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Ergothioneine protects against neuronal injury induced by cisplatin both in vitro and in vivo

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ABSTRACT

The neuroprotective effects of ergothioneine (EGT) against cisplatin toxicity were investigated both in vitro and in vivo. For in vitro study, two types of neuronal cells, primary cortical neuron (PCN) cells and rat pheochromocytoma (PC12) cells, were incubated with EGT (0.1–10.0 μM) for 2 h followed by incubation with 0.5 μM cisplatin for 72 h. Results show that cisplatin markedly decreased the proliferation of PC12 cells and strongly inhibited the growth of axon and dendrite of PCN cells, but these effects were significantly prevented by EGT. For in vivo study, CBA mice were orally administered with 2 or 8 mg EGT/kg body weight for 58 consecutive days and were injected i.p. with 5 mg cisplatin/kg body weight on days 7, 9 and 11. We found that EGT significantly restored the learning and memory deficits in mice treated with cisplatin evaluated by active and passive avoidance tests. EGT also significantly prevented brain lipid peroxidation, restored acetylcholinesterase (AChE) activity and maintained glutathione/glutathione disulfide ratio in brain tissues of mice treated with cisplatin. These results demonstrate that EGT protects against cisplatin-induced neuronal injury and enhances cognition, possibly through the inhibition of oxidative stress and restoration of AChE activity in neuronal cells.

1. Introduction

Cisplatin is a potent antitumor agent widely used for the chemotherapy of different solid malignancies (Thigpen et al., 1994; Van Basten et al., 1997; Recchia et al., 2001). However, cisplatin is characterized by some severe side effects such as nephrotoxicity, neurotoxicity, ototoxicity and nausea and vomiting, which frequently hampers its chemotherapeutic efficacy (Screnci and McKeage, 1999; Sweeney, 2001). Cisplatin-caused neurotoxicity occurs in up to 30% of patients and is dose-limiting for cisplatin. Dietrich et al. (2006) pointed out that chemotherapeutic agents, including carmustine (BCNU), cisplatin and cytosine arabinoside (cytarabine), are more toxic for the progenitor cells of the central nervous system (CNS) and for nondividing oligodendrocytes than for cancer cells. When administered systemically in mice, these chemotherapeutic agents are associated with increased cell death and decreased cell division in the dentate gyrus of the hippocampus and in the corpus callosum of the potential CNS (Dietrich et al., 2006). Pedersen et al. (2000) have reported that cisplatin induces severe cognitive malformations in mouse and rat experimental models.

The neurotoxic and cytotoxic effects of cisplatin have been shown to be related to the production of reactive oxygen species (ROS) (Ravi et al., 1995; Rybak et al., 1995) and high levels of Pt–DNA binding and apoptosis of dorsal root ganglion (DRG) neurons (Ta et al., 2006). Complete degeneration of the spiral ganglion following exposure to cisplatin has been reported (Anniko and Sobin, 1986). In vivo studies have shown an increase in the cochlear activity of superoxide dismutase (SOD), H_2O_2, and malondialdehyde (MDA), and a decrease in glutathione (GSH) and GSH reductase activity after exposure to cisplatin, suggesting that PCN cells cisplatin-induced neurotoxicity is the result of increased oxidative stress (Rybak et al., 1995). Gabaizadeh et al. (1997) also indicated that intraneuronal levels of ROS play a key role in cisplatin-induced neuronal cell death. Thus, antioxidant molecule will be effective in preventing cisplatin-induced damage to neurons.

Recently, great efforts have been put forward to the neuroprotective effects of dietary food or chemoprotective agents to reduce the toxic effects of cisplatin. These compounds are able to protect neuronal cells in various in vivo and in vitro models through different intracellular targets (Mendonça et al., 2009; Gerritsen van der Hoop et al., 1994; Hol et al., 1994; Tredici et al., 1994). Chemoprotective agents such as GSH, methionine and *para*-aminobenzoic

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acid have been used and have shown their efficacies in different experimental models (Bohm et al., 1999; Basinger et al., 1990; Esposito et al., 1993).

Ergothioneine (2-mercaptohistidine trimethylbetaine; EGT) is formed in some bacteria and fungi but not in animals (Melville et al., 1955). In humans, ergothioneine is only absorbed through consumption of plant diet, primarily by consumption of edible mushrooms. Blood concentrations of EGT in humans have been estimated to be in the range of 1–4 mg/100 ml blood (46–184 μM) and have long half-life in the human body (Touster, 1951; Melville, 1958), while the EGT concentrations in bovine and porcine ocular tissues are reported to be 2.96 ± 0.2 and 8.69 ± 1.57 mmol/mg tissues, respectively (Shires et al., 1997).

In vitro studies have shown that EGT is radioprotective and that it scavenges singlet oxygen, hydroxyl radical, hypochlorous acid and peroxyl radicals as well as inhibits peroxynitrite-dependent nitration of proteins and DNA (Aruoma et al., 1997; Dubost et al., 2007). In addition, Jang et al. (2004) have reported that EGT is neuroprotective because EGT protects rat pheochromocytoma (PC12) cells from oxidative and nitrosative cell death caused by Aβ. In vivo studies have shown that EGT protects retinal neurons from N-methyl-D-aspartate-induced excitatoxicity (Moncaster et al., 2002) and protects against diabetic embryopathy in pregnant rats (Guijarro et al., 2002). EGT also confers cellular homeostasis in neuronal cells challenged with the prooxidant mixture of *N*-acetylcysteine/hydrogen peroxide (Aruoma et al., 1999). Although EGT has not been shown to protect against cisplatin-induced neuronal injury, we hypothesized that it could do so and do it effectively, based on the literature data and our own preliminary in vitro data. The study reported here was conducted to explore the protective effects of EGT against cisplatin-induced neuronal damage both in vitro and in vivo.

