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ORIGINAL PAPER

Beneficial antioxidant properties of betaine against oxidative stress mediated by levodopa/benserazide in the brain of rats

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Abstract The present study was designed to evaluate antioxidant effects of betaine in the brain following administration of levodopa and benserazide, which are routinely used in the treatment of Parkinson's disease. Sprague–Dawley male rats were divided into levodopa (LD), Betaine (Bet.), levodopa plus betaine (LD/Bet.), levodopa plus benserazide (LD/Ben.), levodopa plus betaine-benserazide (LD/Bet.-Ben.) and control groups. The experimental groups received LD 300 mg/kg, Bet. 1.5 % w/w of the total diet, Ben. 75 mg/kg and distilled water to controls for 10 consecutive days, orally. The concentration of plasma total homocysteine significantly increased in LD/Ben.-treated rats when compared to the other groups. Brain glutathione peroxidase (GPx) activity and glutathione content both elevated with betaine treatment in LD/Bet. and LD/Bet.-Ben groups. Superoxide dismutase activity was also higher in controls and betaine-treated rats in comparison with LD and LD/Ben. groups. Likewise, catalase activity significantly increased in control and betaine groups when compared to LD- and LD/Ben.-treated rats. In contrast, brain lipid peroxidation significantly increased in response to LD and LD/Ben. treatments. Regarding metabolism of LD in peripheral tissues, serumic dopamine concentration significantly increased in LD-treated rats in comparison with LD/Ben. group. The present results show beneficial antioxidant and methyl donor properties of betaine versus oxidative stress and hyperhomocysteinemia induced by levodopa and benserazide in an animal model.

Keywords Brain · Parkinson's · Betaine · Homocysteine · Levodopa · Benserazide

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Introduction

The evidence for the occurrence of oxidative stress in Parkinson's disease (PD) is overwhelming, and there is little doubt that oxidative stress leads to an increase in oxidative damage in the brain [1–6]. The degradation of dopamine by monoamine oxidase-B (MAO-B) to produce hydrogen peroxide (H_2O_2) further emphasizes how oxidative stress might arise in the substantia nigra (SN) of the brain [7]. Enzymatic oxidation of dopamine to H_2O_2 caused increased formation of oxidized glutathione, suggesting the occurrence of oxidative stress and impairment of a major antioxidant system [8]. Importantly, H_2O_2 also converts by Fenton reactions to produce the highly toxic hydroxyl radical in the presence of the high levels of iron

normally found in SN [9]. Oxidative stress may also be intimately linked to other processes associated with cell death, such as mitochondrial dysfunction, inflammation, excitotoxicity, and the toxic effects of nitric oxide [7]. Although the brain has defenses against reactive oxygen species (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) [10, 11], there has not yet been agreement over whether changes occur in GPx, SOD, and CAT activities, as well as GSH content in substantia nigra pars compacta (SNC) for PD patients.

Homocysteine (Hcy) is a neuro and vascular toxic sulphur-containing intermediate product [11], and passes across the blood–brain barrier (BBB) [12, 13]. Hcy has various consequences for neural cells: oxidative stress, activation of caspases, mitochondrial dysfunction and increase of cytosolic calcium, which contribute to apoptosis [11, 14, 15]. Hcy also inhibits the expression of antioxidant enzymes which might potentiate the toxic effects of ROS [11, 16, 17]. In addition, autooxidation of Hcy is known to generate ROS which overload oxidative stress in neurodegenerative disorders [11]. Because the adverse effects of Hcy are most likely related to its prooxidant properties [11, 18], a direct involvement of the amino acid in PD progression is hypothesized. In this sense, Hcy is considered as a predictor for dementia and Alzheimer's disease [13, 19, 20]. Previous reports also suggest that elevated plasma Hcy levels may be a risk factor for neuropsychiatric disorders such as dementia, depression and PD [11, 21]. It is well known that catabolism of levodopa (LD), as the most effective dopaminergic drug for PD, interferes with Hcy metabolism. Indeed, there is experimental evidence that LD administration increases tHcy levels in plasma [22]. 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 patients [23, 24]. Inhibition of dopa decarboxylase results in the elevation of tHcy by catechol-*O*-methyltransferase (COMT) [25]. Therefore, hyperhomocysteinemia is a major problem in LD or LD/benserazide (LD/Ben.) treatment.

