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Diosgenin Ameliorates Cognition Deficit and Attenuates Oxidative Damage in Senescent Mice Induced by D-Galactose

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Abstract: This study attempted to access the neuroprotective effect of diosgenin on the senescent mice induced by d-galactose (D-gal). The mice in the experiments were orally administered with diosgenin (1, 5, 25 and 125 mg/kg), for four weeks from the sixth week. The learning and memory abilities of the mice in Morris water maze test and the mechanism involved in the neuroprotective effect of diosgenin on the mice brain tissue were investigated.

Diosgenin (5, 25 and 125 mg/kg, p.o.) showed significantly improved learning and memory abilities in Morris water maze test compared to D-gal treated mice (200 mg/kg, ten weeks). Diosgenin also increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and decreased the malondialdehyde (MDA) level in the brain of D-gal treated mice.

These results indicated that diosgenin has the potential to be a useful treatment for cognitive impairment. In addition, the memory enhancing effect of diosgenin may be partly mediated via enhancing endogenous antioxidant enzymatic activities.

Keywords: *Diosgenin*; Cognition Deficit; Aging; D-gal.

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Introduction

Aging is a complicated multifactorial process associated with physiological decline. Cognitive deficits are the main clinical symptoms of brain aging in human beings (Sharps and Gollin, 1987). Memory decline is a characteristic of aging and age-related neurodegenerative disorders which lead to a progressive loss of cognitive function, especially in spatial memory (Barnes *et al.*, 1980). Moreover, oxidative stress and reactive oxygen species (ROS) have been proposed to be major causes of aging (Olanow, 1993; Valko *et al.*, 2007). It is noted that the formation of ROS has been an important step leading to neuronal death in a variety of age-related neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (De Iuliis *et al.*, 2005). Furthermore, the neuron is particularly susceptible to oxidative damage resulting from the production of ROS, which oxidizes various biological macromolecules within the neuron and ultimately results in cell death. Since the oxidative damage may play a role in the aging process, including the associated cognitive decline, age-related impairment in spatial learning and memory may be alleviated by antioxidant treatment (Socci *et al.*, 1995).

D-Galactose (D-gal) causes the accumulation of ROS or stimulates free radical production indirectly by the formation of advanced glycation end-products *in vivo*, thus finally resulting in oxidative stress (Zhang *et al.*, 2005). In addition, repeated injection of D-gal could induce aging-like symptoms in animals, such as abnormal alterations in biochemistry markers, loss in propagating ability, retrograde changes in neural cells and memory impairments (Shen *et al.*, 2002; Lu *et al.*, 2006). Therefore, mice injected with D-gal have been used for pharmacological studies *in vivo* on brain aging. Some studies have further showed that D-gal induced aging-related changes, including increased production of ROS (Zhang *et al.*, 2007) and decreased antioxidant enzyme activities (Wei *et al.*, 2005).

Yam (*Dioscorea* spp.) is an important edible tuber plant used widely in traditional Chinese medicine to promote health and produce functional foods in Taiwan (Liu *et al.*, 1995). Steroidal saponins are the major physiologically active compounds in yam (Liu *et al.*, 1995; Hu *et al.*, 1996, 1997; Yang *et al.*, 2003). They usually exist as glycosides in nature and have many biological activities, such as hemolytic (Santos *et al.*, 1997; Zhang *et al.*, 1999), hypocholesterolemic (Malinow, 1985; Sauvaire *et al.*, 1991), hypoglycemic (Kato *et al.*, 1995), anti-thrombotic (Zhang *et al.*, 1999; Peng *et al.*, 1996), anti-neoplastic (Hu *et al.*, 1996, 1997), antiviral (Aquino *et al.*, 1991), and anti-cancer (Ravikumar *et al.*, 1979; Sung *et al.*, 1995) activities. Our previous study found that yam (*Dioscorea pseudojaponica* Yamamoto) ameliorates cognition deficits and attenuates oxidative damages in the brain of aging mice induced by d-galactose (Chiu *et al.*, 2009). Diosgenin, obtained from yam saponins after hydrolysis, is a principal starting material for industrial production of steroidal drugs (Djerassi, 1992; Chen and Wu, 1994; Morgan and Morynihan, 1997). Some studies reported that diosgenin suppressed cholesterol absorption and increased cholesterol secretion through biliary excretion (Cayen and Dvornik, 1979; Uchida *et al.*, 1984; Accatino *et al.*, 1998; Kamisako and Ogawa, 2003). Diosgenin possesses antioxidative and hypolipidemic effects on the model of high-cholesterol

