Betaine protects cerebellum from oxidative stress following levodopa and benzerazide administration in rats

Masoud Alirezaei 1, 2*

1 Division of Biochemistry, School of Veterinary Medicine, Lorestan University, Khorram Abad, Iran
2 Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorram Abad, Iran

Introduction

It is well known that oxidative stress plays a major role in the neurodegenerative process that underlies Parkinson’s disease (PD) (1, 2). Various experimental studies have also shown that levodopa (LD), the most effective dopaminergic agent for PD, may paradoxically contribute to neuronal damage through formation of free radicals and reactive oxygen species (ROS) (1, 3, 4). In this regard, a previous report indicated increased hydroxyl radical formation in blood cells of PD patients under treatment with LD when compared with both untreated PD patients and healthy subjects (5). Homocysteine (Hcy) is a neuro and vascular toxic sulfur-containing intermediate product. Because the adverse effects of Hcy are most likely related to its prooxidant properties (6), a direct involvement of the amino acid in this phenomenon was hypothesized. Moreover, it has been shown that the elevated plasma Hcy levels found in PD patients treated with LD are associated with a nearly two-fold increased prevalence of coronary artery diseases (7, 8). Previous reports also suggest that elevated plasma Hcy levels may be a risk factor for neuropsychiatric disorders such as stroke, dementia, depression, and PD (6, 8).

Although the brain has defenses against ROS including dietary free radical scavengers (ascorbate, α-tocopherol), the endogenous tripeptide glutathione (GSH), and antioxidant enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT), there is considerable evidence that oxidative damage directly or indirectly, due to free radical production and ROS, can lead to brain injury (6, 9). Hcy passes the blood-brain-barrier (BBB) (10, 11), additional functional disturbance of the BBB leads to an unprotected exposure of the brain to Hcy. Hcy has various consequences for neural cells: oxidative stress, activation of caspases, mitochondrial dysfunction and increase of cytosolic calcium, which contribute to apoptosis (6, 12, 13). Hcy also inhibits the expression of antioxidant enzymes, which might potentiate the toxic effects of ROS (6, 14-16). In
addition, autooxidation of Hcy is known to generate ROS which overload oxidative stress in neurodegenerative disorders (6). In this sense, Hcy is considered a predictor for dementia and Alzheimer’s disease (11, 17, 18). Therefore, the high occurrence of hyperhomocysteinemia in brain disorders and its easy treatment, make Hcy an interesting amino acid in the prevention of neurodegenerative disorders (6).

LD is metabolized by four major metabolic pathways as follows: decarboxylation to dopamine, O-methylation to 3-o-methyl dopa, transamination, and oxidation (19). As Hcy synthesis represents a secondary reaction product of O-methylation of LD to 3-O-methylldopa (3-OMD), one may hypothesize that catecho1-O-methyltransferase (COMT) inhibitors and/or vitamin supplementation may exert a certain preventive effect on the onset of axonal polyneuropathy during LD treatment (20). A previous report demonstrated elevated total homocysteine (tHcy) levels with concomitant tolcapone (as a COMT inhibitor) and/or vitamin intake (21). From this point of view, it is concluded that COMT inhibition and vitamin supplementation only provide a limited impact on tHcy accumulation. In recent years, LD has been used in combination with a dopa decarboxylase inhibitor (DDI), such as benserazide to reduce its peripheral metabolism so as to avoid peripheral toxicity and to enhance its brain penetration in PD (22, 23). However, hyperhomocysteinemia has been considered one of the side effects of this treatment protocol in PD.