Betaine (trimethylglycine) transfers a methyl group via the enzyme betaine homocysteine methyl transferase (BHMT) to become dimethylglycine [11, 26, 27]. BHMT is the only known enzyme that uses betaine as a substrate, mediates the transfer of a methyl group from betaine to Hcy, and converts Hcy to methionine (Supplementary file 1) [11, 28]. The formation of methionine from Hcy can occur either via betaine or via 5-methyltetrahydrofolate [11, 29]. Animal studies have shown that both pathways are equally important and that betaine is a vital methylating agent [11, 30, 31]. Although betaine can across the BBB, Hcy remethylation to methionine catalyzed by BHMT occurs mainly in liver [32, 33]. Thus, one may hypothesize that betaine administration can exert as a methyl donor to reduce tHcy level in the brain. Our previous reports are in agreement with this hypothesis, with betaine being demonstrated to be a vital methylating and antioxidant agent for prevention of hyperhomocysteinemia and oxidative stress in cerebellum, testis, ovary and liver of rats [11, 26, 27, 34].

In line with the previous experimental research, we hypothesized that, through betaine treatment as an antioxidant agent, it may be possible to prevent the onset of oxidative stress in forebrain and brainstem regions. For this purpose, the oral administration of betaine concomitant with LD and benserazide, as new drugs for PD treatment, were applied in an experimentally induced oxidative stress and hyperhomocysteinemia in rats. We also investigated how tHcy of plasma and dopamine of serum varied with betaine, LD, and benserazide therapy in rats.

Materials and methods

Materials

The GPx and SOD kits were obtained via Randox® (Antrim, UK). Dopamine hydrochloride, methanol, thiobarbituric acid (TBA) and glutathione (GSH) were supplied from Merck (Darmstadt, Germany). Benserazide (as a dopa-decarboxylase inhibitor) was obtained from Roche®, New Zealand. Betaine (Betafin® 96 %) was prepared from Biochem (Lohne, Germany). The homocysteine enzymatic kit was prepared by Axis® Homocysteine (Axis-Shield, UK). Levodopa was kindly prepared from JALINOOS® Pharmacy (Karaj, 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-h light/dark photocycle with food and tap water supplied ad libitum. All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for Lorestan University of Medical Sciences (Khorramabad, Iran). All the experimental procedures were carried out between 08:00 and 17:00 hours to prevent circadian rhythm changes between days. The rats were divided into six equal groups, with weight gain and food consumption were determined at 5-day intervals, and 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 were treated with LD (3 × 100 mg/kg p.o. at 08:30, 12:30 and 16:30 hours), the betaine (Bet.) group received betaine (1.5 % w/w of the total diet at 08:00 hours), the levodopa plus betaine (LD/Bet.) group were treated by LD (3 × 100 mg/kg p.o. at 4-h intervals) plus betaine (1.5 % w/w of the total diet at 08:00 hours), the levodopa plus benserazide (LD/Ben.) group received LD and benserazide [3 × (LD 100 mg + Ben. 25 mg) at 4-h intervals], and the final group, levodopa plus betaine-benserazide (LD/Bet.-Ben.), were treated via LD, benserazide and betaine based on the above-mentioned dosage. Betaine, LD, and benserazide were dissolved daily in distilled water before administration. Doses of levodopa and benserazide were determined according to a previous report [21], and betaine will be found in our previous work [11, 26, 27]. Two hours after the last gavage, the rats were sacrificed by light diethyl ether (Dagenham, UK) anesthesia. Blood samples were collected via cardiac puncture in order to provide serum and plasma. The cerebellum and the brain were carefully cleaned by phosphate buffer and dissected with a scalpel. The brain, sera and plasma samples were stored at −70 °C for biochemical analysis, up to 2 months later. For evaluation of histological results, the left hemisphere samples of the experimental groups were removed and after processing stained with hematoxylin and eosin.

