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Research Report

Effects of cysteamine on MPTP-induced dopaminergic neurodegeneration in mice

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ABSTRACT

Cysteamine is a degradation product of the amino acid cysteine and a reduced form of cystamine. Cysteamine exhibits strong antioxidant activity and has been implicated in the treatment of neurodegenerative disorders such as Huntington's disease. In the present study, we investigated whether cysteamine confers protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity in the dopaminergic neurons in a mouse model for Parkinson's disease (PD). The loss of dopaminergic (DA) neurons and reduction in striatal DA concentrations induced by MPTP was ameliorated to a significant extent by pretreatment with low (20 mg/kg/day), but not high (75 mg/kg/day), dose of cysteamine 4 days prior to and subsequently along with the MPTP treatment. Consistently, the increased production of pro-oxidants, such as reactive oxygen species (ROS) and malondialdehyde (MDA), was significantly suppressed by low dose of cysteamine. Conversely, the reduction in GSH level caused by MPTP exposure was significantly attenuated by pretreatment of cysteamine. In addition, the inhibited secretion of the brain-derived neurotrophic factor (BDNF) by neurons derived from substantia nigra pars compact (SNpc) of MPTP-treated mice was significantly restored by cysteamine administration. Our results demonstrate that cysteamine at low dose confers potent neuroprotection against MPTP-induced toxicity of dopaminergic neurons, and may become a potential therapeutic strategy for PD.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the loss of dopaminergic neurons and, consequently, the depletion of dopamine (DA) in its striatal projections (rev. by Schapira 2009). Although the etiology of PD remains unknown (Wolters, 2006), mitochondrial dysfunction, oxidative stress and other mechanisms are believed to be pivotal factors involved in the death of dopaminergic neurons. In particular, oxidative stress and mitochondrial dysfunctions are known to be crucial factors in the initiation and progression of various neurodegenerative disorders (Wong et al., 1999; Albanese et al., 1993). For example, decreases in activities of mitochondrial complex I

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Abbreviations: PD, Parkinson's disease; DA, dopamine; MPP+, 1-methyl-4- phenylpyridinium ions; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GSH, glutathione; MDA, malondialdehyde; SNpc, substantia nigra pars compact; ROS, reactive oxygen species; SOD, superoxide dismutase; BDNF, brain-derived neurotrophic factor; TH, tyrosine hydroxylase; DAB, diaminobenzidine tetrahydrochloride; HPLC, high performance liquid chromatography; ECD, electrochemical detector; PFA, paraformaldehyde; TBS, Tris-buffered saline; ELISA, enzyme immunoassay system assay; DCDHF-DA, 2′, 7′-dichlorodihydrofluorescein diacetate; TBARS, thiobarbituric acid reactive species; ANOVA, analysis of variance

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and superoxide dismutase (SOD) and glutathione (GSH) level, and increases in malondialdehyde (MDA) which catalyzes the Fenton reaction, have been observed in idiopathic PD (rev. by Shadrina and Slominskii, 2008; Bharath and Andersen, 2005). In addition, decreased release of the brain-derived neurotrophic factor (BDNF) observed in the striatum contributes to the exacerbation of PD (rev. by Kuipers and Bramham, 2006).

Existing therapies for PD are mainly designed for symptom management and so far there is no treatment available to attenuate the progression of the disease. The ultimate cure for the disease requires approaches to protecting the DA neurons against damages and to halting or slowing the continuous functional decline of these cells at early pathological stages. One strategy is to recover the mitochondrial function and increase the reductase activities by antioxidant reagents that allow for elimination of excessive reactive oxygen species (ROS) from and inhibition of oxidative stress in the neurons. A number of such reagents have been tested for their potentials for protecting DA neurons against oxidative cell stress in rodent models of PD (Cleren et al., 2008; Bagh et al., 2008; Cai et al., 2008; Chung et al., 2007; Wang et al., 2009; Rojas et al., 2009). However, for efficacy and safety concerns, none of them has been suggested for a clinical application.