fed rats (Son *et al.*, 2007). It has stronger preventive and therapeutic activities than the total saponin of *Dioscorea panthaica* in the hypercholesterolemia induced by cholesterol in mice or rats (Ma *et al.*, 2002). Diosgenin possesses the protective action in isoproterenol-induced myocardial infarction (Jayachandran *et al.*, 2009). Moreover, in some pre-clinical and mechanistic studies, diosgenin has played a significant role as chemopreventive and therapeutic agent against some cancers by over-expressing HER2 gene (Raju and Mehta, 2009; Chiang *et al.*, 2007). By growth inhibition and induction of apoptosis, diosgenin is an inhibitor of human colon carcinoma cells (Raju and Bird, 2007). In an antitumor study, diosgenin possessed significant antitumor activity on S-180, HepA, and U14 transplant mice *in vivo* and L929, HeLa, and MCF cells *in vitro* (Wang *et al.*, 2002). The diverse medical properties attributed to the diosgenin and the presence of antioxidant and free radical scavengers prompted us to investigate the anti-aging effect of diosgenin.

To our knowledge, there is no previous study of the anti-aging effects of diosgenin in animal models of mice. Therefore, it is necessary to investigate the effect of diosgenin on animal model for developing neuroprotective drugs. As rodents chronically injected with D-gal have been used as an animal aging model for brain aging or anti-aging pharmacology research (Wei *et al.*, 2005), and it was also reported that D-gal could impair neurogenesis in the dentate gyrus, a process similar to the natural aging in mice (Zhang *et al.*, 2005), we addressed this issue and investigated the mechanism underlying the neuroprotective effect of diosgenin on the cognition of aging mice induced by D-gal and antioxidant parameters in the mouse brain.

Materials and Methods

Reagents

Diosgenin was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). The assay kits for SOD and glutathione peroxidase (GSH-Px) were purchased from Randox Laboratory Ltd. All the other chemicals were analytical grade.

Animals

Male ICR mice (18–22 g) were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, Taiwan and housed in standard cages at a constant temperature of $22 \pm 1\,^{\circ}C$, and relative humidity $55 \pm 5\%$ with 12 h dark-light cycle (08:00 $\sim$ 20:00) for at least one week before the experiment. They were fed with food and water *ad libitum*. Mice were randomly divided into five groups and habituated to subcutaneous injection each day with D-gal at a dose of 200 mg/kg, or vehicle (0.9% saline) for ten weeks respectively.

All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on Animal Research at China Medical University.

Morris Water Maze (MWM)

The process of MWM consisted of a four-day learning and memory training on day 5. Behavioral testing was performed in the water maze (Morris, 1984; Villarreal *et al.*, 2002), which was a black circular tank 100 cm in diameter and 50 cm in depth. The tank was divided virtually into four equal quadrants and an escape platform was hidden 1.0 cm below the surface of the water in a fixed location in the 3rd quadrant of the pool. After a day's training, a trial was started by placing the mice into the pool close to the rim, facing the wall of the tank in one of the four quadrants. Mice were given four trials per session for five days, with each trial having a ceiling time of 60 s and a trial interval of approximately 30 s. After climbing onto the platform, the animal remained there for 30 s before the next trial. If the mice failed to reach the escape platform within 60 s, it was gently placed on the platform and allowed to remain there for 30 s. Latencies to escape from the water maze (finding the submerged escape platform) were recorded. Following the acquisition phase (five days), a probe test was conducted by removing the platform. The time spent and the numbers of crossing in the target quadrant, which had previously contained the hidden platform, were recorded.

