Research Report

Protective effect of L-theanine on chronic restraint stress-induced cognitive impairments in mice

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\textbf{A R T I C L E I N F O}

Article history:
Accepted 31 January 2013
Available online 5 February 2013

Keywords:
Chronic restraint stress
L-theanine
Cognitive impairments
Brain oxidative alterations
Neuroendocrine changes

\textbf{A B S T R A C T}

The present work was aimed to study the protective effect of L-theanine on chronic restraint stress (CRS)-induced cognitive impairments in mice. The stress was produced by restraining the animals in well-ventilated polypropylene tubes (3.2 cm in diameter × 10.5 cm in length) for 8 h once daily for 21 consecutive days. L-theanine (2 and 4 mg/kg) was administered 30 min before the animals subjected to acute immobilized stress. At week 4, mice were subjected to Morris water maze and step-through tests to measure the cognitive function followed by oxidative parameters and corticosterone as well as catecholamines (norepinephrine and dopamine) subsequently. Our results showed that the cognitive performances in CRS group were markedly deteriorated, accompanied by noticeable alterations in oxidative parameters and catecholamine levels in the hippocampus and the cerebral cortex as well as corticosterone and catecholamine levels in the serum. However, not only did L-theanine treatment exhibit a reversal of the cognitive impairments and oxidative damage induced by CRS, but also reversed the abnormal level of corticosterone in the serum as well as the abnormal levels of catecholamines in the brain and the serum. This study indicated the protective effect of L-theanine against CRS-induced cognitive impairments in mice.

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1. Introduction

In the competitive world of modern technology, mental and emotional stress has become an unavoidable part of life and is known to have deleterious effects on physical and mental well-being. Stress is a crucial determinant for maintenance of health and disease (Jacobson and Spolsky, 1991; Gilgun-Sherki et al., 2001). Stress either due to internal or external stimuli disturbs physiological homeostasis and causes neurobehavioral alteration (Masood et al., 2003; Masood et al., 2004). It has been reported that restraint stress is an easy and convenient method for psychological and physical stress resulting in restricted mobility and aggression (Romanova et al., 1994; Singh et al., 1999).
Severe and prolonged stress precipitates affective disorders and causes impairment in learning and memory. The main physiological responses to chronic stress include the hypothalamic–pituitary–adrenal axis (HPA) and the sympathoadrenomedullary system (SAM), through which the levels of corticosterone and catecholamine were altered (Cohen and Hamrick, 2003). Enhancement of corticosterone (CORT) levels via the hyperactivity of the HPA axis resulted in impaired performances of the cognitive function, including learning and memory, and spatial recognition (Beane et al., 2002; Harvey et al., 2006). However, the secretion of serum catecholamines conducted by the SAM system, e.g., noradrenaline (NA) and dopamine (DA), were considered as an immediate response in fighting against restraint stress (Sachser, 1987; Chen et al., 2011). Furthermore, there have been many reports (Shaheen et al., 1993; Kovacs et al., 1996; Liu and Mori, 1999; Olivenza et al., 2000) suggesting that free radicals play an aberrant role in the mechanism of stress. As previously shown (Shaheen et al., 1993; Liu and Mori, 1999; Matsumoto et al., 1999), stress can stimulate numerous pathways leading to an increased production of free radicals. Oxidative stress is widely accepted as a contributor to neuronal vulnerability (Langley and Ratan, 2004; Lin and Beal, 2006; Ohta and Ohsawa, 2006). The putative role of oxygen radicals and radical-derived reactive oxygen species in neurodegeneration and cognitive decline has been well reviewed (Sayre et al., 2008; Gallagher et al., 1996; Berr et al., 2000). The brain, though comprising a very small part of body mass consumes an appreciable amount of the oxygen, is extremely susceptible to reactive oxygen species induced damage.

