Diosgenin ameliorates cognition deficit and attenuates oxidative damage in senescent mice induced by D-galactose

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Running title: Memory enhancing effect of *Diosgenin*

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Abstract

This study attempted to access the neuroprotective effect of diosgenin on the senescent mice induced by D-gal. The mice in the experiments were orally administered with diosgenin (1, 5, 25 and 125 mg/kg, 4 weeks, from sixth week). The learning and memory ability 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 ability in Morris water maze test compared with D-gal treated mice (200 mg/kg, 10 weeks). Diosgenin (5, 25 and 125 mg/kg, p.o.) also increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and decreased the malondialdehyde (MDA) level on the brain of D-gal treated mice.

To sum up, these results indicated that diosgenin had the potential to be a useful treatment for cognitive impairment. The memory enhancing effect of diosgenin may be partly mediated via enhancing endogenous antioxidant enzymatic activities.

Key words: *Diosgenin*, cognition deficit, aging, D-gal

Introduction

Aging is a complicated multifactorial process associated with physiological decline. Cognitive deficits are main clinical symptoms of brain aging in human beings (Sharps and Gollin,1987). Memory decline is 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). Formation of ROS has been proposed to be 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). The neuron is particularly susceptible to oxidative damage resulted from the production of ROS. ROS oxidizes various biological macromolecules within the neuron and ultimately resulting 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 (AGE) 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, deduction 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 included increased production of ROS (Zhang et al.,2007) and decreased antioxidant enzyme activities (Wei et al.,2005).

Yam (*Dioscorea* spp.) is an important tuber plant for edible and used widely in traditional Chinese medicine to promote human health and provided for functional foods

produced in Taiwan (Liu et al.,1995). Steroidal saponins are the major physiologically active compounds in yam (Liu et al., 1995; Hu et al., 1996; Hu et al., 1997; Yang et al., 2003). They usually exist as glycoside in nature and 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; Hu et al., 1997), antiviral (Aquino et al., 1991), anti-cancer (Ravikumar et al., 1979; Sung et al., 1995) and activities have been observed. Our previous study finds that yam (*Dioscorea pseudojaponica* Yamamoto) ameliorates cognition deficits and attenuates oxidative damage 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 againsts hypercholesterolemia induced by cholesterol in mice or rats is stronger than total saponin of Dioscorea panthaica in the preventive and therapeutic activity (Ma et al.,2002). Diosgenin possesses the protective action in isoproterenol-induced myocardial infarction (Jayachandran et al.,2009). Moreover, in some preclinical and mechanistic studies, diosgenin have been played a significantly role of chemopreventive and therapeutic agent against several 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 antitumor study, diosgenin possesses significantly antitumor activity on S-180, HepA, U14 transplant mice in vivo and L929, HeLa, 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 on 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 drug. Rodent chronically injected with D-gal has been used as an animal aging model for brain aging or anti-aging pharmacology research (Wei et al., 2005). It was reported that D-gal could impair neurogenesis in the dentate gyrus, a process similar to the natural aging in mice (Zhang et al., 2005). In the present report, 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 mice brain.

Materials and Methods

Reagents

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

Subjects

Male ICR mice (18 g–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±1 ℃, and relative humidity 55±5% with 12 h dark-light cycle (08:00 ~ 20:00) for at least 1 week before the experiment. They were fed with food and water ad libitum. After 1-week acclimatization to the home cage, mice were randomly divided into five groups and habituated to subcutaneous injection each day with D-gal (Sigma-Aldrich,

MO, USA) at dose of 200 mg/kg, and vehicle (0.9% saline) for 10 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 4-day learning and memory training on day 5. Behavioral testing was performed in the water maze (Morris,1984; Villarreal et al.,2002), which was black circular tank with 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 one day's training, a trial was started by placing the mice into the pool close to the rim, facing the wall of the tank into one of the four quadrants. Mice were given four trials per session for 5 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 (5 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.

Preparation of brain tissue and homogenates

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 solution (PBS). Brains were homogenized in 1/5 (w/v) PBS containing a protease inhibitor cocktail (Sigma–Aldrich) with 10 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 glutathione peroxidase (GSH-Px) activities, MDA levels and protein contents. The second part of homogenates was solicited four times for 30 sec with 20 sec intervals using a DELTA ultrasonicator (DC400H), then it was centrifuged at 5000 g for 10 min at 4 ℃. The supernatants were collected and stored at -70 ℃ for determination of superoxide dismutase (SOD) enzyme activities.

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 (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 was 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 levels 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 Glutathione peroxidase (GSH-Px) activity

The method of Flohe and Gunzler (1984) was adopted here and *GSH-Px* enzyme activity was determined at 37℃. The reaction mixture was composed of 500ul phosphate buffer, 100ul 0.01M GSH (reduced form), 100ul 1.5mM NADPH and 100ul GSH-Rd (0.24 units). One hundred microliter of the tissue extract was added to the reaction mixture and incubated at 37℃ for 10 min. Then 50ul of 12mM t-butyl hydroperoxide was added to 450ul tissue reaction mixture and measured at 340nm for 180s. The molar extinction coefficient of 6.22×10^{-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 malondialdehyde (MDA) levels

The level of MDA in brain tissue homogenates was determined according to the method used in Uchiyama and Mihara (1978). Half a milliliter of each homogenate was mixed with 3 ml of H_3PO_4 solution (1%, v/v) followed by the addition of 1ml of thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95 ℃ in a water bath for 45 min. The colored complex was extracted into n-butanol, and the absorption at 532 nm was measured using tetramethoxypropane as 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 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 with the D-gal treatment.