Cysteamine (2-aminoethanthiol) is a promising and safe antioxidant compound that has been approved by the U S Food and Drug Administration (FDA) to treat nephropathic cystinosis. It is a degradation product of the amino acid cysteine and a reduced form of cystamine and constitutes the terminal region of the coenzyme A (CoA) molecule from which it is liberated (rev. by Tsai 2006). Biochemically, cysteamine cleaves the disulfide bond with cystine to produce molecules that can escape the metabolic defect in cystinosis and cystinuria. It also plays crucial roles in antioxidant defenses and energy homeostasis in cystinosis via preventing lipid peroxidation and protein carbonylation, improving activities of superoxide dismutase and glutathione peroxidase (Dubinsky and Gray, 2006; Kessler et al., 2008), and normalizing creatine kinase and pyruvate kinase (Rech et al., 2008). More recent studies found that cysteamine and cystamine exhibits neuroprotective effects in a mouse model of Huntington's disease (HD) through inhibiting the transglutaminase activity and enhancing the BDNF level (Karpuj et al., 2002). Moreover, a phase I study using cysteamine in HD patients showed the drug to be safe and tolerable (Dubinsky and Gray, 2006), suggesting it might be a promising drug for treating neurodegenerative disorders. However, such effects have not been tested either experimentally or clinically in PD. The current study was designed to explore the antioxidant effects of cysteamine on the survival and function of dopaminergic neurons and its potential protective roles against MPTP-induced parkinsonism in a mouse model.

2. Results

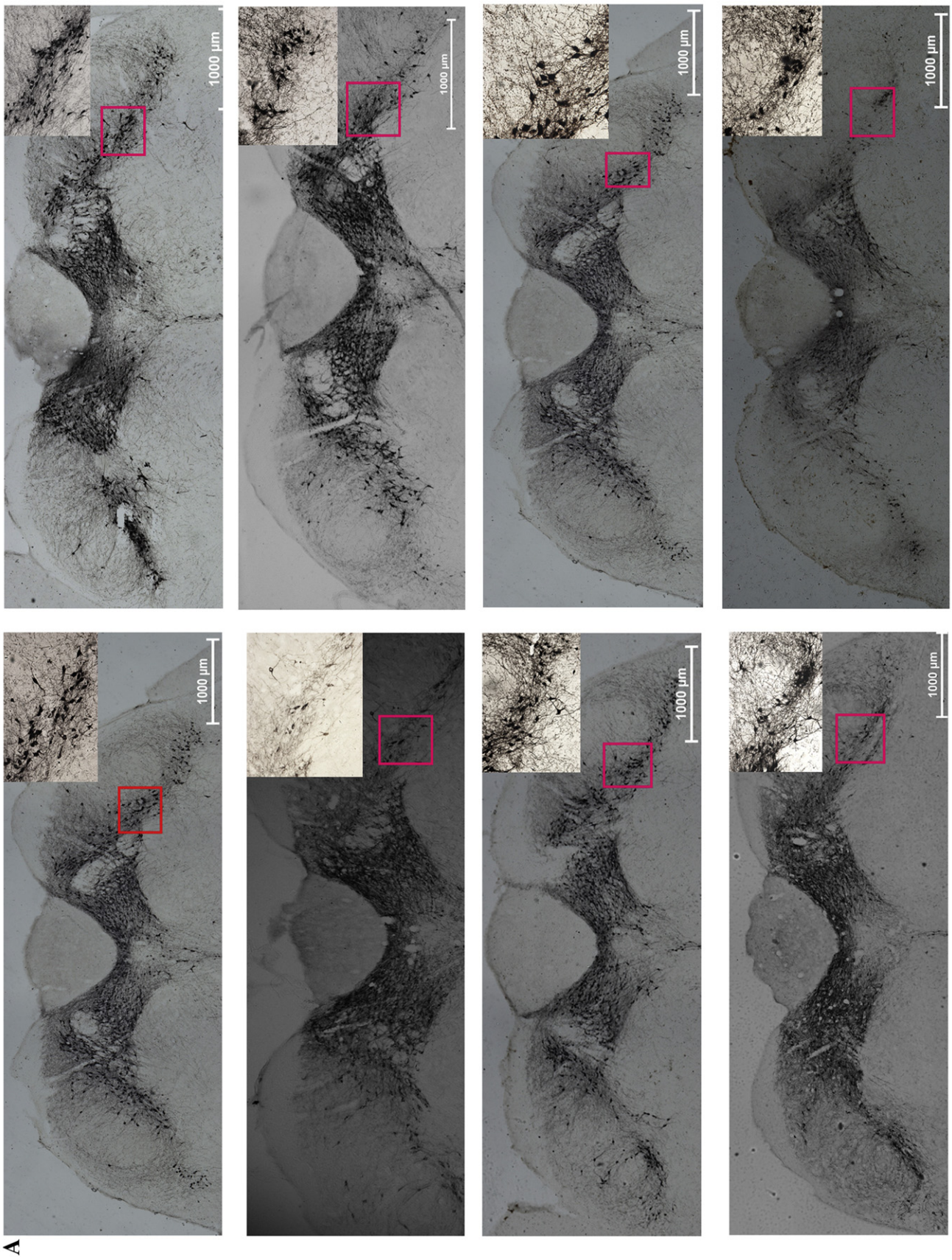
2.1. *Low dose of cysteamine prevented MPTP-mediated loss of TH-IR neurons in the substantia nigra pars compacta (SNpc) and loss of striatal dopamine concentration*