Tissue Homogenates

After examination of the memory behavior, animals were deeply anesthetized and sacrificed. Brains were promptly dissected and perfused with 50 mM (pH 7.4) ice-cold phosphate buffer saline (PBS). Brains were homogenized in 1/5 (w/v) PBS containing a protease inhibitor cocktail (Sigma-Aldrich, USA) with ten strokes in a homogenizer. Homogenates were divided into two portions. The first part was directly centrifuged at 8000 g for 10 min to obtain the supernatant. Supernatant aliquots were used to determine brain GSH-Px activity, malondialdehyde (MDA) level and protein contents. The second part of homogenate was solicited four times for 30 s with 20 s intervals using a DELTA ultrasonicator (DC400H), then it was centrifuged at 5000 g for 10 min at 4°C. The supernatants were collected and stored at −70°C for determination of superoxide dismutase (SOD) enzyme activity.

Assay of SOD Activity

The assay for total SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine-xanthine oxidase system (Oyanagui, 1984). The hydroxylamine nitrite produced by the oxidation of oxymine had an absorbance peak at 550 nm. SOD activity was measured according to the method described by McCord and Fridowich (1969). Solution A was prepared by mixing 100 ml of 50 mM PBS (pH 7.4) containing 0.1 mM EDTA and 2 mmol of cytochrome c with 10 ml of 0.001N NaOH solution containing 5 mmol of xanthine. Solution B contained 0.2 U xanthine oxidase/ml and 0.1 mM EDTA. Fifty microliters of a tissue supernatant was mixed with 2.9 ml of solution A and the reaction was started by adding 50 ml of solution B. Change in absorbance at 550 nm was monitored in a

spectrophotometer (Roche Mira Plus, USA). SOD activities were expressed as units per mg protein with reference to the activity of a standard curve of bovine SOD under the same conditions.

Assay of GSH-Px Activity

The method of Flohe and Gunzler (1984) was adopted and *GSH-Px* enzyme activity was determined at 37°C. The reaction mixture was composed of 500 ul phosphate buffer, 100 ul each of 0.01 M GSH (reduced form), 1.5 mM NADPH and GSH-Rd (0.24 units). One hundred microliters of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Then 50 μl of 12 mM *t*-butyl hydroperoxide was added to the mixture and measured at 340 nm for 180 s. The molar extinction coefficient of $6.22 \times 10^{-3}\,M^{-1}\,cm^{-1}$ was used to determine GSH-Px enzyme activity. One unit of activity is equal to the mM of NADPH oxidized/min per mg protein.

Measurement of MDA Levels

The level of MDA in brain tissue homogenates was determined according to the method used by Uchiyama and Mihara (1978). Half a milliliter of each homogenate was mixed with 3 ml of phosphate solution (1%, v/v) followed by the addition of 1 ml of thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95°C in a water bath for 45 min. The colored complex was extracted with *n*-butanol, and the absorption at 532 nm was measured using tetramethoxypropane as a standard. MDA levels were expressed as nmol per milligram of protein. Protein concentration was measured by Lowry method (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

Statistical Analysis

Data were represented as the mean ± SEM. Data were analyzed with one-way ANOVA followed by Scheffe's multiple range test. The criterion for statistical significance was $p < 0.05$. All statistical analysis was carried out by using SPSS for Windows (SPSS Inc.).

Results

Morris Water Maze Test

The Morris water maze is a validated test used for the assessment of spatial learning and memory in mice. The results of the present study showed that the D-gal treated mice had significant cognitive deficits. As shown in Fig. 1, the mean latency to find the platform declined progressively during the training days in all animals. However, the D-gal group mice had longer latencies finding the platform throughout the training days than normal mice ($p < 0.01$), showing poorer learning performance due to chronic administration of D-gal. Diosgenin (5, 25, 125 mg/kg) treatment significantly shortened this prolongation of mean latency ($p < 0.01 - 0.001$) compared to the D-gal treatment.