2. Materials and methods

2.1. Chemicals

Chemicals including *n*-butanol, thiobarbituric acid, 1,1,3,3-tetraethoxypropane and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Cell culture and cell viability assay

PC12 cells (BCRC 60048), a rat pheochromocytoma, were obtained from Food Industry Research & Development Institute (Hsin Chu, Taiwan) and maintained in 10% fetal bovine serum and 90% RPMI with 4 mM L-glutamine containing 1.5 g/L sodium bicarbonate, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days. Cells were seeded at a density of 1×10^5 cells/well onto a 12 well-plate (FALCON, Becton Dickinson, NJ, USA) 24 h prior to drug treatment. Various concentrations of EGT (0.1∼10 μM) were added to cells 2 h prior to cisplatin treatment. After incubation, cells were washed with phosphate buffered saline (PBS). Viable cell numbers were determined 24, 48 and 72 h after the addition of cisplatin and EGT by means of the Trypan blue exclusion method using a hemocytometer.

2.3. Primary cultured of rat cortical neuron (PCN) cells

Cultured cortical cells were prepared from the cerebral cortices of one-day-old Sprague–Dawley rats as previously described (Huang et al., 2000). After the brain was dissected, the blood vessels and meninges were removed under microscope. Then, the cortices were placed in ice-cold DMEM, and minced. The tissue chunks were incubated with papain solution (100 U/ml papain, 0.5 mM EDTA, 0.2 mg/ml cysteine, 1.5 mM $CaCl_2$, DNase I) at 37 °C for 20 min to dissociate the cells. The reactions were terminated by adding heat-inactivated horse serum. After the cell suspension was centrifuged at 200*g*, the pellet was resuspended in DMEM supplemented with 10% horse serum. Cells were plated onto poly-D-lysin-coated Petri dishes, and incubated at 37 °C in a humidified incubator with 5% CO_2. Two hours after plating, the medium was replaced with neurobasal containing B27, 25 μM glutamine and 0.5 mM glutamine. On the 4th day in vitro, the medium was changed and replaced with neurobasal/B27 without glutamate. The PCN cells were grown for another 10 days to permit the growth of axon and dendrite. Morphological changes were conducted using a phase-contrast inverse microscope (IMT-2, Olympus Co. Ltd., Tokyo, Japan).

2.4. Animal experimental design

CBA mice (6–7 weeks old) were used for the experiments. The mice were provided with food and water *ad libitum* and received i.p. 5 mg cisplatin/kg body weight (bw) with or without oral supplementation of EGT (2 or 8 mg/kg bw, p.o.) or melatonin (10 mg/kg bw, p.o.) in five experimental groups. *Group 1*: control (0.9% NaCl, i.p., 10 mL/kg bw + 0.9% NaCl, p.o., 10 mL/kg bw); *group 2*: cisplatin + 0.9% NaCl, p.o.; *group 3*: cisplatin + melatonin; *group 4*: cisplatin + EGT (2 mg/kg bw, p.o.); *group 5*: cisplatin + EGT (8 mg/kg bw, p.o.). Cisplatin, EGT and melatonin were all prepared in saline. Mice were given daily by gastric feeding (10 mL/kg bw) of EGT or melatonin for 58 consecutive days. Cisplatin solution (10 mL/kg bw, i.p.) was administered consecutively on days 7, 9 and 11. Control animals received equal amounts of 0.9% NaCl to replace cisplatin (i.p.) or EGT (p.o.). The experimental schedule is shown in Fig. 1. All experimental procedures involving animals were conducted in accordance with National Institutes of Health (NIH) guidelines. This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the Chung Chou Institute of Technology.

2.5. Passive avoidance test

Passive avoidance was measured using a Gemini Avoidance System (San Diego Instrument, San Diego, CA) which consists of two-compartment shuttle chambers with a constant current shock generator. On an acquisition trial, each mouse was placed into the start chamber, which remained darkened. After 20 s, the chamber light was illuminated and the door was opened for mouse to move into the dark chamber freely. Immediately it entered the dark chamber, the door was closed and an inescapable scrambled electric shock (0.5 mA, 0.5 s) for three times (5 s interval) was delivered through the floor grid. Twenty-four hours later, each mouse was again placed in the start chamber again (retention trial). The interval between the placement in the lighted chamber and the entry into the dark chamber was measured as latency in both acquisition and retention trials (maximum 180 s) (Jhoo et al., 2004). The longer latency time the mouse stays in the light room, the better learning memory the mouse has.

2.6. Active avoidance test

Active avoidance was also measured using a Gemini Avoidance System (San Diego Instrument, San Diego, CA). Mice received five learning trials per day for 3 days. On the first trial, mice were placed individually into the large compartment of the apparatus with the door closed and accommodated there for about 10 s. A light (60 W, 10 s) was switched on alternately in the two compartments and used as a conditioned stimulus (CS). The CS preceded the onset of the unconditioned

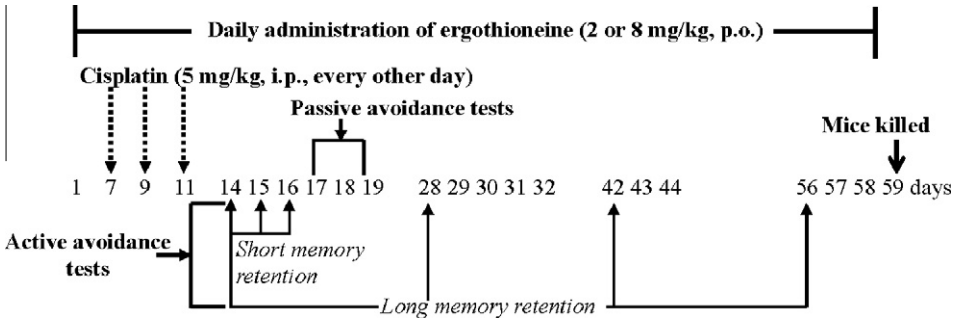

Fig. 1. The animal experimental schedule.