In the present study we used betaine (trimethyl-glycine), as a vital methylating agent for prevention of hyperhomocysteinemia and oxidative stress according to our previous reports (24-26). Betaine transfers a methyl group via the enzyme betaine homocysteine methyl transferase (BHMT) to become dimethylglycine (6). It is well-known that BHMT is the only known enzyme that utilizes betaine as a substrate and transfers a methyl group from betaine to Hcy; thereafter Hcy converts to methionine (supplementary file) (24, 25). The formation of methionine from Hcy can occur either via betaine or via 5-methyltetrahydrofolate. Animal studies have shown that both pathways are equally important and that betaine is a vital methylating agent (26). Although betaine can cross the BBB, Hcy remethylation to methionine catalyzed by BHMT occurs mainly in liver (6). Herein, we investigated possible neuroprotective effects of betaine on cerebellum in an animal model. To achieve this goal, we measured lipid peroxidation marker (TBARS), GPx activity and GSH content, which are indicators of oxidative stress, in cerebellum of rats chronically treated by LD and benserazide. We also determined valuable effects of betaine in prevention of hyperhomocysteinemia in rats.

**Materials and Methods**

**Materials**

Dopamine hydrochloride, methanol, thiobarbi-turic acid (TBA), and glutathione (GSH) were supplied by the Merck Chemical Company (KGaA, Darmstadt, Germany). The GPx kit was obtained via Randox ® Company (Antrim, UK). Benserazide [as a DDI] was obtained from Roche® Company, New Zealand. Betaine (Betatin® 96%) was prepared from Biochem Company (Brinckstrasse 55, D-49393 Lohne, Germany). The Hcy enzymatic kit was prepared by Axis® Homocysteine (Axis-Shield AS, UK). LD kindly provided by JALINOOS® Pharmacy (Kara, Alborz province, Iran). Other chemicals used were of analytical grade.

**Experimental design**

A total of 42 adult male Sprague-Dawley rats (weighing 150–170 g) were housed in temperature-controlled conditions under a 12:12 light/dark photoperiod with food and tap water supplied ad libitum. All rats were treated humanely and in compliance with the recommendations of the Animal Care Committee for Lorestan University of Medical Sciences (Khorramabad, Iran). All experimental procedures were carried out between 8:00 am and 5:00 pm for prevention of circadian rhythm changes. The rats were divided into six identical groups (n=7 per group), weight gain and food consumption were determined at 5 day intervals, and they were treated daily for 10 consecutive days orally by gavage in the following order: the control group received 1 ml distilled water, the levodopa (LD) group was treated with LD (3 × 100 mg/kg PO at 8:30 am, 12:30 pm, and 4:30 pm), the betaine (Bet) group received betaine (1.5% w/w of the total diet at 8.00 am), the levodopa plus betaine (LD/Bet) group were treated by LD (3 × 100 mg/kg PO at 4 hr intervals) plus betaine (1.5% w/w of the total diet at 8.00 am), the levodopa plus benserazide (LD/Ben) group received levodopa (3 × (LD 100 mg+Ben 25 mg ) at 4 hr intervals), and the final group, levodopa plus betaine-benserazide, (LD/Bet-Ben) was treated via levodopa+benserazide and betaine. LD, betaine, and benserazide were dissolved in distilled water before administration, daily. Doses of LD and benserazide were determined according to a previous report (8), and betaine was found in our previous works (6, 24, 25). 2 hr after the last gavage, the rats were sacrificed upon light diethyl ether anesthesia (Dagenham, UK). Blood samples were collected via cardiac puncture, in order to provide serum and plasma, separately. The cerebellum was removed and carefully cleaned by cold phosphate buffer (0.1 mol/l, pH 7.4). The samples were stored at −70 °C until biochemical analysis.

**Measurement of dopamine concentration**

Dopamine concentration in serum was measured by the HPLC method as described previously (19). In brief, serum (250 µl) was extracted with 125 µl of 2
TBARS of plasma. The concentration was expressed as micromoles per liter in a study. A mixture of phosphate buffer (KH₂PO₄) at pH = 2.5 and methanol with the ratio of 50/50 (v/v), and flow rate of 1 ml/min was used as mobile phase. The temperature of the column was maintained at 30 °C by a column oven (CTO-10AS VP). Dopamine concentration was expressed as millimol per milliliter (mmol/ml) of serum.

