroke. First National Health and Nutrition Examination Survey epidemiologic follow-up study. Stroke 1995;26:1166–70.

Haghdoost-Yazdi H, Fraidouni N, Faraji A, Jahanihashemi H, Sarookhani M. High intake of folic acid or complex of B vitamins provides anti-Parkinsonism effect: no role for serum level of homocysteine. Behav Brain Res 2012;233(2):375–81.

Hancock DB, Martin ER, Stajich JM, Jewett R, Stacy MA, Scott BL, et al. Smoking, caffeine, and nonsteroidal anti-inflammatory drugs in families with Parkinson disease. Arch Neurol 2007;64:576–80.

Hefti F, Melamed E, Sahakian BJ, Wurtman RJ. Circling behavior in rats with partial, unilateral nigro-striatal lesions: effect of amphetamine, apomorphine, and DOPA. Pharmacol Biochem Behav 1980;12(2):185–8.

Jacques PF, Bostom AG, Wilsin PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine in the Framingham Offspring cohort. Am J Clin Nutr 2001;73:613–21.

Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. Neurology 1996;47:161–70.

Jia H, Liu Z, Li X, Feng Z, Hao J, Li X, et al. Synergistic anti-Parkinsonism activity of high doses of B vitamins in a chronic cellular model. Neurobiol Aging 2010;31:636–46.

Kuhn W, Roebroek R, Blom H, Van Oppenraaj D, Przuntek H, Kretschmer A, et al. Elevated plasma levels of homocysteine in Parkinson's disease. Eur Neurol 1998;40:225–7.

Kumar R, Agarwal ML, Seth PK. Free radical-generated neurotoxicity of 6-hydroxydopamine. J Neurochem 1995;64:1701–7.

Lamberti P, Zoccolella S, Iliceto G, Armenise E, Fraddosio A, de Mari M, et al. Effects of levodopa and COMT inhibitors on plasma homocysteine in Parkinson's disease patients. Mov Disord 2005;20(1):69–72.

Lau LM, Koudstaal PJ, Van Meurs JBJ, Uitterlinder AG, Hofman A, Breteler MMB. Methylenetetrahydrofolate reductase C677T genotype and PD. Ann Neurol 2005;57:927–30.

Martins PJF, Galdieri LC, Souza FGA, Andersena ML, Benedito-Silva AA, Tufika S, et al. Physiological variation in plasma total homocysteine concentrations in rats. Life Sci 2005;76:2621–9.

Miller JW, Shukitt-Hale B, Villalobos-Molina R, Nadeau MR, Selhub J, Joseph JA. Effect of L-Dopa and the catechol-O-methyltransferase inhibitor Ro 41-0960 on sulfur amino acid metabolites in rats. Clin Neuropharmacol 1997;20(1):55–66.

Nikkhah G, Rosenthal C, Falkenstein G, Roedter A, Papazoglou A, Brandis A. Microtransplantation of dopaminergic cell suspensions: further characterization and optimization of grafting parameters. Cell Transplant 2009;18:119–33.

Nutrient Requirements of Laboratory Animals4th ed. Washington, D.C.: National Academy Press; 1995

O'Suilleabhain PE, Sung V, Hernandez C, Laccritz L, Dewey RBJ, Bottiglieri T, et al. Elevated plasma homocysteine level in patients with Parkinson disease: motor, affective, and cognitive associations. Arch Neurol 2004;61:865–8.

Obeid R, Herrman W. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. FEBS Lett 2006;580:2994–3005.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. San Diego, CA: Academic Press; 2007.

Prins ND, Den Heijer T, Hofman A, Koudstaal PJ, Jolles J, Clarke R, et al. Homocysteine and cognitive function in the elderly: the Rotterdam Scan Study. Neurology 2002;59:1375–80.

Przedborski S, Levivier M, Jiang H, Ferreira M, Jackson-Lewis V, Donaldson D, et al. Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. Neuroscience 1995;67(3):631–47.

Quadri P, Fragiacomo C, Pezzati R, Zanda E, Tettamanti M, Lucca U. Homocysteine and B vitamins in mild cognitive impairment and dementia. Clin Chem Lab Med 2005;43:1096–100.