To examine the effect of cysteamine on the number of TH–immunoresponsive (TH-IR) nigral neurons, histochemistry analysis was performed in cells derived from the substantia nigra pars compacta (SNpc) using anti-TH monoclonal antibody. As shown in Fig. 1, the number of TH-IR nigral neurons significantly decreased in the MPTP- induced mice (166.65 ± 58.15) (A-2 and B), as compared with control mice (295.65 ± 47.47) ($P=0.000$) (A-1 and B). However, the loss of TH-IR neurons from mice injected with cysteamine (20 mg/kg/day) 4 days prior to and subsequently along with MPTP-treatment (CS20 mg/kg/day + MPTP) was ameliorated (262.45 ± 32.75) to a significant extent ($P=0.000$, compared with MPTP alone) (A-5 and B). Interestingly, the higher dose of cysteamine (CS75 mg/kg/day + MPTP) did not attenuate the loss of number (172.10 ± 42.69) of TH-IR neurons (compared with MPTP alone, $P=0.713$) (A-6 and B), and treatment with high dose of cysteamine alone (CS 75 mg/kg/day) even promoted the loss of TH-IR neurons (185.05 ± 29.24) (compared with non-treated control, $P=0.000$) (A-4 and B). In contrast to pretreatment, simultaneous treatment of cysteamine at either low or high dose with MPTP (MPTP + CS20mg/kg/day or MPTP + CS75mg/kg/day) did not suppress the reduction of number of TH-IR neurons (179.5 ± 68.11 and 174.05 ± 45.78, respectively) induced by MPTP (both $P>0.05$, compared with MPTP alone) (**A-7**, A-8 and B).

Similarly, the striatal DA concentration (ng/mg protein) of MPTP-treated mice (26.38 ± 6.47) was significantly decreased as compared with control (57.62 ± 12.70) ($P=0.000$). Pretreatment with low dose of cysteamine (20 mg/kg/day) daily for 4 days before and along with MPTP significantly protected against MPTP-induced striatal DA depletion (47.96 ± 10.57) ($P=0.000$). But pretreatment with high dose of cysteamine (75 mg/kg/day) did not attenuate the loss of striatal DA concentration (25.65 ± 7.71) ($P=0.89$, compared with MPTP alone), and treatment with high dose of cysteamine alone (CS 75 mg/kg/day) even significantly decreased the DA concentration (32.22 ± 4.71) ($P=0.000$, compared with non-treated control**).** Simultaneous treatment with cysteamine (20 mg/kg/day or 75 mg/kg/day) during MPTP-treatment did not ameliorate the MPTP-induced striatal DA depletion (36.27 ± 16.42 and 25.08 ± 8.10, respectively) (Fig. 2).

2.2. *Cysteamine decreases the concentrations of ROS and MDA while increasing the concentration of GSH in the SNpc of MPTP-treated mice*