Lack of satisfactory treatment of the cognitive deficits usually accompanying stress, depression, and associated mental problems present a constant challenge for psychopharmacology research. Anti-anxiety or hypno-sedative agents, commonly used for the management of stress, have several disadvantages and ill effects. Therefore, employment of safe natural products can be an ideal choice. L-theanine, one of the major amino acids contained in green tea, has been a focus of attention due to its biochemical characteristics, Yokogoshi et al. (1998a, 1998b) reported that L-theanine could pass through the blood-brain barrier, and that it increased by 1 h at the latest in serum, the liver, and the brain after administration, thereafter decreasing sharply in the serum and liver but only beginning to decrease in the brain 5 h after administration. Furthermore, another study reported that L-theanine could influence the secretion and function of neurotransmitters in the central nervous system even at 30 min after oral administration (Terashima et al., 1999). L-theanine has also been demonstrated to have antioxidative properties (Serrano and Klann, 2004; Yokozawa and Dong, 1997; Cho et al., 2008) and neuroprotective effects against ischemia (Nishida et al., 2008; Kakuda, 2002; Egashira et al., 2004) and Parkinson-related neurotoxicants (Yokozawa and Dong, 1997). In further support, L-theanine has been shown to improve memory function (Egashira et al., 2007; Nathan et al., 2006) and prevent memory impairment induced by cerebral ischemia (Yamada et al., 2008), moreover, L-theanine is known to block the binding of L-glutamic acid to glutamate receptors in the brain and oral intake of L-theanine could cause anti-stress effects via the inhibition of cortical neuron excitation (Kimura et al., 2007). However, the protective effect of L-theanine on CRS-induced spatial cognitive impairments and the mechanisms of cognitive improvement are yet to be reported. Therefore, the aim of this study was to evaluate the neuroprotective effect of L-theanine on stress-induced cognitive impairments in mice. Meanwhile, the neuroendocrine changes and alterations in anti-oxidative status associated with chronic restraint stress were also determined.

2. Results

2.1. Effects of L-theanine on behavioral assessment

2.1.1. Spatial recognition and learning

To examine whether L-theanine could attenuate the CRS-induced cognitive impairments, we tested the learning and memory using the Morris water maze (MWM) test and the results are shown in Fig. 1. The mean escape latency for the trained rats was decreased over the course of the learning trials in all the groups (Fig. 1A), and from the third day onwards there was a significant difference in transfer latency...
between the CRS and the control mice \([F(4,45) = 10.512, P < 0.001]\). However, treatment with L-theanine at doses of the 2 mg/kg and 4 mg/kg \((P < 0.05)\) significantly decreased the transfer latency as compared to the CRS mice. However, there was no significant difference in escape latency between the L-theanine (4 mg/kg) group and the control group.

In the probe trial of the MWM study, which assesses how well the animals have learned and consolidated the platform location during the four days of training, the animals showed a significant difference (Fig. 1B). The percentage of time spent in the target quadrant was significantly \(F_{(4,45)} = 35.439, P < 0.001\) lower in CRS animals as compared to the control group. However, the latencies of L-theanine treatment at doses of 2 mg/kg \((P < 0.05)\) and 4 mg/kg \((P < 0.001)\) showed a significantly longer stay in the target quadrant compared with the CRS group.

2.2. Effects of L-theanine on the passive avoidance response in step-through test

Significant effects of CRS on the latency to the initial entry into the dark compartment were observed in the step-through test (Fig. 2). Post-hoc comparisons showed that exposure to CRS significantly decreased the latencies during the training and retention trials \([F(4, 45) = 64.41, F(4, 45) = 25.512, P < 0.001]\) compared with the control group, the latencies of L-theanine treatment groups \((2 \text{ mg/kg} \text{ and } 4 \text{ mg/kg})\) were evidently increased \((P < 0.01)\) (Fig. 2A). Similarly, the number of mistakes in L-theanine treatment group at doses of 2 mg/kg and 4 mg/kg only had a tendency to decrease and at the dose of 4 mg/kg the differences were statistic \([F(4,45) = 21.874, F(4, 45) = 16.88, P < 0.05]\) (Fig. 2B).