Measurement of dopamine concentration

Dopamine concentration in serum was measured by HPLC method as described previously [35]. In brief, 250 µl of serum was extracted with 125 µl of 2 M $HClO_4$ and the extract was centrifuged for 10 min at 500*g* (Centrifuge 5415 R; Rotofix, Germany). The supernatant (20 µl) injected into HPLC system. Analytical reversed phase high performance liquid chromatography (LC-10AD VP; Shimadzu, Japan) with a UV–Vis detector (SPD-10AVP) and reverse phase column (RP-18, 250 × 4.6 mm, 5 µm) was used. A mixture of phosphate buffer (KH_2PO_4) 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 homocysteine of plasma, which refers to the sum of protein-bound, free-oxidized, and reduced species of homocysteine, was determined by the Axis® Homocysteine enzymatic kit [11, 36, 37]. tHcy concentration was expressed as micromoles per liter (µmol/l) of plasma.

Tissue preparation for biochemical analysis

Forebrain and brainstem (approximately <1 g of mesencephalic sections) of rats were taken according to our previous report [1]. The samples were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH 7.4), containing 5 mM EDTA, and debris were removed by centrifugation at 2000 RPM for 10 min (MA & MK method). Supernatants were recovered and used for GPx, SOD, and CAT assay, TBARS and GSH concentrations, and protein measurement. Protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as a standard [38].

Measurement of lipid peroxidation

The amount of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the brain homogenates. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described previously [8], as also reported by Alirezaei et al. [11, 26, 27]. In short, 40 µl of homogenate was added to 40 µl of 0.9 % NaCl and 40 µl of deionized H_2O, 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 M hydrochloride 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 h. 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; WPA, Cambridge, UK) read at 532 nm, using an extinction coefficient of 1.56×10^5/M cm. The blanks for all 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 milligram of tissue protein (nmol/mg protein).

Measurement of GPx activity

The activity of glutathione peroxidase (GPx) was evaluated with a Randox® GPx detection kit according to the manufacturer's instructions, as reported by Alirezaei et al. [11, 26, 27]. GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidised 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

milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein (U/mg protein).

Measurement of total glutathione (GSH content)

Total glutathione was measured by the model as described previously [39], as reported by Neamati et al. [40]. In brief, 20 µl tissue homogenates were prepared in 20 mM EDTA, pH 4.7, and 100 µl of the homogenate or pure GSH was added to 0.2 mM Tris–EDTA (1.0 ml, pH 8.2) buffer (Fluka, Switzerland) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman's reagent (10 mM DTNB in methanol). After 30 min of incubation at room temperature, the 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 a millimolar extinction coefficient of 13.6/M cm and a molecular weight of 307 g. Results for GSH content were expressed as micromoles per milligram of tissue protein (µmol/mg protein).

Measurement of SOD activity

The activity of superoxide dismutase (SOD) was evaluated with the Randox® SOD detection kit according to the manufacturer's instructions, as reported by Alirezaei et al. [11, 26, 27]. The role of SOD is to accelerate the dismutation of the toxic superoxide (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured as the degree of inhibition of this reaction. One unit of SOD is that which causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve, and expressed as unit per milligram of tissue protein (U/mg protein).

Measurement of CAT activity

Tissue catalase activity was assayed using the method as described previously [41], and reported by our laboratory [11, 26, 27]. The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2, and a 20- to 50-µl sample. The reaction was initiated by the addition of H_2O_2 and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6/M cm. The CAT activity was expressed as the unit defined as µmol of H_2O_2 consumed per min per mg of tissue protein (U/mg protein).

Statistical analysis

Statistical analysis was performed using the statistical package GraphPad PRISM version 5 (GraphPad Software, San Diego, CA, USA). All variables were tested for normal and homogeneous variances by Leven's statistic test. All results are presented as mean ± SEM. The statistical differences were applied among the 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 + benserazide (levodopa plus dopa-decarboxylase inhibitor) significantly increased tHcy in plasma of the LD/Ben. group compared to the other groups, while administration of betaine in LD/Bet. group could suppress the increase of tHcy ($P < 0.05$). Moreover, tHcy in the LD-treated rats was significantly higher as compared to the control, betaine, and LD/Bet. groups ($P < 0.05$; Fig. 1).