stimulus (US) by 5 s. The US was an electric shock (0.3 mA for 5 s) applied to the floor grid. If the animal avoided the US by running into the dark compartment within 5 s after the onset of the CS, the microprocessor recorder unit of the shuttle-box recorded an avoidance response. Each mouse was given 5 trials daily for 3 days with a mixed intertrial interval of 20 s. The activity level was also assessed by measuring the number of crossings between the chambers when no shock was present (intertrial crossing). The recorder unit of automated shuttle-box continuously recorded this parameter during all experimental period (15 trials). The results were expressed as the mean percent avoidance responses for each daily shuttle-box session. The more avoidance number and less latency time the mouse escapes, the better learning memory the mouse has.

2.7. Preparation of brain tissue homogenates

After completion of treatment with EGT (on day 59), the mice were killed by decapitation under CO_2 anesthesia. All following procedures were carried out at 0–4 °C. Whole brains, except cerebellum, were homogenized using an Ultra Turax homogenizer in 1:10 (w/v) buffer (5.0 mM Tris base, 150 mM NaCl and 20 mM EDTA, pH 7.5). The homogenates were sonicated for 30 s in a disruptor (Bronson Sonic, NY) and centrifuged at 16,000*g* for 10 min. The supernatant was kept at −70 °C until use.

2.8. Determination of GSH/GSSG ratio in brain tissues

GSH/GSSG ratios in brain tissue homogenates were assayed according to the HPLC method of Schofiled and Chen (1995) with some modification. In brief, after adding100 μL of 10% perchloric acid to precipitate the protein of brain homogenates (1.0 mL), the supernatants were treated with iodoacetic acid, neutralized with an excess of $NaHCO_3$, and incubated in the dark at 40 °C for 1 h. A volume (0.2 mL) of 3% (v/v) 2,4-dinitrofluorobenzene was added to the reaction mixtures and allowed to react at 40 °C for 4 h in the dark. After centrifugation (3000*g* for 15 min), a portion (25 μL) of the supernatant was applied onto HPLC column and the GSH/GSSG content was measured at 365 nm.

2.9. Determination of thiobarbituric acid-reactive substances (TBARS) in brain tissues

Lipid peroxidation in brain tissue homogenates was determined as MDA using the thiobarbituric acid (TBA) assay (Buege and Aust, 1978). Butylated hydroxytoluene (10 μL, 50 mM) was added to the tissue homogenate (1.0 mL) to terminate the peroxidation reaction and then mixed with 1 mL of 7.5% (w/v) cold trichloroacetic acid (TCA) to precipitate proteins. The supernatant was allowed to react with 1 mL of 0.8% (w/v) TBA in a boiling water bath for 45 min. After cooling, levels of MDA were determined at 555 nm with excitation at 515 nm using 1,1,3,3-tetraethoxypropane as the standard. MDA levels were expressed as nmol per mg of protein.

2.10. Determination of acetylcholinesterase (AChE) activity and EGT contents in brain tissues

AChE activity in brain tissues was measured by the spectrophotometric method as developed by Ellman et al. (1961). For determination of EGT contents, we used the method outlined in detail by Dubost et al. (2007) for measuring EGT in mushrooms. In short, analysis was carried out using an HPLC with separation carried out on two Econosphere C18 columns (Thermo scientific Associates, IL) with each column being 250 × 4.6 mm, 5 μm particle size connected in tandem. The isocratic mobile phase was 50 mM sodium phosphate in water with 3% acetonitrile and 0.1% triethylamine adjusted to a pH of 7.3 with a flow rate of 1 ml per min. The injection volume was 20 μl, with the column temperature being ambient. EGT was quantified by monitoring absorbance at 254 nm using the authentic standard. Data were expressed as mmol of EGT per milligram of protein (mmol/mg protein). Protein concentrations were determined using a standard commercial kit (Bio-Rad Laboratories Ltd.) with serum bovine albumin as standard.

2.11. Statistical analysis

Data are expressed as means ± SD and analyzed using one-way ANOVA followed by LSD Test for multiple comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS Inc.); a P value <0.05 is considered statistically significant.

3. Results

3.1. Effect of EGT on cisplatin-induced cytotoxicity in PC12 cells

To choose an appropriate concentration of cisplatin to induce PC12 neuron cell damage, we incubated PC12 cells with different concentrations (0.5, 1.0 and 5.0 μM) of cisplatin for 24, 48 and 72 h. As shown in Fig. 2, cisplatin resulted in a dose-dependent decrease in the cell number of PC12 cells. In cells treated with 1.0 or 5.0 μM cisplatin for 24, 48 and 72 h, cell proliferation was almost completely inhibited, pretreatment of PC12 cells with 0.1–10.0 μM EGT for 2 h provided no protective effect at these concentrations of cisplatin. However, when PC12 cells were treated with 0.5 μM cisplatin for 24, 48 and 72 h after pretreatment with 0.1–10.0 μM EGT for 2 h, we found that EGT effectively prevented the antiproliferative effect of cisplatin, and the effect of EGT was concentration- and time-dependent (Fig. 3).