![Fig. 2 – (Section 2.1.2). Effects of L-theanine on the passive avoidance response in step-through test. Step-through latency is shown (A). Number of mistakes is illustrated (B). Data are reported as mean ± SEM \((n = 10)\). \(^*P < 0.001\) as compared to the control group; \(^{\#}P < 0.05\), \(^{\#\#}P < 0.01\), \(^{\#\#\#}P < 0.001\), as compared to the CRS group.](image)

2.3. Effect of L-theanine on CORT increment in the serum

As shown in Fig. 3, the CORT levels in serum were significantly higher in the CRS group than in the control group \([F(4,35) = 54.931, P < 0.001]\), while a marked decline in the serum CORT levels was observed in the L-theanine treatment groups at doses of 2 mg/kg \((P < 0.05)\) and 4 mg/kg \((P < 0.001)\). However, there was no significant difference between the L-theanine (4 mg/kg) group and the control group.

2.4. Effects of L-theanine on biogenic catecholamine reduction in the serum and the brain

As shown in Fig. 4, CRS significantly decreased the levels of NA \([F(4,35) = 23.053, P < 0.001]\) and DA \([F(4,35) = 11.898, P < 0.001]\) as compared to the control group. However, L-theanine treatments at doses of 2 mg/kg and 4 mg/kg resulted in a significant elevation in NA \((P < 0.05, P < 0.001)\) and DA \((P < 0.05, P < 0.001)\) levels in the serum as compared to the CRS mice, respectively (Fig. 4A, B). In addition, CRS significantly decreased the NA levels in both the hippocampus \([F(4,35) = 52.459, P < 0.001]\) and the cerebral cortex \([F(4,35) = 52.688, P < 0.001]\) as compared to the control group (Fig. 5). However, L-theanine at doses of 2 mg/kg and 4 mg/kg significantly inhibited the depletion of NA levels in the hippocampus \((P < 0.05, P < 0.001)\) and the cerebral cortex \((P < 0.001, P < 0.001)\) in CRS mice, respectively (Fig. 5A). Similarly, CRS dramatically decreased the levels of DA in the hippocampus \([F(4, 35) = 20.690, P < 0.001]\) and cerebral cortex \([F(4, 35) = 68.731, P < 0.001]\), whereas L-theanine treatments \((2 \text{ mg/kg} \text{ and } 4 \text{ mg/kg})\) resulted in a significant elevation in DA levels in both the hippocampus \((P < 0.05, P < 0.001)\) and the cerebral cortex \((P < 0.05, P < 0.001)\) as compared to CRS mice (Fig. 5B). However, L-theanine per se did not alter catecholamine levels in both the serum and different brain areas as compared to the control mice.

![Fig. 3 – (Section 2.2). Effects of L-theanine on the CRS-induced CORT increment in the serum. Data are reported as mean ± SEM \((n = 8)\). \(^{\#\#}P < 0.001\), as compared to the control group; \(^{\#}P < 0.05\), \(^{\#\#}P < 0.001\), as compared to the CRS group.](image)
2.5. Effects of L-theanine on parameters of oxidative stress in brain

2.5.1. Effects of L-theanine on CRS-induced changes in lipid peroxidation
Effects of chronic treatment with L-theanine on lipid peroxidation (LPO) are depicted in Fig. 6A. Malondialdehyde (MDA) levels were significantly increased in the cerebral cortex \( F(4,35) = 22.571, P < 0.001 \) and hippocampus \( F(4,35) = 25.45, P < 0.001 \) of CRS mice as compared to the control animals. However, L-theanine (2, 4 mg/kg) treatment significantly inhibited the elevation of MDA levels as compared to CRS mice \( \text{P} < 0.001, \text{P} < 0.001 \) and hippocampus \( \text{P} < 0.05, \text{P} < 0.01 \). However, L-theanine per se did not alter MDA levels in different brain areas of control mice.