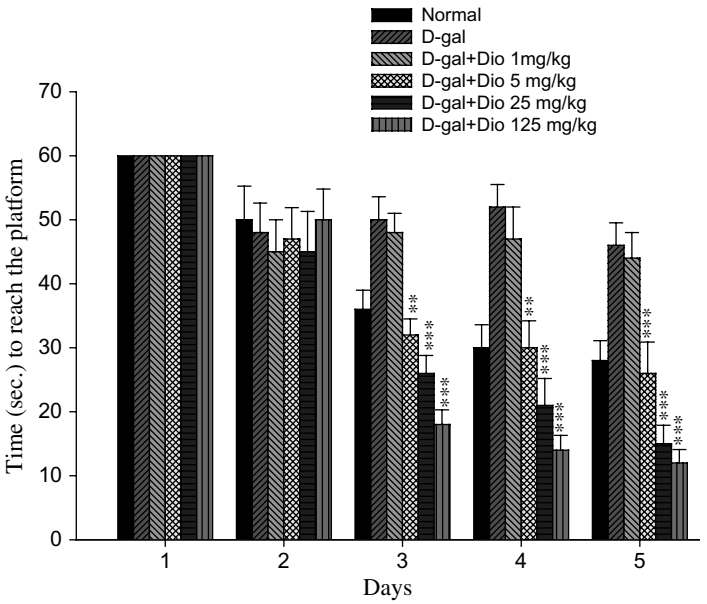

Figure 1. Effects of diosgenin on time (sec) to reach the platform in senescent mice induced by D-gal. Values are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to D-Gal group ($n = 10$).

On the probe trial, the D-gal group mice failed to remember the precise location of the platform, spending significantly less time to target quadrant than the normal group. (Fig. 2, $p < 0.01$ vs. normal group). The mean percentage time spending in the target quadrant was increased by the administration of diosgenin, (Fig. 2, $p < 0.01 - 0.001$ vs. D-gal group), suggesting that diosgenin reversed the memory deficits induced by D-gal. Furthermore, the numbers of target crossing was significantly reduced in D-gal group mice ($p < 0.001$), pointing to a spatial navigation deficit. Diosgenin treatment significantly reversed these spatial navigation deficits as seen in Fig. 3 ($p < 0.05 - 0.01$ vs. D-gal group). All results revealed that diosgenin could improve the ability of spatial learning and memory in D-gal treated mice.

Effects of Diosgenin on SOD, GSH-Px Activities and MDA Content in D-gal-Treated Mouse Brain

Compared to the mice of normal group, SOD activity in the brain was significantly declined in mice of D-gal group. Diosgenin (25, 125 mg/kg) could increase the SOD activities (Fig. 4). There was no significant difference between the normal and the diosgenin groups. The activity of GSH-Px in brain of model group mice was significantly lower compared to that of the normal group (Fig. 5, $p < 0.001$). Diosgenin treatment resulted in a significant elevation in the enzyme activity. There was no significant difference between the control and the diosgenin groups.

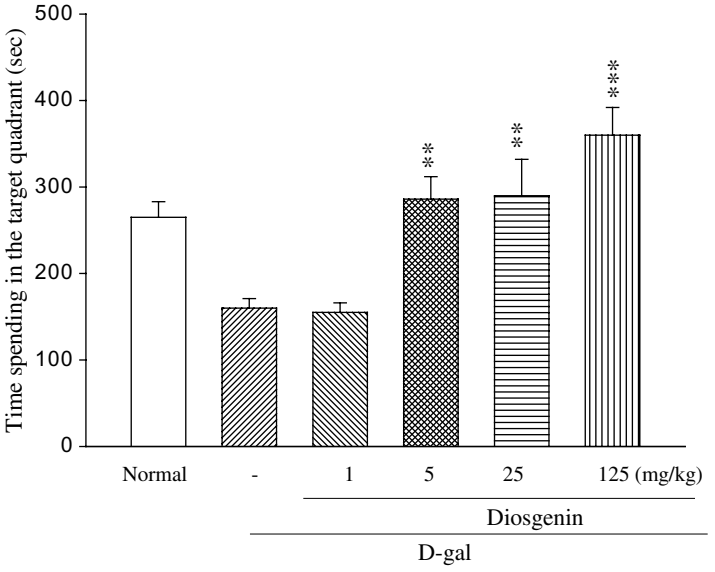

Figure 2. Effects of diosgenin on time spending in the platform area (memory frequency) in the brain of senescent mice induced by D-gal. Values are expressed as mean ± SEM. $^{\#\#}p < 0.01$ as compared to Normal group. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to D-Gal group ($n = 10$).