Measurement of tHcy concentration

Total Hcy of plasma which refers to the sum of protein-bound, free-oxidized, and reduced species of Hcy was determined by the Axis® Homocysteine enzymatic kit according to the manufacturer’s instructions, by enzyme immunoassay method (6, 27, 28). The sample volume was used was 25 µl. Absorbance was measured at a wavelength of 450 nm using an ELISA reader (STAT FAX 2100, USA). All estimations were performed in duplicate and the intra assay coefficient of variation was <10%. tHcy concentration was expressed as micromoles per liter (µmol/l) of plasma.

Tissue preparation for protein measurement, TBARS and GSH content and GPx assay

Rat cerebella were thawed and manually homogenized in cold phosphate buffer (0.1 mol/l, pH 7.4), containing 5 mmol/l EDTA, and debris were removed by centrifugation at 2000× g for 10 min. Supernatants were recovered and used for GPx assay, TBARS and GSH concentrations and protein measurement. Protein content of tissue homogenates was determined using the colorimetric method of Lowry with bovine serum albumin as a standard (29).

Measurement of lipid peroxidation

The amount of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the cerebellum. Tissue TBARS was determined by following its production as described previously (30) and reported in our previous works (6, 24, 25). In short, 40 µl of homogenate was added to 40 µl of 0.9% NaCl and 40 µl of deionized H₂O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µl of cold 0.8 mol/l hydrochloric acid, containing 12.5% trichloroacetic acid. Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 hr. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1500× g in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) read at 532 nm, using and extinction coefficient of 1.56×10⁵/M/cm. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nanomoles per mg of tissue protein (nmol/mg protein).

Measurement of GPx activity

The activity of GPx was evaluated using Randox® GPx detection kit according to the manufacturer’s instructions, as reported in our previous works (6, 24, 25). GPx catalyzes the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSGS) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance was measured spectrophotometrically against blank at 340 nm. One unit (U) of GPx was defined as 1 µmol of oxidized NADPH per min per mg of tissue protein. The GPx activity was expressed as unit per mg of tissue protein (U/mg protein).

Measurement of total glutathione (GSH content)

Total GSH was measured by the model as described previously (31), which was reported in our previous work (32). In brief, 20 µl of tissue homogenates was prepared in 20 mmol/l EDTA, pH 4.7, and 100 µl of the homogenate or pure GSH was added to 0.2 mol/l Tris–EDTA (1.0 ml, pH 8.2) buffer (Fluka, Switzerland) and 20 mmol/l EDTA, pH 4.7 (0.9 ml), followed by 20 µl of Ellman’s reagent (10 mmol/l DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. The blank was prepared with the same method; however, instead of 100 µl of the tissue homogenates, 100 µl of distilled water was applied. Both the blank and sample reaction mixtures were read against water at 412 nm. GSH concentration was calculated on the basis of millimolar extinction coefficient of 13.6/M/cm and molecular weight of 307 g. Results for GSH content were expressed as micromoles per mg of tissue protein (µmol/mg protein).

Statistical analysis

All variables were tested for normal and homogeneous variances by the Leven’s test. All results are presented as mean±SEM. Statistical analysis was performed using the statistical package GraphPad PRISM version 5 (GraphPad Software Inc., San Diego, CA, USA) (24, 25). The statistical differences were tested among all groups by one-way analysis of variance (ANOVA) with Tukey’s post hoc analysis. A calculated P-value of less than 0.05 was considered statistically significant.
Results

Treatment of rats with levodopa plus dopamine decarboxylase inhibitor (LD+benserazide) significantly increased tHcy of the LD/Ben group compared to the other groups, while administration of betaine to the LD/Bet group could suppress tHcy increase (P<0.05). Moreover, tHcy in the LD/Bet-Ben group was significantly lower compared to the LD/Ben-treated rats (P<0.05; Figure 1).