2.5.2. Effects of L-theanine on CRS-induced changes in the anti-oxidant profile
The reduced glutathione (GSH) levels \( F(4,35) = 30.517, P < 0.001; F(4,35) = 47.428, P < 0.001 \) and enzyme activities of superoxide dismutase (SOD) \( F(4,35) = 23.373, P < 0.001; F(4,35) = 37.344, P < 0.001 \) and catalase (CAT) \( F(4,35) = 72.798, P < 0.001; F(4,35) = 29.922, P < 0.001 \) significantly decreased in the cerebral cortex and hippocampus of CRS rats as compared to the control group (Fig. 6B–D). This reduction was significantly improved by the treatment with L-theanine in both cerebral cortex and hippocampus of CRS mice. However, L-theanine per se did not influence the endogenous anti-oxidant profile.

3. Discussion
Today stress is one of the major mental disorders affecting a large number of the population, which disturbs normal physiological equilibrium of the body by producing adverse effects on the nervous, endocrine, biochemical, and immune systems (Ray et al., 2003). Exposure to chronic restraint stress in rats and psychosocial stress in humans has been shown to alter cognitive functions such as learning and memory and have been linked to the pathophysiology of mood and anxiety disorders (Xu et al., 2009). The main finding of the present study was that chronic restraint stress can impair cognitive function and that harmful effect of chronic stress can be prevented by L-theanine treatment, suggesting that L-theanine has potential therapeutic effect in protecting against CRS-induced cognitive impairments in mice.

L-theanine has been shown to exert a neuroprotective effect against cerebral ischemia (Kakuda, 2002; Egashira, et al., 2004, 2007) and Parkinson-related neurotoxins (Cho et al., 2008). In addition, recent investigations have demonstrated that L-theanine can cause anti-stress effects via the inhibition of cortical neuron excitation (Kimura et al., 2007), and...
improve memory function (Nathan et al., 2006; Yamada et al., 2008) and prevent memory impairment induced by repeated cerebral ischemia in rats (Egashira et al., 2008). To investigate whether l-theanine could cure CRS-induced impairments of cognitive function, the cognitive function of mice exposed to CRS with or without daily l-theanine treatment was determined using MWM test and step-through test. In the MWM test, mice treated with l-theanine shortened the time spent by mice to reach the platform compared with the CRS group, and when the invisible platform is removed stressed mice treated with l-theanine swam in the target quadrant area for a longer time than stressed controls. In the step-through test, l-theanine treated mice exhibited increased step-through latencies as compared with the stressed controls. These findings suggest that l-theanine can relieve CRS-induced impairment of cognitive function. This is the first report that has shown that l-theanine treatment attenuate CRS-induced cognitive impairments.