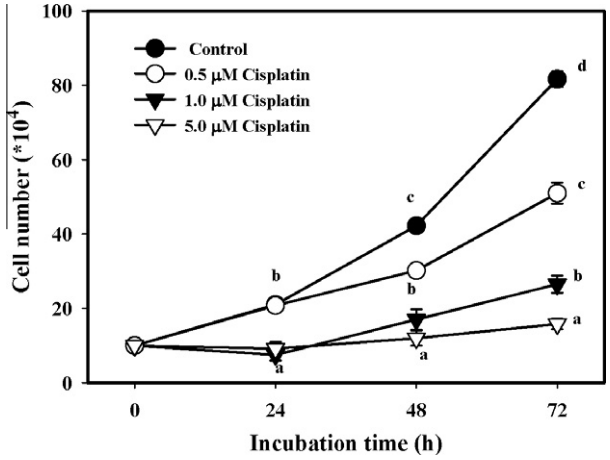

Fig. 2. The in vitro antiproliferative effect of cisplatin in PC12 cells. Cells were incubated with saline (control) or cisplatin (0.5, 1.0 or 5.0 μM) for 24, 48 and 72 h. Cell viability was estimated by Trypan blue dye exclusion method. Values (means ± SD of triplicate tests) not sharing a superscript letter are significantly different ($P < 0.05$).

3.2. Effects of EGT on morphological changes in primary cultured rat cortical neuron cells treated with cisplatin

To determine the effect of cisplatin and EGT on cell morphology and the growth of axons and dendrites, we used primary cultured rat cortical neuron cells incubated with 0.5 μM cisplatin. We found that incubation with cisplatin (0.5 μM) for 72 h resulted in marked inhibition of neuritis outgrowth of rat cortical neuron cells (Fig. 4B). However, when the cells were treated with EGT (0.1 to

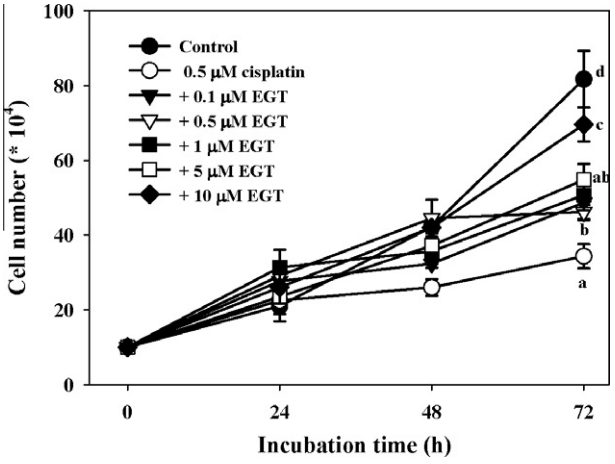

Fig. 3. The in vitro protective effect of ergothioneine (EGT) against cisplatin-induced growth inhibition of PC12 cells. Cells were incubated with 0.5 μM cisplatin and 0.5, 1.0 or 5.0 μM EGT for 24, 48 and 72 h. Cell viability was estimated by Trypan blue dye exclusion method. Values (means ± SD of triplicate tests) not sharing a superscript letter are significantly different ($P < 0.05$).

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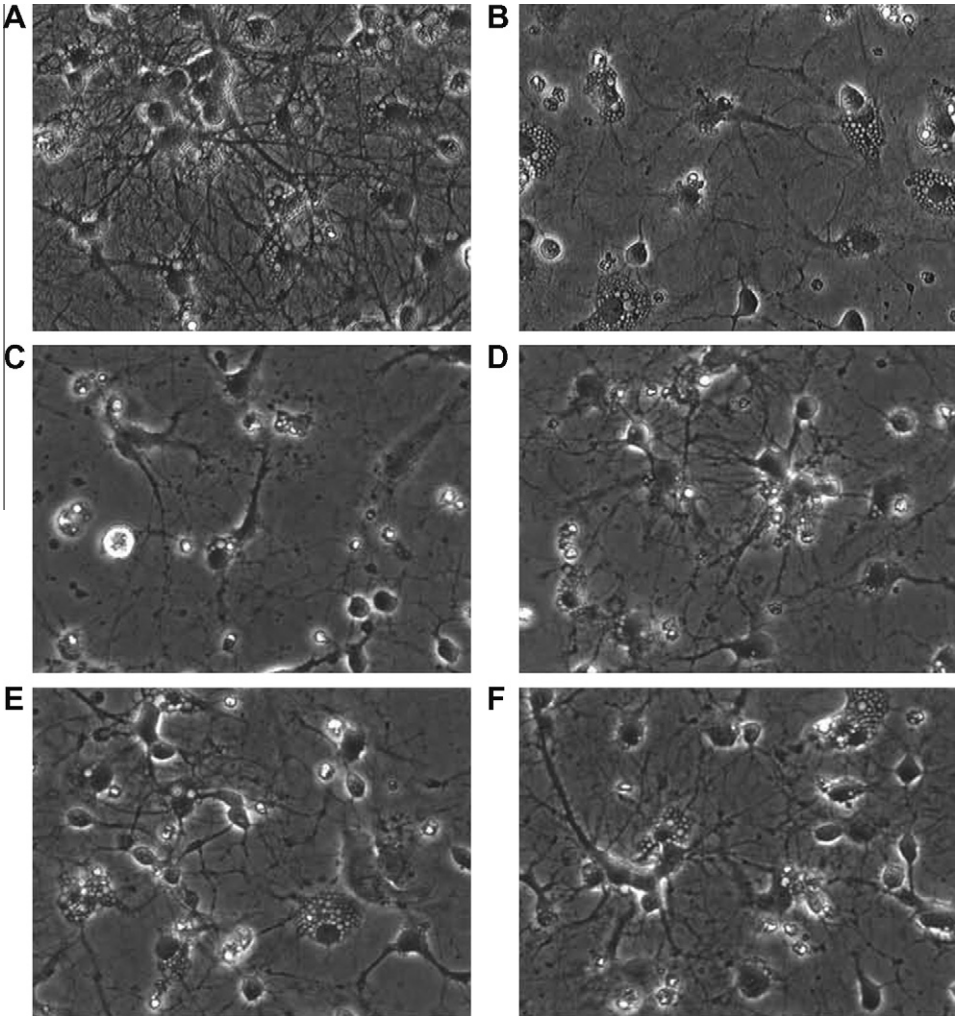

Fig. 4. Neuroprotective effect of ergothioneine (EGT) on cisplatin-induced morphological change of primary cultured rat cortical neuron cells. EGT (0–10 μM) was applied to neurons 2 h prior to the treatment with 0.5 μM cisplatin. The photomicrographys were taken under a phase contrast microscope (100×). (A) Control; (B) neurons treated with cisplatin (0.5 μM); (C–F) neurons treated with 0.5, 1.0, 5.0 and 10.0 μM of EGT prior 2 h cisplatin-treated, respectively.

