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

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ABSTRACT

Objective(s): The aim of the present study was to evaluate antioxidant and methyl donor effects of betaine in cerebellum following levodopa and benserazide administration in rats.

Materials and Methods: Sprague-Dawley male rats were treated with levodopa (LD), betaine (Bet), levodopa plus betaine (LD/Bet), levodopa plus benserazide (LD/Ben), levodopa plus betaine-benserazide (LD/Bet-Ben), and the controls with vehicle for 10 consecutive days, orally.

Results: Treatment of rats with LD and benserazide significantly increased total homocysteine in plasma of the LD/Ben group when compared to the other groups. Lipid peroxidation of cerebellum increased significantly in LD-treated rats when compared to the other groups. In contrast, glutathione peroxidase activity and glutathione content in cerebellum were significantly higher in the betaine-treated rats when compared to the LD and LD/Ben groups. Serum dopamine concentration increased significantly in LD-treated rats in comparison with the LD/Ben group. LD/Bet-treated rats also demonstrated significantly higher dopamine levels when compared to the LD/Ben group.

Conclusion: We observed valuable effects of Bet in combination with LD and benserazide, which routinely were used for Parkinson’s disease (PD) treatment, in experimentally-induced oxidative stress and hyperhomocysteinemia in rats. Therefore, it seems that Bet is a vital and promising agent regarding PD for future clinical trials in humans.

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).
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 catechol-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 (trimethylglycine), 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, thiobarbituric 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 (Betafin® 96%) was prepared from Biochem Company (Brinkstrasse 55, D-49393 Lohn, 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+benserazide (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
mol/1 HClO₄, and the extract was centrifuged for 10 min at 500 ×g (Centrifuge 5415 R; Rotofix 32 A, Germany), the supernatant (20 μl) injected into the HPLC system. Analytical reversed phase high performance liquid chromatography (Shimadzu, LC-10AD VP, Japan) with a UV-Vis detector (SPD-10AVP) and reverse phase column: RP-18, 250 × 4.6 mm, 5 μm, was used in this 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-10 AS VP). Absorbance was measured at a wavelength of 450 nm.

TBARS and GSH concentrations and protein measurement

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Measurement of TBARS

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 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).

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Tissue preparation for protein measurement

Tissue preparation for protein measurement

TBARS and GSH content and GPx assay

Rat cerebellums 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 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 (GSSG) 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).

Statistical analysis

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 dopa-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 GSH 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.

Figure 4. The effects of levodopa, betaine and benserazide treatments on glutathione content (GSH) in control and treated rats (n=7 per group). Values represent mean±SEM of GSH (micromoles per milligram protein of cerebellum tissue). *; denotes a significant difference (P<0.05) versus levodopa and LD/Bet groups. **; denotes a significant difference (P<0.05) versus levodopa and LD/Ben groups. NS; not significant. LD; levodopa, Bet; betaine, Ben; benserazide.

Figure 5. The effects of levodopa, betaine and benserazide treatments on dopamine levels in control and treated rats (n=7 per group). Values represent mean±SEM of dopamine (mmol/ml serum). *; denotes a significant difference (P<0.05) versus levodopa and LD/Bet groups. NS; not significant. LD; levodopa, Bet; betaine, Ben; benserazide.

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 adjutives 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...
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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, H$_2$O$_2$ 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.

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