Stress can be considered as a physiological response to a threat, which usually activates a specific neuroendocrine pathway known as the hypothalamic–pituitary–adrenal (HPA) axis (de Kloet et al., 2005). The secretion of the neurohormones, i.e., corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP), recognized as the most important physiological substances involved in basal and stress-induced adrenocorticotrophic hormone (ACTH) and glucocorticoid secretion, is controlled by a variety of brain monoamines and peptides such as catecholamines, serotonin, and neuropeptide Y (Marcilhac et al., 1998). The neurotransmitters NE and DA are important monoamines which are widely distributed in brain, and their functional role is well established during stressful conditions (Tsigos and Chrousos, 2002; Carrasco and Van de Kar, 2003). Changes in their activity lead to alteration in the HPA axis homeostasis, leading to development of various psychological and physiological disorders (Sheikh et al., 2007). Plasma CORT level, being an immediate stress effector, is considered to be an important marker to evaluate stress response. High glucocorticoid levels actually signal the presence of a stress condition that might perturb homeostasis, and chronic high levels can themselves be related to health risks and impairment of physiological functions. For example, chronic stress mediated by glucocorticoids impairs cognitive and learning abilities in vertebrates (Sapolsky et al., 2000). In our study, serum CORT level was markedly increased after chronic restraint stress, while pretreated with l-theanine markedly decreased the serum CORT level compared with the stressed controls. It has also been postulated that cognitive dysfunction and behavioral depression, induced by stress, may be induced by similar neurochemical mechanisms, including depletion of monoamines by sustained stress (Bhattacharya et al., 2002). NA and DA are the important monoamines which are widely distributed in brain and their functional role is well established during stressful conditions (Tsigos and Chrousos, 2002; Carrasco and Van de Kar, 2003). Evidence suggested that neurotransmitter systems are also involved in learning and memory processes, and a substantial part of learning and memory impairments is due to changes in neurotransmission (Wenk et al., 1987). In our present study, a significant reduction was observed in levels of catecholamines including NA and DA in the brain and the serum of animals subjected to CRS. However, l-theanine exhibited considerable reversal effect on the CRS-induced variations of the values of NA and DA as shown by their quantification. Consequently, l-theanine treatment could attenuate the cognitive impairments, which might be further attributed

Fig. 6 – (Section 2.4). Effects of l-theanine on lipid peroxide (A), reduced glutathione (B), superoxide dismutase (C), and catalase (D) levels in the brain. Data are reported as mean ± SEM (n = 8). aP < 0.001, as compared to the control group; bP < 0.05, cP < 0.01, dP < 0.001, as compared to the CRS group.
to its normalizing effect on the levels of CORT and catecholamines.

It has been shown that chronic administration of glucocorticoids in mammal causes increased levels of lipid peroxidation and decreased antioxidant capacity in different body tissues (Behl et al., 1997; McIntosh et al., 1998a, 1998b; Orzechowski et al., 2002). Also, psychological stress induces oxidative stress by reducing antioxidant defenses (Zafir and Banu, 2009). Oxidative stress causes cellular damage and accelerates neurodegeneration by inducing the reactive oxygen species (ROS) that oxidize vital cellular components such as lipids, proteins and DNA (Marzatico et al., 1998; Suh et al., 2000). L-theanine itself does not have antioxidant properties, but intake of L-theanine was thought to be effective against the tissue changes with GSH level reduction (Sadzuka et al., 2005). In the present study, we found that decrease of GSH in mouse brain induced by chronic restraint stress was also prevented by L-theanine. Although GSH depletion may enhance the peroxidation process, it alone is not sufficient to induce lipid peroxidation. On the other hand, organisms per se have enzymatic and non-enzymatic defences, including CAT and SOD against the ROS-induced lipid peroxidation (Mates et al., 1999). It has been well documented that chronic restraint stress decreased the antioxidant capacity of mouse brain by inhibiting the activity of the antioxidant enzymes. Our study demonstrated L-theanine significantly prevented the decrease of activities of SOD and CAT in mice induced by CRS. These findings indicated that L-theanine protected mice against CRS-induced oxidative stress via multiple routes. Thus, we guess that during the exposure to chronic restraint stress continuous consumption of L-theanine reduced oxidative stress in the brain, leading to the prevention of the decline in learning and memory.

In summary, continuous consumption of L-theanine throughout the whole period of chronic restraint stress reduced oxidative stress and CORT levels, prevented the decline in monoaminergic content. It also improved cognitive decline, all of which were induced by chronic physical restraint stress. All of these suggest that L-theanine can be of therapeutic value for stress related disorders, and the mechanisms underlying its anti-stress action may be related with its antioxidant activity along with its ability to regulate HPA axis and the levels of catecholamines.