10 μM) for 2 h followed by incubation with 0.5 μM cisplatin for 72 h, the damage to axon and dendrite induced by cisplatin was effectively prevented by EGT (Fig. 4C–F).

3.3. Effect of EGT on body weights of mice treated with cisplatin

As shown in Fig. 5, cisplatin alone significantly decreased body weights of CBA mice starting on day 14 of the experiment (i.e., 3 days after completion of cisplatin treatment) until day 58, as compared to the control mice. However, supplementation with EGT (2 or 8 mg/kg bw) or melatonin (10 mg/kg bw) resulted in substantial recovery of body weights, and no significant differences in body weights were found on days 28 and 58 in these groups of mice, as compared with the control mice.

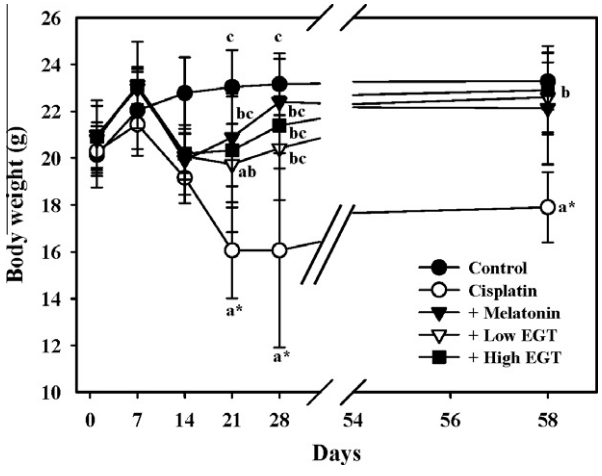

Fig. 5. Body weights in cisplatin-treated mice supplemented with or without ergothioneine (EGT). Two groups of mice were injected i.p. with saline solution (control group) or cisplatin solution (cisplatin group) without administration of test materials. The other cisplatin-treated mice were supplemented with melatonin (+Melatonin, 10 mg/kg/day), low-dosage EGT (+Low EGT, 2 mg/kg/day) and high-dosage EGT (+High EGT, 8 mg/kg/day). Values (means ± SD, $n = 9$ mice per group) obtained at the same wk not sharing a superscript letter are significantly different ($P < 0.05$). Asterisks represent significant differences ($P < 0.05$) from the 0 week cisplatin-treated group.

3.4. Passive avoidance test

The passive and active avoidance tests were used to evaluate the memory and learning ability in this animal model. Step-through latency from light chamber to dark chamber was used as a marker to evaluate the memory and learning ability in the passive avoidance test. As shown in Fig. 6, all the mice would immediately go into the dark chamber in the first trial (day 17) because of skototaxis, but the electric shock in the dark chamber

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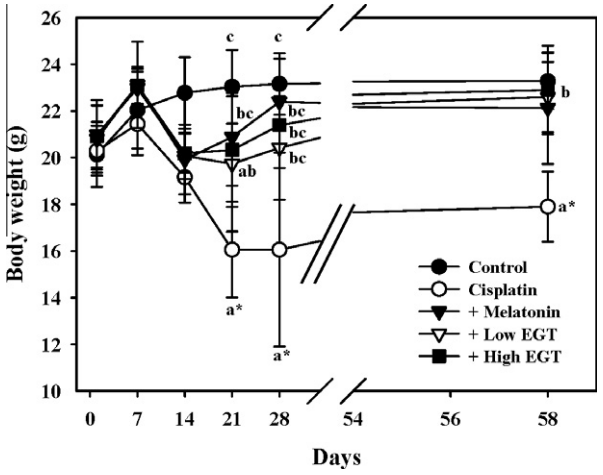

Fig. 6. Effect of ergothioneine (EGT) on step-through latency of multiple-trail passive-avoidance test in cisplatin-treated mice. The tests were carried out from day17 to day 19. Two groups of mice were injected i.p. with saline solution (control group) or cisplatin solution (cisplatin group) without administration of test materials. The other cisplatin-treated mice were supplemented with melatonin (+Melatonin, 10 mg/kg/day), low-dosage EGT (+Low EGT, 2 mg/kg/day) and high-dosage EGT (+High EGT, 8 mg/kg/day). Values (means ± SD, $n = 9$ mice per group) not sharing a superscript letter are significantly different ($P < 0.05$). Asterisks represent significant differences ($P < 0.05$) from the cisplatin-treated group on day 16.

should intimidate and prevent mice with normal memory ability from going into the dark chamber the next time. Therefore, the step-through latency among each group would show significant difference in the next trial. The results of the second and the third trials (on days 18 and 19) clearly indicated that mice treated with cisplatin alone spent significantly shorter period of time staying in the light chamber than the mice of the control group ($P < 0.05$). However, mice treated with low- and high-dosage EGT or with melatonin stayed significantly longer in the light chamber than those treated with cisplatin alone ($P < 0.05$). Although the differences in step-through latency were not significantly different among the mice supplemented with EGT or melatonin, the mice supplemented with high-dosage EGT tended to stay longer than those with low-dosage EGT and the control mice.