To examine the effects of cysteamine on oxidative-stress conditions, both pro- and anti-oxidants were measured in dopaminergic neurons derived from MPTP-treated mice. As shown in Table 1, the levels of both malondialdehyde (MDA) and reactive oxygen species (ROS) were significantly elevated after MPTP exposure (MDA: $P=0.0001$; ROS: $P=0.005$). These decreases were suppressed by pretreatment with both high (MDA: $P=0.005$; ROS: $P=0.04$) and low dose of cysteamine (MDA: $P=0.001$; ROS: $P=0.04$), respectively. In contrast, activity of superoxide dismutase (SOD), the scavenger for H_2O_2, was not significantly changed. Level of GSH (nmol TNB/mg protein) was significantly decreased in the SNpc of the MPTP-treated mice (0.55 ± 0.36) as compared with control group (3.52 ± 1.44) ($P=0.016$), which was partially restored (3.16 ± 1.28) by pretreatment with the low dose of cysteamine ($P=0.033$) but not the high dose group ($P=0.356$) (Fig. 3).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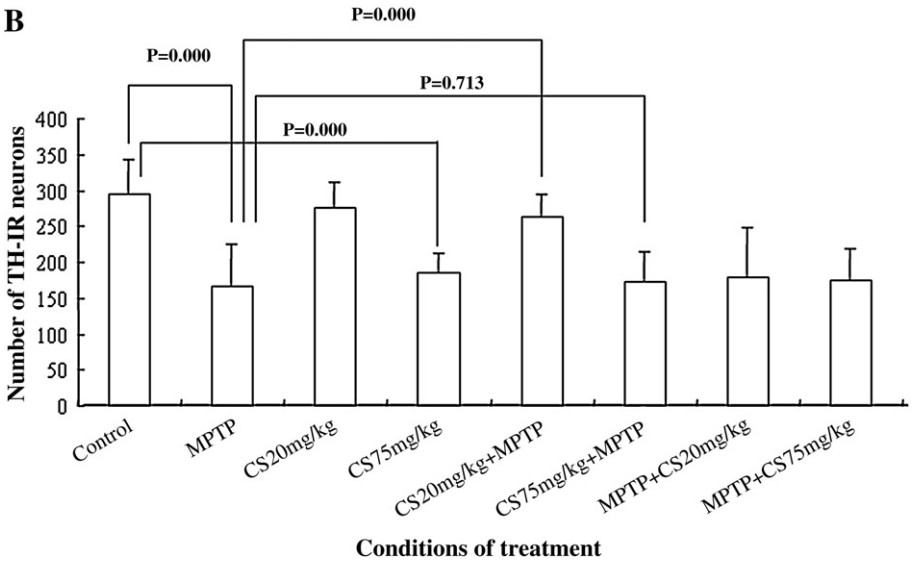

Fig. 1 – Cysteamine increases the number of TH–immunoresponsive (TH-IR) neurons in the substantia nigra of MPTP-treated mice. Coronal sections of substantia nigra (SN) derived from mice with different treatment conditions were stained with anti-TH monoclonal (3D5) antibody and TH-IR nigral neurons was visualized by microscope (40×) (A). A-1: Saline control; A-2: MPTP alone; A-3: Cysteamine 20 mg/kg/day alone; A-4: Cysteamine 75 mg/kg/day alone; A-5: Cysteamine 20 mg/kg/day pretreatment followed by MPTP; A-6: Simultaneous treatment of MPTP and cysteamine 20 mg/kg/day; A-7: Cysteamine 75 mg/kg/day treatment followed by MPTP; A-8: Simultaneous treatment of MPTP and Cysteamine 75 mg/kg/day. TH-IR neuronal count were presented as mean ± SD and their numbers were compared among different treatment groups (B).

2.3. *Cysteamine increases BDNF secretion in the SNpc of MPTP-induced mice*

To examine whether cysteamine can affect production of brain-derived neurotrophic factor (BDNF), the level of BDNF protein concentration of BDNF was measured in the substantia nigra by ELISA. As shown in Fig. 4, MPTP-treatment resulted in a significant reduction of BDNF secretion (39.55 ± 7.82) as compared to control (78.34 ± 16.09) ($P=0.016$). This reduction was attenuated by low dose of cysteamine (74.22 ± 7.79, $P=0.014$ as compared to MPTP-treated mice), but not high dose (55.43 ± 12.15, $P=0.242$). Interestingly, treatment with low dose of cysteamine alone also stimulated an augmentation of BDNF secretion (103.36 ± 30.48) ($P=0.033$, compared to control). In contrast, simultaneous administration of cysteamine with MPTP did not reverse the decrease of BDNF by MPTP (both $P>0.05$).

3. Discussion

Significant oxidative damages to lipids, proteins, and DNA, along with decreased GSH level, have been widely observed in the striatum and substantia nigra of patients and animal models of PD. Oxidative cell stress occurs as a result of an imbalance between the generation of ROS and the mechanisms of oxidation defense and oxidant scavenging (Stack et al., 2008). In the present study, we revealed that pretreatment with low dose (20 mg/mg/day) of cysteamine evidently decreases the MPTP-induced formation of ROS and MDA while augmenting the intracellular level of GSH, resulting in elevated striatal DA level and reduced neuronal loss in the MPTP-treated mice. As GSH is a major free radical scavenger and a highly sensitive indicator of cell viability, these results demonstrated that cysteamine confers potent protection for the DA neurons by acting in both directions to restore the balance of oxidant accumulation and clearance. However, it did not influence the activity of SOD, another important oxidant scavenger present in mitochondria.