Religa D, Czyzewski K, Styczynska M, Peplonska B, Lokk J, Chodakowska-Zebrowska M, et al. Hyperhomocysteinemia and methylenetetrahydrofolate reductase

polymorphism in patients with Parkinson's disease. Neurosci Lett 2006;404:56–60.
Rodriquez M, Barroso-Chinea P, Abdala P, Obeso J, González-Hernández T. Dopamine cell degeneration induced by intraventricular administration of 6-hydroxydopamine in the rat: similarities with cell loss. Exp Neurol 2002;169:163–81.
Rogers JD, Sanchez-Saffon A, Frol AB, Diaz-Arrastia R. Elevated plasma homocysteine levels in patients treated with levodopa: association with vascular disease. Arch Neurol 2003;60:59–64.
Sachdev PS, Valenzuela M, Wang XL, Looi JC, Brodaty H. Relationship between plasma homocysteine levels and brain atrophy in healthy elderly individuals. Neurology 2002;58:1539–41.
Sadakierska-Chudy A, Haduch A, Gołembiowska K, Daniel WA. Effects of low doses of intracerebroventricular 6-OHDA on the levels of monoaminergic neurotransmitters in rat brain structures. Pharmacol Rep 2010;62(6):1225–30.
Schwarting RK, Huston JP. The unilateral 6-hydroxydopamine lesion model in behavioral brain research. Analysis of functional deficits, recovery and treatments. Prog Neurobiol 1996;50(2-3):275–331. [Review].
Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217–46.
Selhub J. The many facets if hiperhomocysteinemia: studies from the Framingham Cohorts. J Nutr 2006;136:1726–30.
Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476–83.
Siniscalchi A, Gallelli L, Mercuri NB, Ibbadu GF, Sarro G. Role of lifestyle factors on plasma homocysteine levels in Pakinson's disease patients treated with levodopa. Nutr Neuosci 2006;9:11–6.
Siniscalchi A, Mancuso F, Gallelli L, Ferreri Ibbadu G, Biagio Mercuri N, De Sarro G. Increase in plasma homocysteine levels induced by drug treatments in neurologic patients. Pharmacol Res 2005;52(5):367–75. [Review].
Soto-Otero R, Mendez-Alvarez E, Hermida-Ameijeiras A, Munoz-Patino AM, Labendeira-Garcia JL. Autooxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem 2000;77:1605–12.
Szot P, Franklin A, Sikkema C, Wilkinson CW, Raskind MA. Sequential loss of LC noradrenergic and dopaminergic neurons results in a correlation of dopaminergic neuronal number to striatal dopamine concentration. Front Pharmacol 2012;3:184.
Tatton NA. Increased caspase 3 and bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. Exp Neurol 2000;166:29–43.
Tiemeier H, van Tuijl HR, Hofman A, Meijer J, Kiliaan AJ, Breteler MM. Vitamin B12, folate, and homocysteine in depression: the Rotterdam Study. Am J Psychiatry 2002;159:2099–101.
Todorovic Z, Dzoljic E, Novakovic I, Mirkovic D, Stojanovic R, Nesic Z, et al. Homocysteine serum levels and MTHFH C677T genotype in patients with Parkinson's disease, with and without levopoda therapy. J Neurol Sci 2006;248:56–61.
Ungerstedt U, Arbuthnott GW. Quantitative recording of rotational behavior in rats after 6-hydroxydopamine lesions of the nigrostriatal dopamine system. Brain Res 1970;24(3):485–93.
Xing H, Peng H, Xuebing C, Sun S. Effect and mechanism of homocysteine on Parkinson's disease induced by 6-OHDA. J Nanjing Med Univ 2008;22(1):12–7.
Yasui K, Kowa H, Nakaso K, Takeshima T, Nakashima K. Plasma homocysteine and MTHFR C677T genotype in levodopa treated patients with PD. Neurology 2000;55:437–40.
Zoccolella S, Lamberti P, Armenise E, de Mari M, Lamberti SV, Mastronardi R, et al. Plasma homocysteine levels in Parkinson's disease: role of antiparkinsonian medications. Parkinsonism Relat Disord 2005;11(2):131–3.