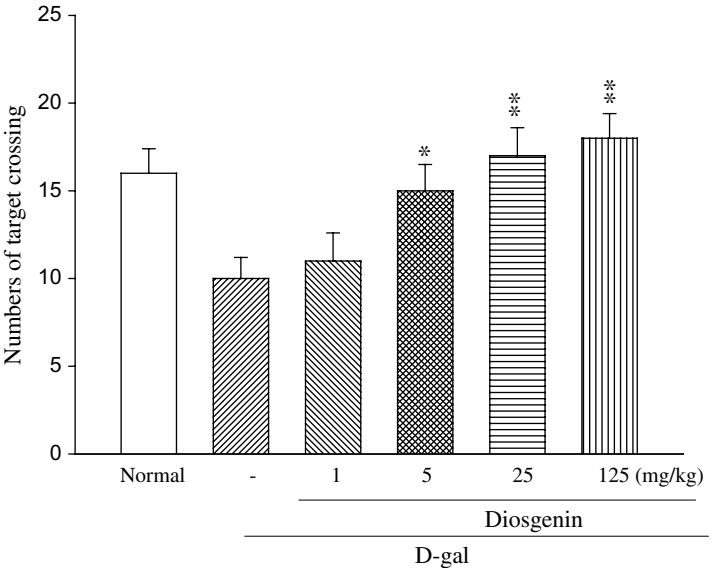

Figure 3. Effects of diosgenin on time spending in the number of target crossing in the platform area in the brain of senescent mice induced by D-gal. Values are expressed as mean ± SEM. $^{\#\#\#}p < 0.001$ as compared to Normal group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to D-Gal group ($n = 10$).

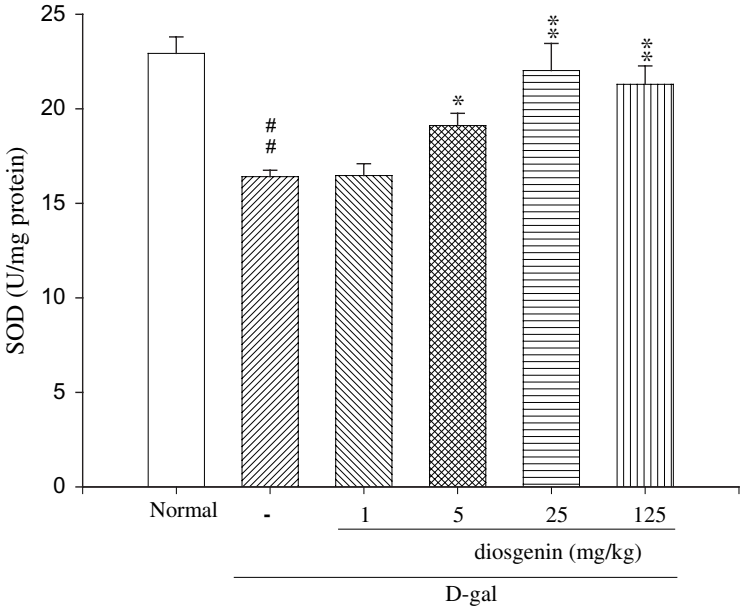

Figure 4. Effect of diosgenin on superoxide dismutase (SOD) activity in the brain of senescent mice induced by D-gal. $^{\#\#}p < 0.01$ as compared to Normal group. $^{**}p < 0.01$ as compared to D-Gal group ($n = 10$).