Lipid peroxidation (TBARS concentration) increased significantly in LD-treated rats when compared to the other groups ($P < 0.05$). The concentrations of TBARS in the LD/Ben.-treated rats also increased significantly as compared to control, betaine, LD/Bet. and LD/Bet.-Ben. groups

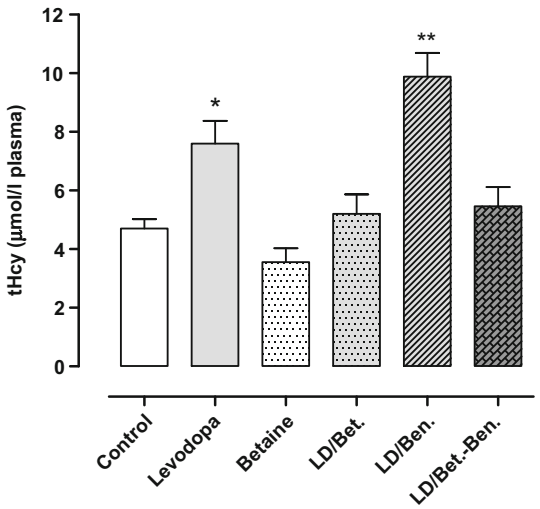

Fig. 1 The effects of levodopa, betaine and benserazide treatments on plasma total homocysteine (tHcy) concentration among the control and treated rats. Values represent mean ± SEM of tHcy (µmol/l of plasma). *$P < 0.05$ versus control, betaine and LD/Bet. groups. **$P < 0.05$ versus control, levodopa, betaine, LD/Bet. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

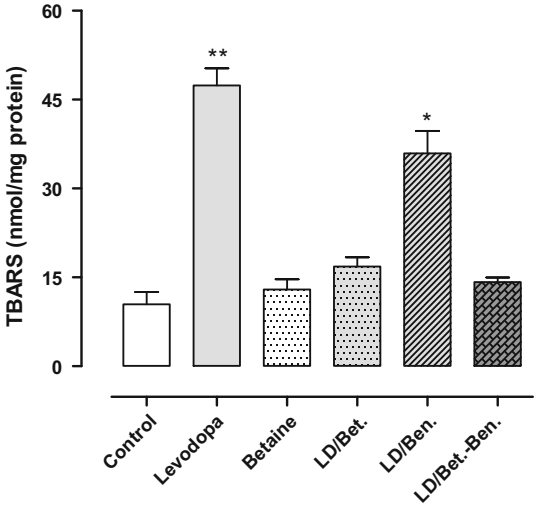

Fig. 2 The effects of levodopa, betaine and benserazide treatments on thiobarbituric acid reactive substances (TBARS) concentration in control and treated rats. Values represent mean ± SEM of TBARS (nanomoles per milligram protein of brain tissue). *$P < 0.05$ versus control, betaine, LD/Bet. and LD/Bet.-Ben. groups. **$P < 0.05$ versus control, betaine, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

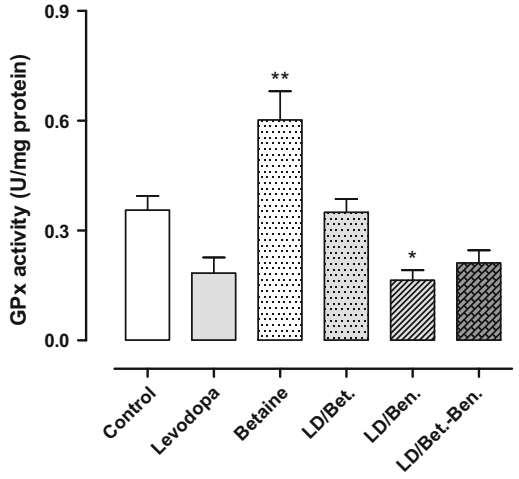

Fig. 3 The effects of levodopa, betaine and benserazide treatments on GPx activity in control and treated rats. Values represent mean ± SEM of enzyme activity (unit/mg protein of brain tissue). *$P < 0.05$ versus control group. **$P < 0.05$ versus control, levodopa, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