Our data also suggested that pretreatment of cysteamine could partially restore the MPTP-induced reduction in BDNF secretion. BDNF is a neurotrophic factor expressed by DA neurons in both substantia nigra and the ventral tegmental area. It plays a key role in the survival and differentiation of midbrain DA neurons (Tinmarla et al., 2009; Tsai 2007) and is required for maintaining the proper number and normal function of DA neurons in substantia nigra (Nagatsu et al., 2000; Zuccato and Cattaneo, 2007). Although it is not tested in this study, the mechanism underlying the upregulation of BDNF by cysteamine might be similar to that suggested by Goggi et al. (2003), and Borrell-Pagès et al. (2006), where cysteamine can directly enhance BDNF secretion from the Golgi apparatus through a heat shock DnaJ-containing protein 1,b (HSJ1b)-dependent mechanism involving transglutaminase inhibition.

It seems that the effects by cysteamine are biphasic and are related to doses of administration. Our results showed that the low (20 mg/kg/day, totally 280 mg/kg) but not high dose of cysteamine (75 mg/kg/day, totally 1050 mg/kg) confers protections against MPTP-induced loss of DA neurons, and the high dose even lead to toxicity to the neurons(Figs. 1A-4, B and 2). This is somewhat similar to the early reports that administration of cysteamine at single doses of 100–200 mg/kg

resulted in a dose-dependent increase of the striatal DA concentration (Oishi et al., 1993), while at a single dose up to 600 mg/kg it caused toxicity accounting for 25% of mortality of mice (Hunyady et al., 2001). But the toxicity observed in our study seemed not to be related to a single dose, since the high single dose used here was 75 mg/kg, which was within the safe range according to the previous studies. These results are consistent with findings of broadly ranging neuroprotective agents, conforming to the quantitative features of the hormetic dose response (rev. by Calabrese 2008a). After extensive review, Calabrese (rev. by Calabrese 2008b) concluded that all neuroprotective chemical assessments are dominated by biphasic dose–response relationships and hormetic dose response may be used to provide an index of the biological plasticity of neuronal system responses to a broad spectrum of growth factors or other modulatory agents and environmental stressor agents.

In summary, the current study demonstrates that low doses of cysteamine confer protection against MPTP-induced degeneration of dopaminergic neurons in the substantia nigra in mouse through the inhibition of oxidation the promotion of BDNF secretion in vivo. In the context of that cysteamine might have a treatment effect on other neurodegenerative disease, such as Huntington and Alzheimer's disease, it is intriguing to further test whether it also has the treatment efficacy in PD.

4. Experimental procedures

4.1. *Drugs and chemicals*

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl), dopamine standard, diaminobenzidine tetrahydrochloride (DAB), 2', 7'-dichlorodihydrofluorescein diacetate (DCDHF-DA), Rabbit anti-tyrosine hydroxylase (TH) affinity purified monoclonal antibody were procured from Sigma-Aldrich. Cysteamine was procured from Walcom-biochem Inc. HPLC-grade acetonitrile and methanol was bought from Fisher Science China. Brain-derived neurotrophic factors (BDNF) was purchased from XiTang Biological Technology (Shanghai, China). Glutathione kit was purchased from Beyotime Institute of Biotechnology (Guangzhou, China). Thiobarbituric acid and superoxide dismutase were obtained from Nanjing Jiancheng Institute of Biological Engineering.

4.2. *Animal and treatment of cysteamine and MPTP-HCl*

Adult male C57/BL mice were purchased from the Animal Center at the Chinese Academy of Sciences in Beijing, and housed five per cage in a light and temperature-controlled room. They were allowed free access to standard food and water ad libitum. All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications Nos. 80-23, revised 1996) and were approved by the institutional animal experimental ethical committee.

C57/BL mice weighted 20–22 g were used. Mice were treated with cysteamine and/or induced by MPTP fresh preparations according to the group assignments. Group assignments ($n=8$/group) were as follows: (1) saline (control) group, (2) MPTP alone (see below), (3) cysteamine (CS) 20 mg/kg/day alone for 2 weeks, (4) CS 75 mg/kg/day alone for 2 weeks, (5) CS 20 mg/kg/day for total 14 days and MPTP was given from days 5 to 9, (6) CS 75 mg/kg/day for total 14 days and MPTP was given from days 5 to 9, (7) simultaneous administration of MPTP and CS 20 mg/kg/day for 5 days, followed by CS alone for additional 9 days, (8) simultaneous administration of MPTP and CS 75 mg/kg/day for 5 days followed by CS alone for additional