4. **Experimental procedures**

4.1. **Animals and housing**

Adult male Kunming mice, 10–12 weeks old and weighing between 22 and 30 g bred in Central Animal House facility of Xuzhou Medical College. The animals were housed under standard laboratory conditions and maintained on a 12 h light/dark cycle and had free access to food and water except during the restraint session. Animals were acclimatized to laboratory conditions before the experiment.

4.2. **Drugs and experimental protocol**

L-theanine supplied as a white powder by Taiyo Kahaku Co., Ltd. (Yokkaichi, Japan) prepared with normal saline, we selected the dose of L-theanine according to similar reports and our previous study (Kim et al., 2009; Yin et al., 2011). Animals were divided into five groups with 10 animals each i.e., control, CRS, CRS+L-theanine (2 mg/kg), CRS+L-theanine (4 mg/kg), L-theanine (4 mg/kg). L-theanine was administered orally (p.o.) followed by restraint stress and accessed by the mice for 3 weeks. All the experiments were carried out between 9:00 and 17:00. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

4.3. **Restraint stress procedure**

Adult mice were subjected to chronic restraint stress as previously described (Egashira et al., 2008). Mice were subjected to 8 h of restraint stress daily for 21 days in well-ventilated polypropylene tubes (3.2 cm in diameter × 10.5 cm in length) deprived of food and water, without physical suppression or pain experience in mice. Mice were returned to their home cages during the stress session. Control mice were housed in a room separately from stressed mice.

4.4. **Behavioral assessment**

4.4.1. **Morris water maze test**

Animals were tested in a spatial version of MWM test (Pawlak et al., 2003; Morris et al., 1982) for five consecutive days after the third week of stressing. The water maze was a circular pool filled with water at room temperature (diameter, 120 cm; height, 60 cm; water temperature, 24 ± 1 °C). A transparent platform (diameter, 10 cm) was hidden 1.5 cm below the surface of water that had been made opaque with white nontoxic paint. The pool was virtually divided into four quadrants, i.e., NE, SE, SW, and NW. The escape platform was kept at a fixed position in the center of a quadrant. The rats received four consecutive daily training trials in the following 5 days, with each trial lasted until either the mouse had found the platform or for a maximum of 90 s. All mice were allowed to rest on the platform for 20 s.

A probe trial was performed (Tuzcu and Baydas, 2006; Kuhad and Chopra, 2007, 2008) on the fifth day wherein the extent of memory consolidation was assessed. In the probe trial, the mice were placed and released opposite the site where the platform had been located. The single-probe trial consisted of a 90 s free swim in the pool without the platform. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning and the percentage of time spent in the former platform was recorded for the probe trial.

4.4.2. **Step-through**

Immediately after the MWM test, the step-through passive avoidance response (Pan et al., 2009) was performed. The step-through task is accepted as a simple and rapid memory test which is in widespread use throughout the world. The apparatus consisted of an illuminated and dark compartment, separated by a vertical sliding door. We initially placed a mouse in the illuminated compartment facing away from the dark compartment for 3 min for environmental adaptation. Then opened the door, the mouse could enter the dark compartment (mice instinctively prefer being in the dark). When the mouse completely entered the dark compartment,
it was given an electric current (36 V). Then the mouse returned to its home cage. Training (0 h) was performed for 5 min as mentioned above, and the test (24 h) was repeated 24 h later. During the training and retention trials, the number of mistakes and the latency to the initial entry into the dark compartment were recorded within 5 min, respectively. The retention trails were set at a limit of 300 s of cut-off time.

4.5. Sample collection

The day after completion of the behavioral tests, blood samples were collected drawn from the orbital sinus in 2 ml Eppendorf tubes. Blood samples were collected followed by centrifuged at 4000 rpm for 15 min at 4 °C and their brains were carefully removed followed by rapid dissection of the hippocampus and the cerebral cortex. The samples of the brain and serum were stored at −80 °C until measurement.