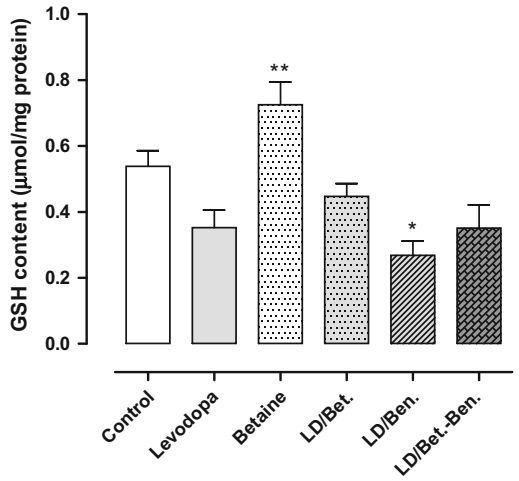

Fig. 4 The effects of levodopa, betaine and benserazide treatments on glutathione content (GSH) in control and treated rats. Values represent mean ± SEM of GSH (micromoles per milligram protein of brain tissue). *$P < 0.05$ versus control group. **$P < 0.05$ versus levodopa, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

($P < 0.05$). Indeed, LD and benserazide treatments both elevated lipid peroxidation significantly in comparison with betaine treatment (Fig. 2).

The mean values (± SEM) of the antioxidant enzyme activities (GPx, SOD, and CAT) and glutathione (GSH content) in mesencephalic sections of the brain are presented in Figs. 3, 4, 5 and 6. The GPx activity (as an antioxidant enzyme) in LD/Ben.-treated rats was significantly lower when compared to the control group ($P < 0.05$). While betaine treatment could increase GPx activity as significantly in the betaine-treated rats in comparison with control, LD, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups ($P < 0.05$; Fig. 3).

In respect to GSH content, as a cofactor for GPx activity, GSH content increased significantly in the betaine group in comparison with LD-, LD/Bet.-, LD/Ben.- and LD/Bet.-Ben.-treated rats ($P < 0.05$). The GSH content in controls was also significantly higher when compared to the LD/Ben. group ($P < 0.05$). Although LD treatment could increase GSH content in the LD group (by a compensatory mechanism) in comparison with LD/Ben.-treated rats, the enhancement was not significant ($P > 0.05$; Fig. 4).

SOD activity (as an antiperoxidative enzyme) was significantly higher in the betaine group compared to the LD-, LD/Bet.-, LD/Ben.- and LD/Bet.-Ben.-treated rats ($P < 0.05$). The controls also indicated significantly higher SOD activity when compared to LD- and LD/Ben.-treated rats ($P < 0.05$; Fig. 5).

CAT activity (another antiperoxidative enzyme) was significantly higher in the betaine group compared to the LD-, LD/Bet.-, LD/Ben.- and LD/Bet.-Ben.-treated rats ($P < 0.05$). The controls also indicated significantly higher CAT activity when compared to LD-, LD/Bet.-, LD/Ben.- and LD/Bet.-Ben.-treated rats ($P < 0.05$; Fig. 6).

In respect of LD metabolism in peripheral tissues, serumic 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 LD/Ben. treatment ($P < 0.05$; Fig. 7).

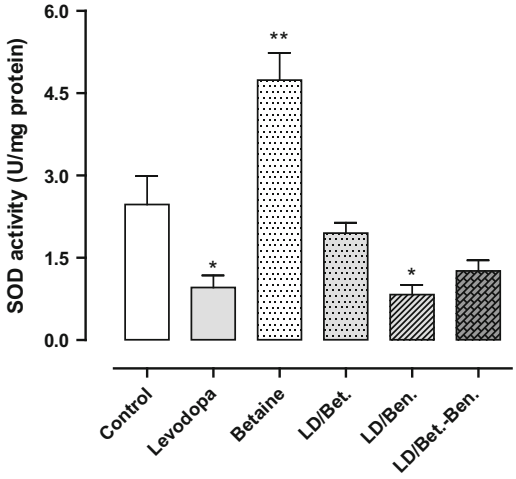

Fig. 5 The effects of levodopa, betaine and benserazide treatments on SOD activity in control and treated rats. Values represent mean ± SEM of enzyme activity (unit/mg protein of brain tissue). $*P < 0.05$ versus control group. $**P < 0.05$ versus levodopa, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