Lipid peroxidation (TBARS concentration) increased significantly in LD-treated rats when compared to the other groups (P<0.05). However, the concentrations of TBARS in the LD/Bet-Ben, control and Bet groups were significantly lower when compared to the LD/Bet-treated rats (P<0.05; Figure 2).

The mean values±SEM of the glutathione peroxidase (GPx) activity and GSH content of the cerebellum tissue from control and experimental groups are presented in Figures 3 and 4. GPx activity was significantly higher in the betaine-treated rats when compared to the LD and LD/Ben groups (P<0.05). While betaine treatment could increase GPx activity as significantly in the LD/Bet group in comparison with LD-treated rats (P<0.05), the enhancement of GPx activity in the LD/Bet-Ben group in comparison with LD/Ben-treated rats was not significant (P>0.05). Regarding GSH molecule as a cofactor for GPx activity, GSH content increased significantly in Bet and LD/Bet groups in comparison with LD-, and LD/Ben-treated rats (P<0.05). Although betaine treatment could increase GSH content in the LD/Bet-Ben group in comparison with LD/Ben-treated rats, the enhancement was not significant (P>0.05; Figures 3 and 4).

Regarding LD metabolism, dopamine concentration increased significantly in LD-treated rats in comparison with the LD/Ben group (P<0.05). Moreover, LD/Bet treatment elevated dopamine level significantly when compared to the LD/Ben treatment (P<0.05; Figure 5).

Discussion

There is abundant evidence for oxidative stress in substantia nigra of brain in PD (1). Nevertheless, it remains unknown whether an increased oxidative load produced by LD would induce oxidative stress in cerebellum tissue of PD patients. The results demonstrated, for the first time, the antioxidant and
methyl donor effects of betaine against LD/Ben-induced oxidative stress in cerebellum of rats. The significant increase of tHcy levels in LD/Ben treated rats and the significant decrease of GPx activity and GSH content in LD and LD/Ben groups in comparison with betaine-treated rats, support the LD/Ben-mediated hyperhomocysteinemia and LD-induced oxidative stress in cerebellum of rats. In addition, in line with the previous reports benserazide administration (as a DDI) was associated with a reduction of dopamine level in serum. Based on the present data LD/Ben, as a new drug for PD, induces hyperhomocysteinemia and this effect was more evident than in LD treatment. In contrast, betaine is not only a methyl donor, but it is also an antioxidant agent versus oxidative stress mediated by LD and LD/Ben in the rat cerebellum. Therefore, it appears that the methyl donor and antioxidant properties of betaine are promising particularly in management of plasma tHcy and oxidative stress in cerebellum tissue.

LD, the most effective drug known in the treatment of PD, has been observed to induce elevations in plasma tHcy concentrations (8). The processes of methyl group transfer are involved in the metabolism of LD (4, 33). The main metabolism of LD is its O-methylation to form 3-OMD. The reaction involves the enzyme COMT, with S-adenosyl methionine (SAM) forms S-adenosyl homocysteine (SAH), which is hydrolyzed to Hcy. Hcy is then metabolized via a remethylation cycle, which leads back to methionine, or a transsulfuration pathway, forming cystathionine (supplementary file). It is well known that catabolism of LD interferes, at various steps, with Hcy metabolism. Indeed, there is experimental evidence that LD administration increases tHcy levels in plasma and is able to increase cerebellar SAH (34, 35). In the present study, betaine as a methyl donor agent that continuously generates SAM could decrease tHcy level as significantly in the LD/Bet group when compared to LD-treated rats. This effect is in agreement with our previous reports (6, 24-26).