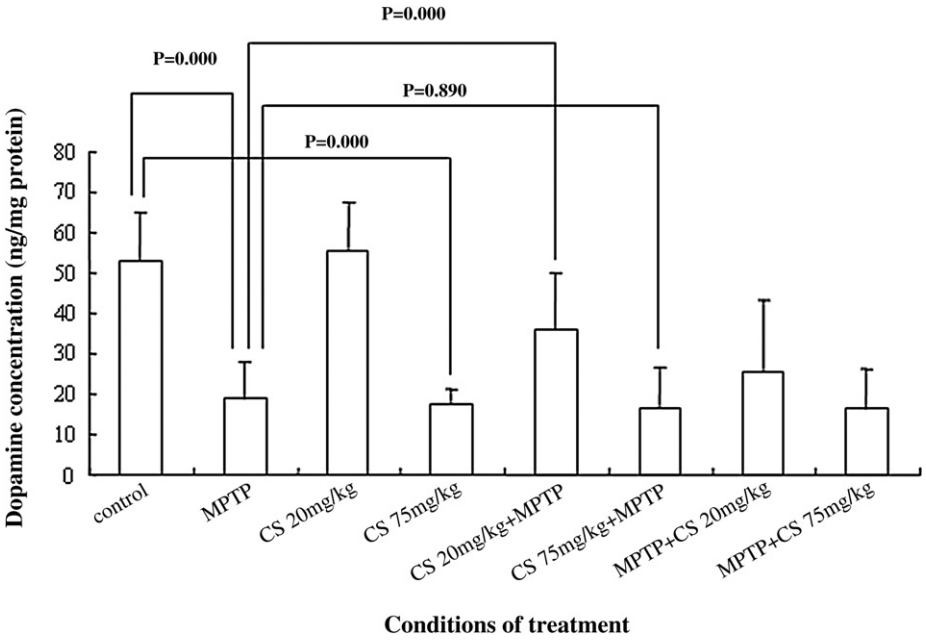

Fig. 2 – Cysteamine protected against MPTP-induced decrease in striatal dopamine (DA) concentrations in mice. Concentrations of DA (ng/mg protein, mean ± SD) in the corpus striatum tissues derived from mice receiving different treatments was measured by high performance liquid chromatography (HPLC) and compared among treatment groups. *P* values as indicated on the figure.

Table 1 – Effect of cysteamine on malondialdehyde (MDA), reactive oxygen species (ROS) and superoxide dismutase (SOD) in the substantia nigra of MPTP-lesion mice ($n=8$).

Species/ enzymes	Control	MPTP	C20mg	C75mg	C20mg+MPTP	C75mg+MPTP	MPTP+C20mg	MPTP+C75mg
MDA (nmol/mg)	7.89±0.65	10.84±2.65*	8.82±2.44	8.90±2.14	8.25±0.90#	8.56±1.08#	9.86±0.91**	9.51±0.57**
ROS (MFI)	13010±261	20207±203*	17460±779*	17980±419*	17411±589*,**	17411±362*,**	19539±431*	21976±489*
SOD (U/mg)	52.55±13.35	57.73±9.57	58.88±17.66	59.13±10.66	62.11±4.74	58.53±5.82	66.77±4.64	59.58±2.24

MDA: *$P<0.05$ compared with the control, **$P<0.01$ compared with MPTP alone, #$P<0.05$ compared with MPTP alone.
ROS: *$P<0.01$ compared with the control, **$P<0.01$ compared with MPTP alone, #$P<0.05$ compared with MPTP alone.
SOD: no significant difference compared with each other.

9 days. The control group was injected with 0.9% saline. MPTP (20 mg/kg) was intraperitoneally (i.p.) injected into the mice twice daily at an interval of 12 h for the initial 2 days and once per day on days 3–5(Tremblay et al., 2006). CS (20 mg/kg/day and 75 mg/kg/day) were injected alone for 14 days.

4.3. *Tissue preparation*

After treatment for 14 days, the animals were sacrificed by decapitation. Tissues were quickly removed from the brain on a Petri dish placed on ice using laboratory microscope (Chan et al., 2007). Bilateral corpus striatum stored at −80 °C until used for HPLC analysis. Bilateral substantia nigra were immediately homogenized using an Ultrasonic Cell Disruptor (Biologics, Model 150 V/T), and which were centrifuged. The supernatant was used for the estimation of various enzyme activities.