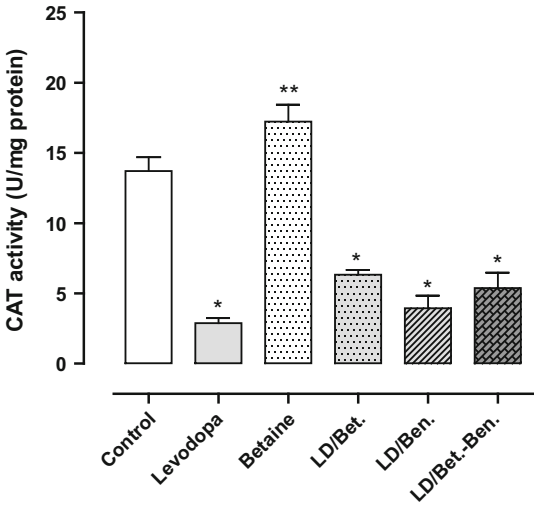

Fig. 6 The effects of levodopa, betaine and benserazide treatments on CAT activity in control and treated rats. Values represent mean ± SEM of enzyme activity (unit/mg protein of brain tissue). $*P < 0.05$ versus control group. $**P < 0.05$ versus levodopa, LD/Bet., LD/Ben. and LD/Bet.-Ben. groups. *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

Regarding histopathological findings, we did not find any histopathological change with levodopa, benserazide and levodopa plus benserazide treatments in the experimental groups (Supplementary file 2).

Discussion

To the best of our knowledge, this study is the first to evaluate the effects of betaine administration on antioxidant status in the mesencephalic section of the brain

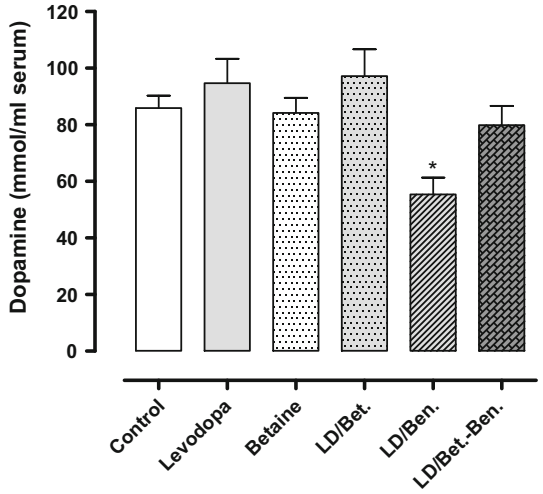

Fig. 7 The effects of levodopa, betaine and benserazide treatments on dopamine level in control and treated rats. Values represent mean ± SEM of dopamine (mmol/ml serum). $*P < 0.05$ versus levodopa and LD/Bet. groups. There was no significant difference among the other groups ($P > 0.05$). *LD* levodopa, *Bet.* betaine, *Ben.* benserazide

where dopaminergic neurons are present. In this study, we showed that betaine elevates antioxidant status and decreases lipid peroxidation in the brain of rats. These beneficial effects of betaine were associated with enhanced GPx, SOD, and CAT activities as well as GSH content subsequently decreasing in the lipid peroxidation process. The present study demonstrated for the first time that levodopa and benserazide treatment (as new drugs for PD) could induce oxidative stress in this area of the brain. Moreover, levodopa plus benserazide also induced hyperhomocysteinemia and this effect was more noticeable than in LD treatment. Betaine can act as an antioxidant agent versus oxidative stress mediated by LD and LD/Ben. treatments. Betaine also demonstrated a methyl donor effect to reduce hyperhomocysteinemia in agreement with our previous reports. Therefore, it appears that the antioxidant and methyl donor properties of betaine are promising, particularly in the management of plasma tHcy and oxidative stress in dopaminergic neurons of the brain.