Putative therapeutic approaches for reduction of tHcy levels include vitamin (B12 and folic acid) supplementation because folic acid and cyanocobalamin catalyze and enhance metabolism of Hcy into methionine (26). A further hypothetical therapeutic alternative would be application of peripheral acting COMT inhibitors as adjunctives to levodopa/dopa decarboxylase inhibitor (LD/DDI) treatment. The COMT inhibitors increase the peripheral bioavailability of LD. The combination of LD with a DDI such as benserazide also reduces decarboxylation of LD in peripheral tissues and increases bioavailability of LD for central nervous system (CNS) (22). In contrast, LD in peripheral tissues increases tHcy in plasma by COMT. However, a previous report indicated an elevation of tHcy levels with concomitant tolcapone (as a COMT inhibitor) (21), it is concluded that COMT inhibitors only provide a limited impact on tHcy increasing. In the present study, LD is metabolized to 3-OMD in the presence of benserazide by COMT. COMT is the essential enzyme for this O-methylation of LD, which demands for a methyl group transfer from the donor SAM (20). As one consequence, SAM is transformed into SAH and then to Hcy. In this setting, tHcy level in LD/Ben-treated rats (Figure 1) well indicates the bioavailability of LD and its conversion to Hcy in comparison with other groups. Thus, we assume that a certain balance between LD and betaine is developed during this treatment protocol. Indeed, betaine supplementation exerted a certain preventive effect on the onset of hyperhomocysteinemia in LD/Bet and LD/Bet-Ben groups.

During the past decade, SAM was used as a co-adjuvant in depression and has a potential
neuroprotective effect in animal models (36). SAM application also increases brain GSH levels via intracellular biochemical pathways of transsulfuration and decreases membrane lipid peroxidation caused by free radical damage, acting as a potential antioxidant drug (6, 36, 37). In theory, good titration and carefully monitoring SAM supplementation with normalizing of reduced SAM levels in PD patients may cause an effective reduction of Hcy levels, while elevated SAM dosage may also worsen motor symptoms or even accelerate neuronal cell death via the apoptotic pathway (22, 38). Betaine is a methylating agent like SAM and it also stabilizes SAM levels via remethylation. In this sense, betaine indicated similar methyl donor effects against ethanol-induced hyperhomocysteinemia in rats and rabbits (6, 26). Betaine treatment may also have some advantages over endogenous SAM application; because SAM application enhances the levels of Hcy, which is undesirable due to the toxicity of this amino acid, whereas betaine treatment decreases Hcy levels by directly inducing the remethylating process, which transforms Hcy into methionine (6, 37, 39). Furthermore, since humans produce little betaine from choline due to lack of choline oxidase (40), betaine is practical for investigations in hyperhomocysteinemia conditions such as neurodegenerative disorders.

The brain is more vulnerable to oxidative stress than other organs due to its low antioxidant protection system and increased exposure of target molecules to ROS (6). The nervous tissue has a high content of polyunsaturated fatty acids, which are easy targets to oxidative damage by free radicals (6, 41). In our study, LD consumption caused significantly increased TBARS concentration (as a lipid peroxidation marker) in the LD-treated rats, and betaine treatment restored this elevated TBARS concentration in the LD/Bet-treated rats to near the LD/Ben group level. On the other hand, there were significant differences between LD and the other groups in TBARS, indicating occurrence of oxidative stress in LD-treated rats. LD/Ben group also indicated increased TBARS level, but this elevation was not significant when compared to the control group. The results of lipid peroxidation were well consistent with our previous report on ethanol-induced oxidative stress in cerebellum of rats (6). Betaine plays a significant role in maintaining the structural and functional integrity of cell membranes. Betaine through its participation in sequential methylation within the cellular membranes maintains a proper balance between phosphatidylethanolamine and phosphatidylcholine, thus sustaining proper membranes and prevents lipid peroxidation (6, 42, 43).