3.5. Active avoidance test

Active avoidance tests were presented as both short memory retention and long memory retention using the shuttle-box active avoidance test. The short memory retention session was conducted for three consecutive days starting on days 14 through 16. As shown in Table 1, all groups of mice in the short memory retention session showed gradual decrease in the escape latency and increase in the number of crossings on the 2nd day (day 15) and 3rd day (day 16) of the learning session, as compared to those of the 1st day (day 14). However, no significant differences were observed in the escape latency and number of crossings of all groups of mice ($P > 0.05$). In other words, supplementation with either EGT or melatonin did not significantly improve the short memory retention.

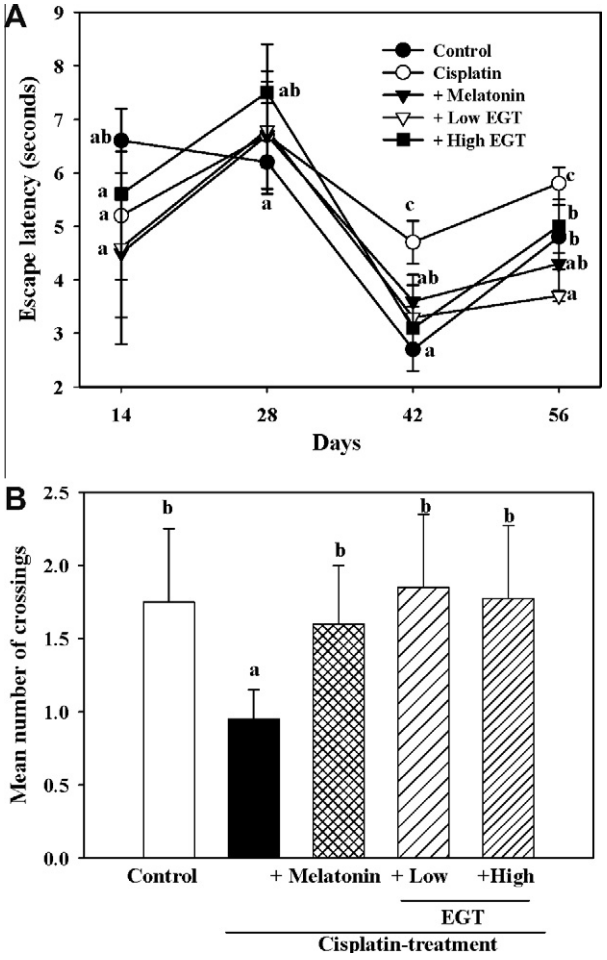

Fig. 7. Effect of ergothioneine (EGT) on step-through latency (A) and mean number of crossings (B) of multiple-trail active-avoidance test in cisplatin-treated mice. The tests were carried out on days14, 28, 42 and 56. Two groups of mice were injected i.p. with saline solution (control group) or cisplatin solution (cisplatin group) without administration of test materials. The other cisplatin-treated mice were supplemented with melatonin (+Melatonin, 10 mg/kg/day), low-dosage EGT (+Low EGT, 2 mg/kg/day) and high-dosage EGT (+High EGT, 8 mg/kg/day). Values (means ± SD, $n = 9$ mice per group) not sharing a superscript letter are significantly different ($P < 0.05$).

Table 1
The effects of ergothioneine (EGT) on active avoidance test (shuttle box) of short memory retention session in cisplatin-treated mice: escape latency and number of crossings.

Groups	Escape latency (sec) Days			Number of crossings Days		
	14	15	16	14	15	16
Control	6.6 ± 0.6^{a}	2.9 ± 0.8^{a}	2.4 ± 0.6^{a}	1.3 ± 0.9^{a}	3.3 ± 0.6^{a}	4.1 ± 0.6^{a}
Cisplatin	5.2 ± 1.2^{ab}	3.8 ± 0.7^{ab}	3.2 ± 0.6^{a}	1.7 ± 1.4^{a}	2.8 ± 0.2^{ab}	3.1 ± 0.6^{a}
+Melatonin	5.0 ± 1.2^{ab}	3.1 ± 1.0^{a}	2.9 ± 0.4^{a}	2.1 ± 1.3^{a}	3.4 ± 0.9^{a}	3.8 ± 0.6^{a}
+Low EGT	4.6 ± 1.8^{ab}	3.2 ± 0.7^{a}	2.6 ± 0.7^{a}	2.2 ± 1.0^{a}	3.3 ± 0.7^{a}	3.9 ± 0.5^{a}
+High EGT	5.6 ± 1.6^{ab}	3.1 ± 0.4^{a}	2.9 ± 1.4^{a}	1.7 ± 1.3^{a}	3.8 ± 0.4^{a}	4.0 ± 0.9^{a}

Two groups of mice were injected i.p. with saline solution (control group) or cisplatin solution (cisplatin group) without administration of test materials. The other cisplatin-treated mice were administered melatonin (+Melatonin, 10 mg/kg/day), low-dosage EGT (+Low EGT, 2 mg/kg/day) and high-dosage EGT (+High EGT, 8 mg/kg/day). Values (means ± SD, $n = 9$ mice per group) not sharing a superscript letter are significantly different ($P < 0.05$).

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The long memory retention session was performed on days 14, 28, 42 and 56. As shown in Fig. 7A, the mice treated with cisplatin alone showed a significant increase in the escape latency on the 42 and 56 days ($P < 0.05$), as compare to those of the control group. In contrast, mice treated with EGT (2 and 8 mg/kg) or melatonin (10 mg/kg) showed a significant decrease in the escape latency ($P < 0.05$) on days 42 and 56, as compared to those treated with cisplatin alone. We also found that the mean number of crossings in mice treated with cisplatin alone was significantly lower than that of control group ($P < 0.05$) (Fig. 7B). EGT and melatonin significantly increased the number of crossings, as compared to those treated with cisplatin alone ($P < 0.05$). However, there was no significant difference between the low-dosage EGT (2 mg/kg) and high-dosage EGT (8 mg/kg) groups ($P > 0.05$).