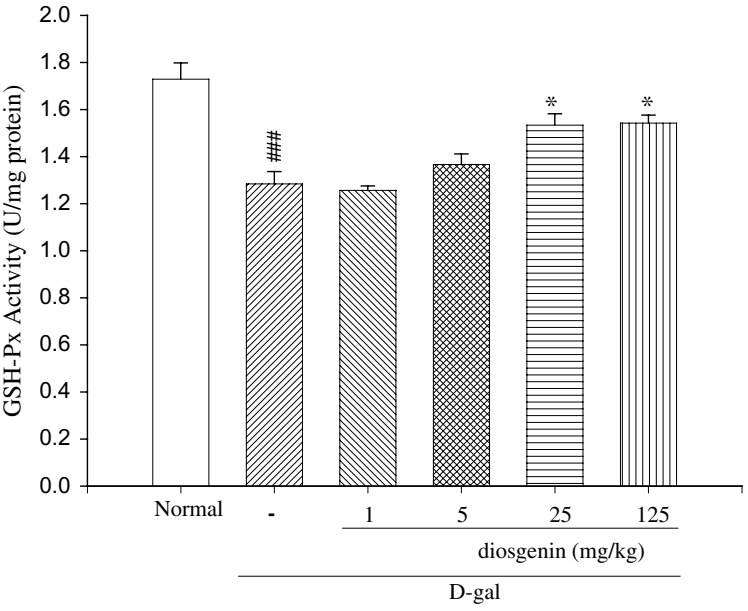

Figure 5. Effect of diosgenin on glutathione peroxidase (GSH-Px) activity in the brain of senescent mice induced by D-gal. $^{\#\#\#}p < 0.001$ as compared to Normal group. $^{*}p < 0.05$ as compared to D-Gal group ($n = 10$).

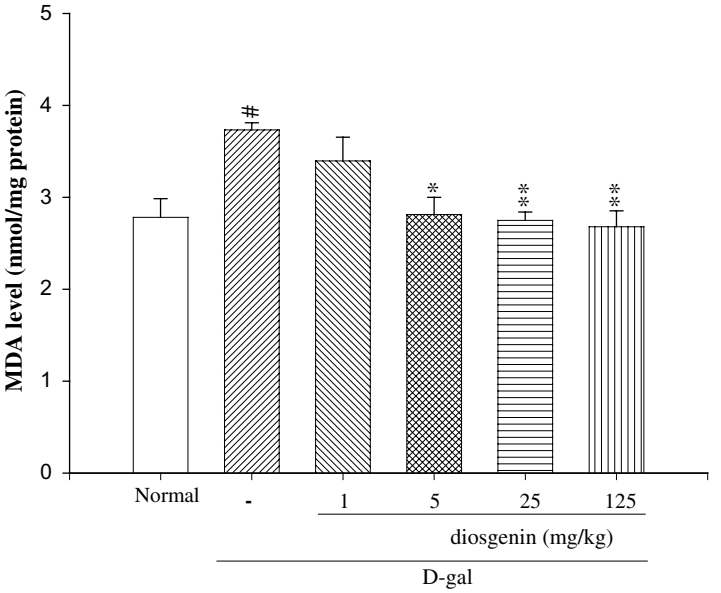

Figure 6. Effect of diosgenin on Malondialdehyde (MDA) level in the brain of senescent mice induced by D-gal. Values are expressed as mean ± SEM. $^{\#}p < 0.05$ as compared to Normal group. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to D-Gal group ($n = 10$).

D-gal group showed a significant increase in MDA level compared to the normal group (Fig. 6, $p < 0.05$). This increase in MDA also attenuated in the brain of diosgenin treated mice ($p < 0.05 - 0.01$). There was no significant difference between the normal and the diosgenin groups.

Discussion

Cognitive deficits, as aging markers, are important clinical symptoms of Alzheimer's and Parkinson's diseases. D-gal, a reducing sugar that can form advanced glycation end product *in vivo*, cannot be further metabolized and accumulated in nerve cells. The free radicals generated from oxidation of D-gal overrun the capacity of cells to clean them. This, consequently, causes the chain reaction of lipid peroxidation and the end products, such as MDA, which combines protein with phospholipid and leads to the injury of cellular membrane and impairment of central nervous system (Hayakawa *et al.*, 1992). These results lead to serious cognitive deficits. Therefore, it is at least partially contributing to the pathological mechanism of the aging model (Song *et al.*, 1999; Tian *et al.*, 2005).