4.4. *Assay of dopamine (DA) by high performance liquid chromatography (HPLC)*

Samples were processed blindly to the treatment conditions for HPLC analysis with electrochemical detector (ECD) using the method by Beyer (Bayer et al., 2002). Bilaterally corpus striatum tissues were sonicated in 1 ml of pre-colded 0.4 N $HClO_4$. The resulting homogenates were centrifuged twice for 12 min at 12,000*g* at 4°C. Mobile phase consisting of 0.035 mol/L anhydrous citric acid, 0.09 mol/L without water-sodium acetate, 0.23 mmol/L sodium alkane sulfonate, 0.13 mmol/L EDTA, 10% methanol (pH4.7). Electrochemical conditions for the experiment were +0.50 V. Separation was carried out at a flow rate of 1.0 ml/min. Samples (20 μl) were automatic injected and were quantified by comparison of the area under the curve (AUC) against reference standards. The concentrations of DA were expressed as ng/mg protein of the brain tissue. Protein content was determined in tissue supernatants by the method of Lowry using bovine serum albumin as a standard (Lowry et al., 1951).

4.5. *Tyrosine Hydroxylase Immunohistochemistry*

Twenty-four hours later the last injection, mice were anesthetized with chloral hydrate (100 mg/□). The mice were subjected to thoracotomy and perfusion with ice-cold 0.9% sodium chloride 50 ml, then with 4% paraformaldehyde (PFA) 100 ml

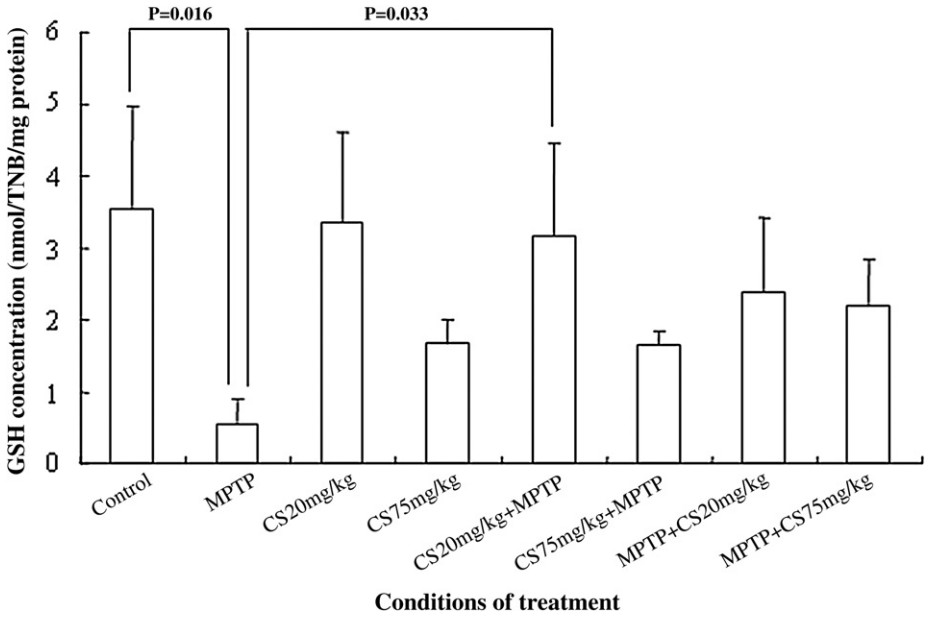

Fig. 3 – Cysteamine protected against MPTP-induced decrease of glutathione (GSH) levels in the SNpc of mice. GSH concentration (nmol TNB/mg protein, mean±SD) of the isolated cells of substantia nigra from mice with different treatments was measured using glutathione reductase and compared among treatment groups. *P* values as indicated on the figure.