Animal models have shown that a number of antioxidants prevent oxidative brain injury through a variety of cellular mechanisms which have described oxidative damage on the CNS (6, 9, 44, 45). GSH antioxidant system plays a fundamental role in cellular defense against ROS. The cellular tripeptide GSH thwarts peroxidative damage by neutralizing the free radicals (42). In the present study, GPx activity and GSH content were increased as significantly in betaine- and LD/Bet-treated rats when compared to LD and LD/Ben groups. It is well known that GPx and CAT are two key antioxidant enzymes that can decompose hydrogen peroxide to water (6). Although, H₂O₂ is not a particularly reactive product, it can be reduced to the highly reactive metabolites hydroxyl radicals (6, 24, 25). The increase in cerebellar GPx activity in betaine-treated rats correlates well with the increase of GSH content as a cofactor for GPx activity in cerebellum. The protective effect of betaine against LD-induced oxidative stress observed in this study may be associated with the restoration of SAM. The increase in SAM concentration contributes through an increase in the supply of substrate needed for the synthesis of GSH (6, 42).

As previously mentioned, LD is metabolized with four major metabolic pathways. The principal path is decarboxylation, whereby LD converts to dopamine via L-dopa decarboxylase (19). In the present study, the co-administration of benserazide with LD results in increased metabolism of LD to 3-O-MD via the enzyme COMT in peripheral tissues, thus, dopamine concentration in LD/Ben-treated rats in comparison with LD and LD/Bet groups is decreased (Figure 5). We measured dopamine concentration in blood to evaluate decarboxylation pathway of LD in peripheral tissues. Because dopamine does not go through BBB, the serum dopamine level after application of LD, betaine and benserazide shall not reflect dopamine concentration in the CNS. In this setting, benserazide treatment prevented LD metabolism to dopamine in the peripheral tissues and is able to enhance dopamine in CNS. However, dopamine measurement in the CNS is needed to clarify the drug’s effects in a future experimental study.

Conclusion

The present study demonstrates that betaine may have a potential as a neuroprotective agent for prevention of LD-induced oxidative damage in cerebellum and benserazide-mediated hyperhomocysteinemia in rats. However, further studies including physiological parameters and histochemical techniques should be performed to validate this hypothesis.

Acknowledgment

This study was financially supported by research project (no. 90/36) of Razi Herbal Medicines.
Animal studies

All rats were treated humanely and in compliance with the recommendations of Animal Care Committee of Lorestan University of Medical Sciences (Khorramabad, Iran).