This study investigated the behavioral manifestations of a mouse aging model induced by D-gal at the most frequently reported dose (200 mg/kg) in ICR mice at first. D-gal at dose of 200 mg/kg could significantly induce behavioral impairment during the MWM test, which has been regarded as one of the most frequently used laboratory tools in spatial learning and memory and neuropharmacological research. MWM typically consists of a

series of spatial learning acquisition training and spatial accuracy memory in probe trial (D'Hooge and De Deyn, 2001). In the present study, subcutaneous administration of D-gal for six weeks caused impairments in memory function and cognitive ability in mice. The results showed that D-gal was suitable for use in the production of the senescent mice model. In our study, chronic administration of D-gal impaired performance of mice in a water maze task and the diosgenin group mice showed a shorter latency, indicating that diosgenin had potential effect to prevent this kind of learning and memory deficits. Accumulated evidence (Fukui *et al.*, 2002; Parle and Dhingra, 2003) has further indicated that treatment with a variety of antioxidants could partially reverse the increase in markers of oxidative stress and the decline in learning and memory.

ROS becomes an active field in aging research because of their potential involvement in many degenerative processes and in many neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Harman, 1992). ROS can be scavenged by endogenous anti-oxidants including SOD and GSH-Px. MDA is a by-product of lipid peroxidation induced by free radicals and is widely used as a biomarker of oxidative stress (Cini *et al.*, 1994). In this study, the activities of SOD and GSH-Px in the brain showed a statistically significant decline in D-gal group mice compared to those in the normal group mice. Treatment with diosgenin for four weeks could improve the activities of GSH-Px and SOD. In addition, an obvious enhancement of the level of MDA was shown in the D-gal group mice, and it could significantly be reduced after diosgenin administration. Therefore, diosgenin sca-venged ROS mainly via increasing the activities of SOD and GSH-Px; consequently, decreased lipid peroxidation.

Injection of D-gal could induce senescent-like symptoms in animals, such as abnormal alterations in biochemistry markers, retrograde changes in neural cells and memory impairments (Shen *et al.*, 2002). As mentioned in the previous study, chronic systemic D-gal exposure would induce memory loss, neurodegeneration, and oxidative damage in mice (Cui *et al.*, 2006). We also found that there were correlations between the antioxidant parameters and cognitive parameters. Significant negative correlations were found between the latency to find the platform on the sixth day and the activities of SOD and GSH-Px in mice brain. The levels of MDA were positively correlated with the latency in the mice brain. All these together suggested that the oxidative damage may play a role in the cognitive decline of the senescent mice induced by D-gal and that diosgenin's function against oxidative stress in brain may be one of the mechanism of its action to ameliorate the impairments of learning and memory.

Diosgenin is an aglycone of the yam steroidal saponins. Many studies indicated that diosgenin has potent hypocholesterolemic (Sauvaire *et al.*, 1991) and hypoglycemic (Kato *et al.*, 1995) effects. Diosgenin is also used as the raw material for industrial production of steroidal drugs (Djerassi, 1992). The mucilaginous material is useful and much of them contain the chemical diosgenin, a precursor of progesterone, cortisone and other medically important steroids. Strikingly, diosgenin significantly increased the activities of all these enzymes and decreased the level of MDA in the brain of D-gal treated mice (Figs. 4–6). Our results strongly suggested that diosgenin could strengthen anti-oxidative defense against free radicals induced by D-gal *in vivo*.

In conclusion, the present findings indicated that chronic administration of D-gal would cause memory impairment and changes of some redox-related biomarkers in mice brain, including decrease in SOD, GSH-Px activities and increase of MDA level. In addition, diosgenin (5–125 mg/kg) significantly improved the cognitive impairment and increased the activities of endogenous antioxidant enzymes in the brain of mice. Therefore, diosgenin may have potential to serve as an anti-aging therapy or as a treatment of neurodegenerative diseases.

Acknowledgments

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