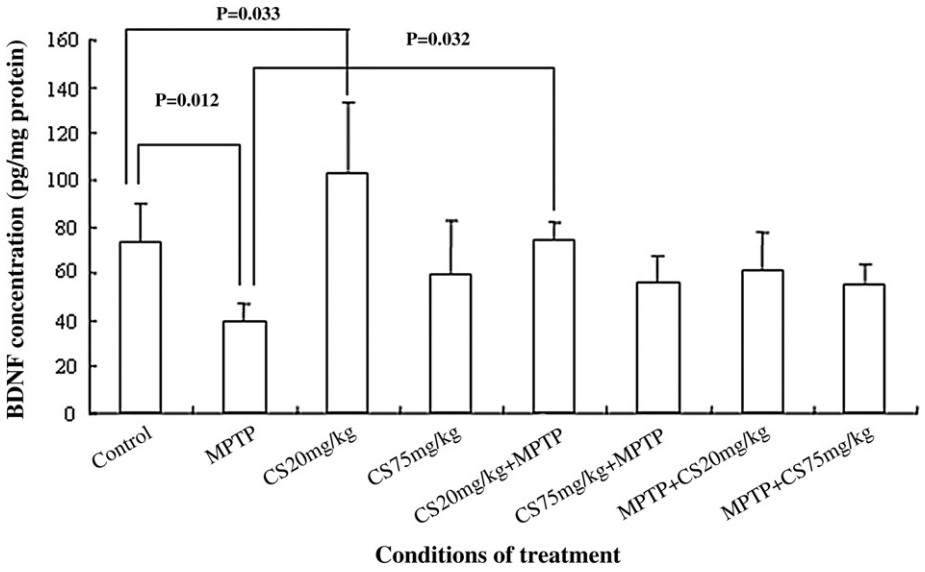

Fig. 4 – Cysteamine attenuated MPTP-induced decrease in the content of brain-derived neurotrophic factor (BDNF) in the SNpc of mice. BDNF concentrations (pg/mg protein, mean ± SD) were measured by an ABC-ELISA method for the isolated cells of substantia nigra from mice with different treatment and compared among treatment groups. *P* values as indicated on the figure.

in 0.01 M PBS through the left ventricular. After fixation, the brain was post-fixed and then cryoprotected in 30% sucrose for 24 h. The brains were sectioned through the substantia nigra (SN) coronally at 30 μm thickness on a cryostat (Leica CM1900, Nussloch, Germany). Sections were collected in 0.01 M PBS and processed free floating. The free floating sections were washed 3 times in Tris-buffered saline (TBS), incubated with H_2O_2 (3%) for 30 min and blocked with 5% normal goat serum (NGS), 0.2% Triton X-100 in TBS. Sections were incubated for 24 h with primary rabbit anti-TH monoclonal antibody (1:10000) (Watanabe et al., 2004). Sections were washed in PBS and incubated for 2 h at room temperature in secondary antibodies: biotinylated goat anti-rabbit (1:500) diluted in blocking buffer. The sections were incubated for 2 h at room temperature in horseradish peroxidase labeled avidin work streptomycin solution (1:500). TH immunoreactivity was visualized in SN after incubation in DAB and Nickel sulfate salt for 5–10 min. Sections were mounted on slides, dried, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX mounting medium and coverslip. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the primary antibody omitted. After TH-staining, the image of the sections from the substantia nigra were digitalized with NIS-Elements imaging software (Nikon). The number of neurons was measured in the bilateral substantia nigra.

4.6. *Measurement of oxidative stress parameters*

The ROS level was measured using 2', 7'-dichlorodihydrofluorescein diacetate (DCDHF-DA). DCDHF-DA which is cell-permeable and non-fluorescent, and, in the presence of ROS it is oxidized inside cells and transformed into a fluorescent compound, 2', 7'-dichlorofluorescein (DCDF), which remains trapped within the cell. To measure the ROS level in substantia nigra, isolated cells (10^5/ml) were incubated with 50 μM DCDHF-DA for 40 min at 37°C and washed twice in PBS. The fluorescence was monitored in microplate reader (Liu et al., 2009). For each sample, 10,000 cells were acquired. Data were expressed as an average of fluorescence intensity of analyzed cell population (mean fluorescence intensity ± SD).

The formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction was determined by the absorbance at 532 nm. The rate of lipid peroxidation was expressed as nmol/mg protein. SOD activity was detected based on its ability to inhibit the superoxide anion free radical of O^{2-} generated by the xanthine/xanthine oxidase system. The absorbance at 550 nm was monitored and SOD levels were expressed as U/mg protein. The total glutathione (reduced and oxidized form of glutathione) was determined using glutathione reductase. Absorbance was measured at 412 nm using microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples. Results were reported as nmol TNB/mg protein.

4.7. *Assay for brain-derived neurotrophic factor (BDNF)*

The concentration of BDNF was measured by an ABC-ELISA method, according to the manufacturer's protocol (Beyotime Institute of Biotechnology). The assay sensitivity was 15 pg/ml and no significant cross reactivity with other related neurotrophins. With BDNF anti-mouse antibody-coated plate in ELISA, BDNF of the standard materials and samples combined with the monoclonal antibody, by adding biotinylated anti-mouse BDNF, the formation of immune complexes connected to the board, horseradish peroxide complex enzyme labeled streptavidin and biotin combination, adding the substrate fluid to show blue, plus the final termination of liquid sulfuric acid, and then measured OD value 450 nm. OD value and BDNF concentration are

proportional to the standard curve obtained the standard BDNF concentrations. Data are represented as pg/mg protein.

4.8. *Statistical analysis*

Comparison among over two groups was performed by one-way ANOVA with a post hoc test. Differences were considered significant when $P<0.05$. Data analyses were analyzed by the Statistical Package for the Social Sciences software (SPSS 16.0 for Windows).

Acknowledgments

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