3.6. Effect of EGT on AChE activity in the brain of cisplatin -treated mice

In mice treated with cisplatin alone, the brain AChE activity was significantly increased in comparison with the control group (Fig. 8). Oral administration with EGT (2 or 8 mg/kg) or melatonin significantly prevented the rise in brain AChE activity induced by cisplatin ($P < 0.05$). However, there was no significant difference in the brain AChE activity between the high-EGT group and the low-EGT group.

3.7. Effect of EGT on antioxidant status in the brain of cisplatin-treated mice

As shown in Table 2, cisplatin significantly decreased EGT contents in mouse brain tissues (31% lower than the control group, $P < 0.05$). Oral administration of EGT significantly restored the loss of EGT levels induced by cisplatin, and the effect of EGT was dose dependent, with the high-EGT group completely recovering the loss of brain EGT levels and reaching a slightly higher level than that of the control mice. Interestingly, melatonin also completely recovered the loss of brain EGT contents, and reached a level that is comparable to that of the high-EGT group, even though the brain EGT contents in the melatonin group and the high-EGT group are not significantly different from those in the control group.

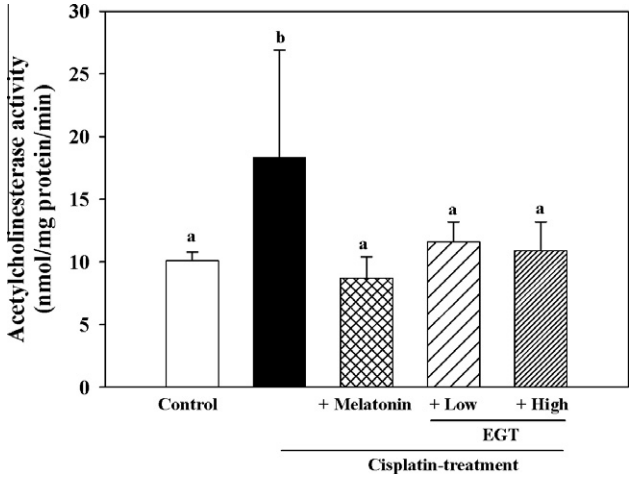

Fig. 8. Effect of ergothioneine (EGT) on the acetylcholinesterase activity in mouse brain tissues. Two groups of mice were injected i.p. with saline solution (control group) or cisplatin solution (cisplatin group) without administration of test materials. The other cisplatin-treated mice were supplemented with melatonin (+Melatonin, 10 mg/kg/day), low-dosage EGT (+Low EGT, 2 mg/kg/day) and high-dosage EGT (+High EGT, 8 mg/kg/day). Values (means ± SD, $n = 9$ mice per group) not sharing a superscript letter are significantly different ($P < 0.05$).

Table 2
The effects of ergothioneine (EGT) on antioxidant status in the brain tissue of cisplatin-treated mice.

Groups	EGT (mmol/mg protein)	MDA (nmol MDA/mg protein)	GSH/GSSG ratio
Control	35.8 ± 6.9^{b}	100 ± 66^{b}	14.5 ± 2.7^{d}
Cisplatin	24.9 ± 6.1^{a}	372 ± 179^{a}	4.5 ± 0.5^{a}
+Melatonin	37.9 ± 2.4^{bc}	71 ± 48^{b}	9.6 ± 0.1^{c}
+Low EGT	27.1 ± 6.0^{ab}	117 ± 65^{b}	7.9 ± 1.9^{b}
+High EGT	37.8 ± 7.5^{bc}	78 ± 64^{b}	10.3 ± 1.0^{c}

Values (means ± SD, $n = 9$ mice per group) not sharing a superscript letter are significantly different ($P < 0.05$).

As also shown in Table 2, cisplatin increased lipid peroxidation in the brain, as evidenced by a 3.7-fold increase in TBARS. Both EGT (at 2 and 8 mg/kg bw) and melatonin completely inhibited cisplatin-induced lipid peroxidation. In contrast, cisplatin markedly decreased the GSH/GSSG ratio (from 14.5 ± 2.7 in the control group to 4.5 ± 0.5), and treatment with EGT significantly and dose-dependently prevented the decline of GSH/GSSG ratio. Interestingly, the GSH/GSSG ratio in the melatonin group (9.6 ± 0.1) was significantly higher than that in the low-EGT group (7.9 ± 1.9) ($P < 0.05$).

4. Discussion

EGT is regarded as an antioxidant due to its ability to reduce the oxidative stress both in vitro (Jang et al., 2004; Aruoma et al., 1999) and in vivo (Guijarro et al., 2002). Although several thiols have been reported to protect against cisplatin toxicity (Somani et al., 1995), it is unclear whether EGT is neuroprotective against cisplatin toxicity. In this study, we conducted both in vitro and in vivo experiments to answer this question. Our in vitro experiments demonstrated that EGT significantly prevented the decrease in cell proliferation of PC12 cells. Cisplatin also strongly inhibited the growth of axon and dendrite of PCN cells, and EGT supplementation significantly prevented the inhibition. Our in vivo experiments in cisplatin-treated CBA mice confirmed that EGT significantly prevented brain lipid peroxidation, and we further demonstrated that EGT restored brain GSH/GSSG ratios and attenuated the rise in AChE activity. We further showed that EGT significantly prevented the learning and memory deficits induced by cisplatin by decreasing the active avoidance time and increasing the successful number of passive and active avoidance by the electric shock in cisplatin-treated mice. To the best of our knowledge, this is the first report to document the neuroprotective effects of EGT on cisplatin-induced memory and learning impairment in mice.