4.6. Measurement of corticosterone, dopamine, and norepinephrine levels

CORT levels in the serum were determined with a commercial enzyme-linked immunosorbent assay (ELISA) kits (Adlitteram Diagnostic Laboratories, USA) for mice according to the manufacturer’s instructions. Similarly, the concentrations of NA and DA in the brain and serum samples were also respectively analyzed with ELISA kits (Adlitteram Diagnostic Laboratories, USA).

4.7. Measurement of oxidative stress

The samples of the brain were thawed and homogenized in volumes (1:9 w/v) of ice cold normal saline. The homogenates were centrifuged for 10 min at 4000 rpm at 4 °C and the supernatants were used for determination of MDA and GSH levels and the activities of SOD and CAT.

4.7.1. Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in brain was done by the method of Wills (1966). The amount of MDA formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nanomol MDA/mg protein, using the molar extension coefficient of chromophore (1.56 × 105 M−1 cm−1).

4.7.2. Superoxide dismutase activity

SOD activity was assayed according to the method of Kono (1978), wherein the reduction of nitroblue tetrazolium chloride was inhibited by the superoxide dismutase which was measured at 560 nm spectrophotometrically. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and post nuclear fraction of brain homogenate. The results were expressed as units/mg protein, with one unit of enzyme defined as the amount of SOD required to inhibit the rate of reaction by 50%.

4.7.3. Reduced glutathione level

GH concentrations were determined by the procedures of Ellman (1959). Briefly, 0.5 ml homogenate was mixed with 1.5 ml 0.15 M KCl and 3 ml deproteinization solution. Each sample was centrifuged at 3000 rpm for 10 min and supernatant was removed, followed by the addition of 2 ml phosphate solution and 0.5 ml DTNB into the 0.5 ml supernatant, with the absorbance read at 412 nm and compared with glutathione standards. The concentration of reduced glutathione was expressed as milligram per gram tissue.

4.7.4. Catalase activity

CAT activity was measured according to method of Aebi (1984). Briefly 0.1 ml of supernatant was added to a cuvette containing 1.9 ml of 50 mmol/L phosphate buffer (pH 7.0). The reaction was started by the addition of 1 ml freshly prepared 30 mmol/L H2O2. The rate of decomposition of H2O2 was measured spectrophotometrically by changes in absorbance at 240 nm. The activity of catalase was expressed as units per milligram protein.

4.8. Statistical analysis

All statistical analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Values were expressed as mean ± SEM (Standard Error of the Mean). For the Morris water maze test, data were analyzed with a Kruskal–Wallis non-parametric ANOVA test. If the results were significant, the intergroup variation was measured by the Tukey’s post hoc test. Statistical significance was set at P < 0.05. For the step-through, biochemical and neuroendocrine assays, differences between groups were analyzed with one-way analysis of variance (ANOVA) followed by the Dunnett’s post hoc test. Results were considered significant if P < 0.05.

Acknowledgments

The authors are cordially indebted to these financial supports: “Qing-Lan” Project of Jiangsu Province, the Industrialization of Scientific Research Promotion Projects of Universities and Colleges in Jiangsu Province (2011-16), the Natural Science Fund for Universities and Colleges in Jiangsu Province (O9KJB350003; 11KJB350005), Laboratory of Biological Therapy for Cancer of Xuzhou Medical College (JSL0803; C0903; C0904), The Science and Technology Plan Projects of Xuzhou (XF11C037; XF11C062; XXZZD1219; XXZZD1227; BRA201205), The Post-doctoral Fund in Jiangsu Province (1201036B), Superiority Academic Discipline Construction Project of Jiangsu Higher Education Institutions, and Xuzhou Public Service Platform Projects of Drug Discovery and Research, Innovation Project of Postgraduates in Jiangsu Province, China (CXLX11-0752). Jiangsu Planned Projects for Postdoctoral Research Funds (2012MS21125).

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