References

1. Jennen P. Oxidative Stress in Parkinson’s Disease. Ann Neurol 2003; 53:S26–S38.
2. Ossig C, Reichmann H. Treatment of Parkinson’s disease in the advanced stage. J Neural Transm 2013; 120:523–529.
3. Basm MA, Morris EJ, Nicklas WJ, Geller HM. L-DOPA cytotoxicity to PC12 cells in culture is via auto-oxidation. J Neurochem 1995; 64:825–832.
4. Blundell F, Fancellu R, Martignoni E, Mangiagalli A, Pacchetti C, Samuele A, et al. Plasma Homocysteine and L-DOPA Metabolism in Patients with Parkinson Disease. Clin Chem 2001; 47:1102–1104.
5. Martignoni E, Blundell F, Goli L, Desideri S, Pacchetti C, Mancini F, et al. Peripheral markers of oxidative stress in Parkinson’s disease. The role of L-DOPA. Free Radic Biol Med 1999; 27:428–437.
6. Alirezaei M, Jelodar G, Niknam P, Ghayemi Z, Nazifi S. Betaine prevents ethanol-induced oxidative stress and reduces total homocysteine in the rat cerebellum. J Physiol Biochem 2011; 67:605–612.
7. 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.
8. Nissinen E, Nissinen H, Larjonmaa H, Vaananen A, Helkamaa T, Reenila I, et al. The COMT inhibitor, entacapone, reduces levodopa-induced elevations in plasma homocysteine in healthy adult rats. J Neural Transm 2005; 112:1213–1221.
9. Dal-Pizzol F, Ritter C, Cassol Jr OL, Rezin GT, Petronilho F, Zugno AI, et al. Oxidative mechanisms of brain dysfunction during sepsis. Neurochir Res 2010; 35:1–12.
10. Lee ES, Chen H, Soliman KF, Charlton GO. Effects of homocysteine on the dopaminergic system and behavior in rodents. Neurotoxicology 2005; 26:361–371.
11. Lutz UC. Alterations in homocysteine metabolism among alcohol dependent patients—clinical, pathobiocchemical, and genetic aspects. Curr Drug Abuse Rev 2008; 1:47–55.
12. Chern CL, Huang RF, Chen YH, Cheng JT, Liu TZ. Folate deficiency-induced oxidative stress and apoptosis are mediated via homocysteine-dependent overproduction of hydrogen peroxide and enhanced activation of NF-κappaB in human Hep G2 cells. Biomed Pharmacother 2001; 55:434–42.
13. Ho PI, Ortiz D, Rogers E, Shea TB. Multiple aspects of homocysteine neurotoxicity: glutamate excitotoxicity, kinase hyperactivation and DNA damage. J Neurosci Res 2002; 70:694–702.
14. Bleich S, Degner D, Sperling W, Bönsch D, Thürauf N, Kornhuber J. Homocysteine as a neurotoxin in chronic alcoholism. Prog Neuropsychopharmacol Biol Psychiatry 2004; 28:453–464.
15. Huang RF, Huang SM, Lin BS, Wei JS, Liu TZ. Homocysteine thiocyanate induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci 2001; 68:2799–2811.
16. Austin RC, Sood SK, Dorward AM, Singh G, Shaughnessy SG, Hamid P, et al. Homocysteine dependent alterations in mitochondrial gene expression, function and structure. Homocysteine and H2O2 act synergistically to enhance mitochondrial damage. J Biol Chem 1998; 273:30808–30817.
17. Seshadri S, Beiser A, Selhub J. Plasma homocysteine as a risk factor for dementia and Alzheimer’s disease. N Engl J Med 2002; 346:476–483.
18. Morris MS. Homocysteine and alzheimer’s disease. Lancet Neurol 2003; 2:425–428.
19. Muzzi C, Bertocci E, Terzuoli L, Porcelli B, Ciarì L, Pagani R, et al. Simultaneous determination of serum concentrations of levodopa, dopamine, 3-O-methyldopa and α-methyldopa by HPLC. Biomed Pharmacother 2008; 62:253–258.
20. Muller T, Juge C, Ehret R, Ebersbach G, Bengel G, Muhlick S, et al. Elevation of total homocysteine levels in patients with Parkinson’s disease treated with duodenal levodopa/carbidopa gel. J Neural Transm 2011; 118:1329–1333.
21. Muller T, Erdmann C, Muhlick S, Bremen D, Prunetek H, Goetze O, et al. Pharmacokinetic behavior of levodopa and 3-O-methyldopa after repeat administration of levodopa/carbidopa with and without entacapone in patients with Parkinson’s disease. J Neural Transm 2006; 113:1441–1448.
22. Muller T, Renger K, Kuhn W. Levodopa-associated increase of homocysteine levels and sural axonal neurodegeneration. Arch Neurol 2004; 61:657–660.
23. Schapira AH. The clinical relevance of levodopa neurotoxicity in the treatment of Parkinson’s disease. Mov Disord 2008; 23:S15–S20.
24. Alirezaei M, Jelodar G, Ghayemi Z. Antioxidant defense of betaine against oxidative stress induced by ethanol in the rat testes. Int J Pept Res Ther 2012; 18:239–247.
25. Alirezaei M, Niknam P, Jelodar G. Betaine elevates ovarian antioxidant enzyme activities and demonstrates methyl donor effect in non-pregnant rats. Int J Pept Res Ther 2012; 18:281–290.
26. Alirezaei M, Saeb M, Javidnia K, Nazifi S, Saeb S. Hyperhomocysteinemia reduction in ethanol-fed rabbits by oral betaine. Comp Clin Pathol 2012; 21:421–427.
27. Golbahar J, Aminzadeh MA, Hamidi SA, Omran GR. Association of red blood cell 5-methyltetrahydrofolate folate with bone mineral density in postmenopausal Iranian women. Osteoporos Int 2005; 16:1894–1898.
28. Karthikeyan G, Thachil A, Sharma S, Kalaivani M, Ramakrishnan L. Elevated high sensitivity CRP levels in patients with mitral stenosis and left atrial thrombus. Int J Cardiol 2007; 122:252–254.
Betaine protects cerebellum from oxidative stress