The substantia nigra (SN) appears to be an area of the brain that is normally subject to a high degree of oxidative stress. Although the reasons for this are not clear, they may relate to the energy metabolism of these cells or to their high content of dopamine and its metabolism [7]. LD, the most effective drug known in the treatment of PD, has been observed to induce elevations in plasma tHcy concentrations [42]. The processes of methyl group transfer are involved in the metabolism of LD [43, 44]. LD is metabolized by four major metabolic pathways as follows: decarboxylation to dopamine, O-methylation to 3-o-methyl dopa, transamination, and oxidation [35].The main metabolism pathway of

LD is its O-methylation to form 3-O-methyldopa (3-O-MD) by catechol-O-methyltransferase (COMT). The reaction involves the enzyme COMT, with S-adenosyl methionine (SAM) forming 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 1). Betaine transfers a methyl group via the enzyme BHMT to become dimethylglycine, and converts Hcy to methionine [11]. 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 [45, 46]. In the present study, betaine as a methyl donor agent that continuously generates SAM could decrease the tHcy level as significantly in the LD/Bet. group when compared to the LD-treated rats.

In recent years, levodopa/benserazide composition has been used as the most effective drug in PD treatment [47]. Another hypothetical therapeutic approach seems to be the application of peripherally acting COMT inhibitors as adjunctives to levodopa/dopa decarboxylase inhibitor (LD/DDI) treatment. In this regard, a previous report indicated an elevation of tHcy levels with concomitant entacapone (a peripherally acting COMT inhibitor) [25]. In the present study, LD is metabolized to 3-OMD in the presence of benserazide by COMT, which is the essential enzyme for this O-methylation of LD, which requires a methyl group transfer from the donor SAM [22]. As one consequence, SAM is transformed into SAH and then to Hcy. In this setting, tHcy result for LD/Ben.-treated rats (Fig. 1) well indicates the bioavailability of LD and its converting 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 acted as a certain preventive agent in the onset of hyperhomocysteinemia in LD/Bet. and LD/Bet.-Ben. groups.

Presently, most treatments for PD are aimed at controlling the symptoms. Although dopamine replacement therapy using agents like LD could effectively relieve symptoms, it does not prevent disease progression [1]. During the past decade, SAM has been used as a co-adjuvant in depression and has a potential neuroprotective effect in animal models [48]. SAM application also increases brain glutathione (GSH) levels via intracellular biochemical pathways of transsulphuration and decreases membrane lipid peroxidation caused by free radical damage, acting as a potential antioxidant drug [11, 48, 49]. In theory, well-titrated and carefully monitored 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 apoptotic pathways [23, 50]. 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 [11, 28]. In the present study, betaine showed a significant decrease in plasma tHcy during supplementation in the dietary intake range of 1.5 % (w/w) of total diet. Furthermore, since humans produce little betaine from choline due to lack of choline oxidase [33], betaine is more practical than choline for investigations regarding the treatment of hyperhomocysteinemia in humans.

The SN is more vulnerable to oxidative stress than other parts of the brain due to its low antioxidant protection system and increased exposure of target molecules to ROS [7, 51]. There are increased levels of lipid hydroperoxides in the SN in the form of malondialdehyde and dopamine metabolites [7]. 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 nearer to the control group (Fig. 2). On the other hand, there were significant differences between the LD and the other groups for the lipid peroxidation marker, indicating the occurrence of oxidative stress in LD-treated rats. The LD/Ben. group also indicated significantly increased TBARS level when compared to the control, betaine, LD/Bet. and LD/Bet.-Ben. groups. Betaine is believed to play a significant role in maintaining the structural and functional integrity of cell membranes [11]. Previous studies have demonstrated that betaine, through its participation in sequential methylation within the cellular membranes, maintains a proper balance between phosphotidyl ethanolamine and phosphotidyl choline, thus sustaining proper membranes and preventing lipid peroxidation [11, 52, 53].