The protective effect of EGT against cisplatin toxicity is likely related to the inhibition of lipid peroxidation and to maintaining the GSH/GSSG ratio in mouse brain. Inhibition of lipid peroxidation can prevent the alteration of the neuronal membrane, thereby normalizing the neuronal glucose transporter, deceased glucose phosphorylation, and neuronal cell death (Pardridge, 1994). As expected, the EGT levels were increased in brain tissues of mice supplemented with EGT, and this free thiol could inhibit lipid peroxidation through maintaining the GSH/GSSG ratio by directly reacting with cisplatin or by reducing GSSG to GSH in brain tissues of mice treated with cisplatin. It has been shown that endogenous thiols such as metallothionein and GSH can limit the amount of the DNA platination by cisplatin (Sadowitz et al., 2002; Hagrman et al., 2003). Indeed, Deiana et al. (2004) also have reported that supplementation with EGT not only protects the organs against lipid peroxidation but conserves the consumption of endogenous GSH and alpha-tocopherol. Importantly, Gründemann et al. (2005) have

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reported that EGT has an organic cation transporter-1 (OCTN1) in the neuronal cells that can pass the blood brain barrier (BBB) to enter the central nervous system. Thus, EGT readily enter the neuronal cells to protect neuronal neuron cell membrane and the cellular transport systems by maintain normal membrane fluidity thereby reducing neuronal cell apoptosis.

Cognitive deficits associated with Alzheimer's disease (AD) are thought to be primarily related to the degeneration of cholinergic neurons in cerebral cortex and hippocampus, resulting in deficits of cholinergic neurotransmission. AChE is responsible for acetylcholine hydrolysis, from both cholinergic and non-cholinergic neurons of the brain (Atack et al., 1983). AChE activity has been shown to be increased within and around amyloid plaques (Ulrich et al., 1990; Morán et al., 1993), to promote the assembly of amyloid beta-peptides into fibrils (Inestrosa et al., 1996) and to increase the cytotoxicity of these peptides (Alvarez et al., 1998). Treatment of AD has targeted the inhibition of AChE to increase concentrations of ACh and improve cognitive and behavior symptoms (Krall et al., 1999). Here, we observed that the AChE activity was increased in the brain of cisplatin-treated mice (Fig. 8). As indicated by Kaizer et al. (2005), the elevation of AChE activity may be due to a direct neurotoxic effect on the plasmatic membrane caused by increased lipid peroxidation, and that such changes may affect the integrity and functionality of the cholinergic system. Thus, we believe that alterations in the lipid membrane could be a decisive factor in changing the conformational state of the AChE molecule, which would result in learning and memory deficits after cisplatin exposure. Furthermore, our observation that EGT was capable of preventing the increase of AChE activity suggests that EGT may play an important role in the maintenance of acetylcholine synaptic levels by preventing the compromise of AChE activity. Thus, the prevention of the restoration in AChE activity may be another mechanistic action by which EGT prevents or improves learning and memory functions of mice.

The doses of EGT used in the present study (2.0 and 8.0 mg/kg/day) appear to be achievable in humans, although there is no estimated intake of EGT in humans today. Take the highest EGT dose (8.0 mg/kg/day) as example: for a mouse whose average body weight is about 25 g, a daily supplemental level of 8.0 mg/kg bw translates into 200 μg EGT/mouse/d (i.e., 8.0 mg/kg bw × 0.025 kg bw). This daily consumption level is equivalent to a human consumption level of 40 mg/day, as calculated by the difference in daily food intake, i.e., 2.5 g (dry weight) for a mouse and 500 g (dry weight) for a person with an energy intake of 2000 kcal/day (Liu et al., 2002). This intake level (40 mg) can easily be achieved by consumption of EGT-rich foodstuff, such as mushrooms which contain up to 2.6 mg EGT per g dry weight (Dubost et al., 2007). Thus, an intake of approximately 15 g dry mushroom per day would achieve an EGT intake of 40 mg. Although little is known about the safety of EGT consumption, no reports in animal studies have shown any deleterious effects so far, including the present mouse study of ours. It should be noted that an early study showed that some diabetic patients had elevated blood EGT levels (Fraser, 1950), and this has led to the speculation that EGT may induce diabetes through chelation of zinc (Epand, 1982). However, later experiments have found no statistical significance in blood EGT levels between diabetics and non-diabetics (Epand and Epand, 1988).

Melatonin, which was used as a positive control in the present study, is the main hormone of the pineal gland has been described to be a potent scavenger of free radicals and stimulates other antioxidant activities by preventing hydroxyl (**.**OH) and peroxyl (ROO**.**) radical formation (Tan et al., 1993; Kaya et al., 1999). Studies have demonstrated that melatonin reduces STZ-induced oxidative stress in diabetic rats (Montilla et al., 1998), protects human red bloods cells from oxidative hemolysis (Tesoriere et al., 1999), and inhibits the vasorelaxant action of peroxynitrite in human umbilical artery (Okatani et al., 1999). In addition, melatonin has been reported to either ameliorate or prevent the nephrotoxicity of cisplatin (Hara et al., 2001; Sener et al., 2000). Our results also showed that the administration of melatonin to cisplatin-treated mice significantly increased the GSH/GSSG ratio and decreased MDA formation in the brain of mice. These observations support that melatonin functions as an antioxidant in vivo.

In summary, we demonstrate that EGT administration protects against cisplatin-induced neurotoxicity both in vitro and in vivo. The present findings along with the fact that EGT has an OCTN1 in the neuronal cells to facilitate its entrance to the central nervous system suggest that EGT has the potential to become a useful neuroprotective agent in humans. Further studies are warranted.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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