29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.
30. Subbarao KV, Richardson JS, Ang LC. Autopsy samples of Alzheimer's cortex show increased peroxidation in vitro. J Neurochem 1990; 55:342-345.
31. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968; 25:192-205.
32. Neamati S, Alirezaei M, Kheradmand A. Ghrelin acts as an antioxidant agent in the rat kidney. Int J Pept Res Ther 2011; 17:239-245.
33. Benson R, Crowell B Jr, Hill B, Doonquah K, Charlton C. The effects of L-Dopa on the activity of methionine adenosyltransferase: relevance to L-Dopa therapy and tolerance. Neurochem Res 1993; 18:325-330.
34. 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; 20:55-66.
35. Liu XX, Wilson K, Charlton CG. Effects of L-DOPA treatment on methylation in mouse brain: implications for the side effects of L-DOPA. Life Sci 2000; 66:2277-2288.
36. De La Cruz JP, Pavi'a J, González-Correa JA. Effects of chronic administration of S-adenosyl-L-methionine on brain oxidative stress in rats. Naunyn Schmiedebergs Arch Pharmacol 2000; 361:47-52.
37. Kanbak G, Arslan OC, Dokumacioglu A, Kartkaya K, Inal ME. Effects of Chronic Ethanol Consumption on Brain Synaptosomes and Protective Role of Betaine. Neurochem Res 2008; 33:539-544.
38. Zhao WQ, Williams Z, Shepherd KR. S-adenosyl-methionine-induced apoptosis in PC12 cells. J Neurosci Res 2002; 69:519-529.
39. Sachdev PS, Valenzuela M, Brodaty H, Wang XL, Looi J, Lorentz L, et al. Homocysteine as a risk factor for cognitive impairment in stroke patients. Dement Geriatr Cogn Disord 2003; 15:155-162.
40. Haubrich DR, Gerber NH. Choline dehydrogenase. Assay, properties and inhibitors. Biochem Pharmacol 1981; 30:2993.
41. Smith AM, Zeve DR, Grisel JJ, Chen WJA. Neonatal alcohol exposure increases malondialdehyde (MDA) and glutathione (GSH) levels in the developing cerebellum. Dev Brain Res 2005; 160:231-238.
42. Kharbanda KK, Mailiard ME, Baldwin CR, Beckenhauer HC, Sorrell MF, Tuna DJ. Betaine attenuates alcoholic steatosis by restoring phosphatidylincholine generation via the phosphatidylethanolamine methyltransferase pathway. J Hepatol 2007; 46:314-321.
43. Ganeshan B, Buddhan S, Anandan R, Sivalumaran R, Anbinezhilan R. Antioxidant defense of betaine against isoprorenaline-induced myocardial infarction in rats. Mol Biol Rep 2010; 37:1319-1327.
44. Vajragupta O, Boonyarat C, Murakami Y. A novel neuroprotective agent with antioxidant and nitric oxide synthase inhibitory action. Free Radic Res 2006; 40:685-695.
45. Wang ZJ, Liang CL, Li GM. Neuroprotective effects of arachidonic acid against oxidative stress on rat hippocampal slices. Chem Biol Interact 2006; 163:207-217.