Animal models have shown that a number of antioxidants prevent oxidative brain injury through a variety of cellular mechanisms, which have been described as oxidative damage on the CNS [10, 11, 54, 55]. The glutathione antioxidant system plays a fundamental role in cellular defense against ROS. The cellular tripeptide GSH thwarts peroxidative damage by neutralizing the free radicals [52]. In the present study, GPx activity and GSH content were decreased significantly in LD-, LD/Bet.-, LD/Ben., and LD/Bet.-Ben.-treated rats when compared to the betaine and control groups (Figs. 3, 4). It is well known that GPx and catalase (CAT) are two key antioxidant enzymes that can decompose hydrogen peroxide to water [11]. The increase in GPx activity in betaine-treated rats correlates well with the increase of GSH content as a cofactor for GPx activity. The observed protective effect of betaine in this study is associated with the restoration of SAM, which contributes to an increase in the supply of

substrate needed for the synthesis of GSH through methylation pathways [11, 52]. It seems that the elevation of GSH content (insignificantly) in the LD group may be a compensatory mechanism that was not able to increase GPx activity in the LD-treated rats. It is well known that LD can act both as a pro-oxidant and an anti-oxidant molecule depending on circumstances. "For example, low concentrations of LD can induce an up-regulation in GSH molecule possibly because the drug acts as a minimal stressor that enhances the production of protective molecules" [24]. In the other hand, although GPx activity and GSH content were both elevated to suppress oxidative stress in the LD-treated group, it was not able to prevent lipid peroxidation in the brain of rats (Figs. 2, 3, 4).

Superoxide dismutase (SOD) as an antiperoxidative enzyme in cells rapidly converts superoxide anion (O^{2-}) to the less dangerous H_2O_2, then GPx and CAT can decompose H_2O_2 to water [26, 27]. The increase in SOD activity in our investigation for the betaine-treated rats (Fig. 5) correlates well with the increase of CAT activity in the SN area from the betaine group (Fig. 6). SOD shifts highly reactive O^{2-} to H_2O_2 and thus prevents the cell membrane damage caused by this highly toxic anion. Thus, in the present study, consumption of levodopa and benserazide significantly decreased the activities of both antiperoxidative enzymes, CAT and SOD, when compared to the betaine-treated rats.

Regarding LD metabolism, LD is metabolized by four major metabolic pathways. The principal path is decarboxylation, whereby LD converts to dopamine via LD decarboxylase [35]. In the present study, LD was administered with a peripherally acting dopa decarboxylase inhibitor, benserazide, in order to prevent its metabolism to dopamine so as to enhance brain penetration. The administration of benserazide with LD results in increased metabolism of LD to 3-O-MD via the enzyme COMT in peripheral tissues, thus decreasing the dopamine concentration in the LD/Ben.-treated rats in comparison with the LD group (Fig. 7). We measured dopamine concentration in blood to evaluate the decarboxylation pathway of LD in peripheral tissues. Although the serum dopamine level cannot reflect the dopamine concentration in the brain and dopamine does not cross the BBB, benserazide treatment prevents LD metabolism to dopamine in the peripheral tissues and is able to enhance LD and dopamine in brain tissue. In the present study, we did not measure the dopamine level in brain tissue, which is as one of the limitations of the study, since the homogenates were consumed for antioxidant parameters and lipid peroxidation marker detection. However, dopamine measurement in brain tissue or cerebrospinal fluid is needed to clarify the drug's effects in an experimentally induced Parkinson's model based on our previous report [1].

Regarding histopathological changes in the brain of rats, our results may be correct since we did not induce the Parkinson's model by injection of 6-hydroxy dopamine and/or other parkinsonism-inducing compounds in rats. Indeed, the aim of the study was to clarify the role of LD and benserazide in LD/Ben.-induced oxidative stress, as well as homocysteine metabolism interactions with antioxidant enzymes and lipid peroxidation markers in the brain of rats. It seems that the measurement of physiological parameters in the current study will not reveal any novel finding since we did not induce Parkinson's. Indeed, we decided to prevent the onset of hyperhomocysteinemia and oxidative stress induced by LD/Ben. via betaine therapy in rats. The determination of physiological parameters such as behavioral tests [1] is another limitation of the study.

Conclusion

The present study demonstrates that betaine may have a potential as a neuroprotective agent for prevention of LD-induced oxidative damage in brain tissue of rats. This effect is highlighted and demonstrated by recognition of betaine as an inhibitor of lipopolysaccharide-induced nitrosative stress in microglial cells [56]. However, further studies including evaluation of physiological parameters in a Parkinsonism animal model should be performed to validate beneficial effects of betaine.

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Conflict of interest The authors declare that they have no conflict of interest.

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