On the probe trial, 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 percent 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 with the normal group mice, the SOD activity in brain significantly declined in D-gal group mice. Diosgenin (25, 125 mg/kg) could increase the activities of SOD (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 when it was compared with the normal group (Fig 5; $P < 0.001$). Diosgenin treatment resulted in a significant elevation in the activity of this enzyme. There was no significant difference between the control and the diosgenin group.

D-Gal group showed significant increase in MDA level compared with the normal group (Fig 6; $P < 0.05$). This increase in MDA was 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.

4. Discussion

Cognitive deficits, a kind of aging marker, 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, can not 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 (LPO) and the end products, such as MDA, which combines protein with phospholipid and lead to the injury of cellular membrane and impairment of central nervous system (Hayakawa et al., 1992). The result leads to serious cognitive deficits. Therefore, it's at least partially contributing to the pathological mechanism of the aging model (Song et al., 1999; Tian et al., 2005).

This study firstly compared the behavioral manifestations of a mouse aging model induced by D-gal at the most frequently reported dose (200 mg/kg) in ICR mice. 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 neuropharmacology 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 to be used in the production of the senescent mice model and these results were in agreement with these findings. 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. Accumulating evidences (Fukui et al.,2002; Parle and Dhingra,2003) have further indicated that treatment with a variety of antioxidants partially reversed the increase in markers of oxidative stress and the decline in learning and memory.

ROS become 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). These ROS can be scavenged by endogenous antioxidants 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 normal group mice. Treatment with diosgennin 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 be significantly reduced after diosgennin administration. Therefore, diosgennin scavenged 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. These suggested that the oxidative damage may play a role in the cognitive decline of the senescent mice induced by D-gal and that diosgennin's funtion against oxidative stress to brain may be involved in 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 a 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 (Fig 4 - Fig 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. Diosgenin (5-125 mg/kg) significantly improved the cognitive impairment and increased the activities of endogenous antioxidant enzymes in the brains of mice. Therefore diosgenin may have potential to serve as an anti-aging therapy or as a treatment of neurodegenerative diseases.

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Fig. 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).

Fig. 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).

Fig. 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).

Fig. 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).

Fig. 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).

Fig. 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).

Fig. 1

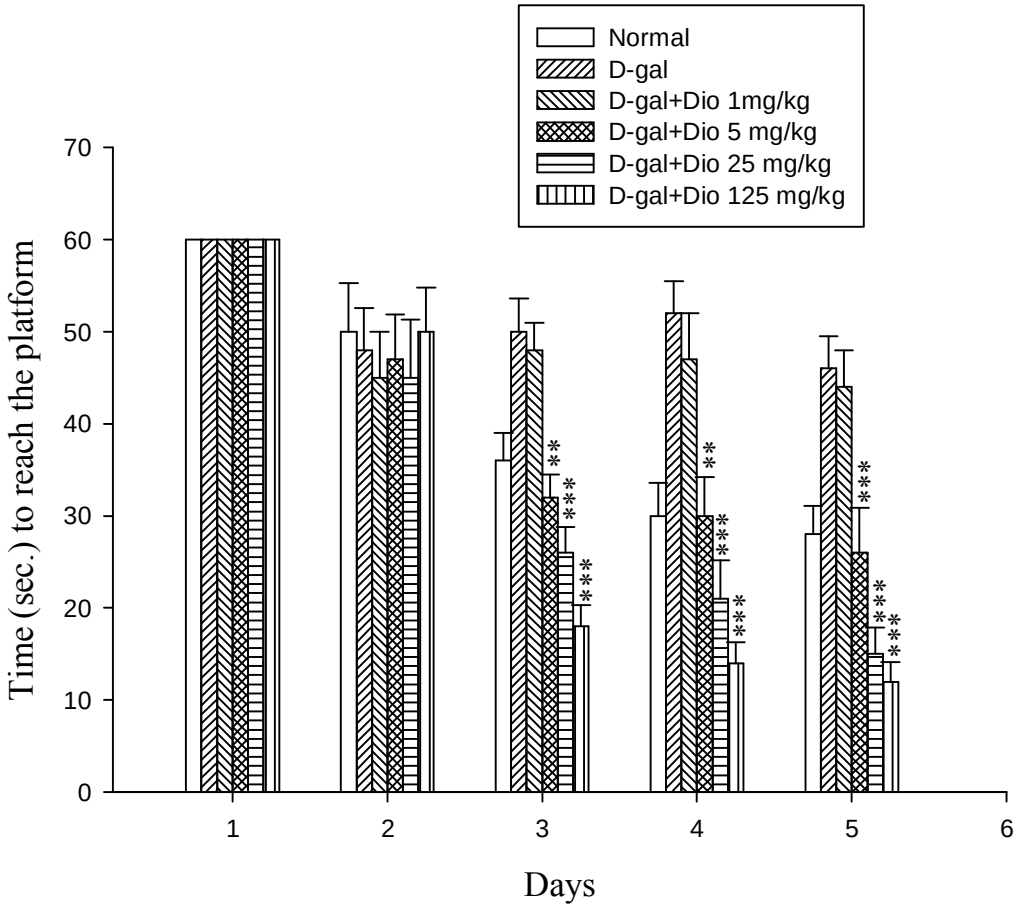
Normal
D-gal
D-gal+Dio 1mg/kg
D-gal+Dio 5 mg/kg
D-gal+Dio 25 mg/kg
D-gal+Dio 125 mg/kg
Time (sec.) to reach the platform
Days
0
10
20
30
40
50
60
70
1
2
3
4
5
6
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Fig. 2

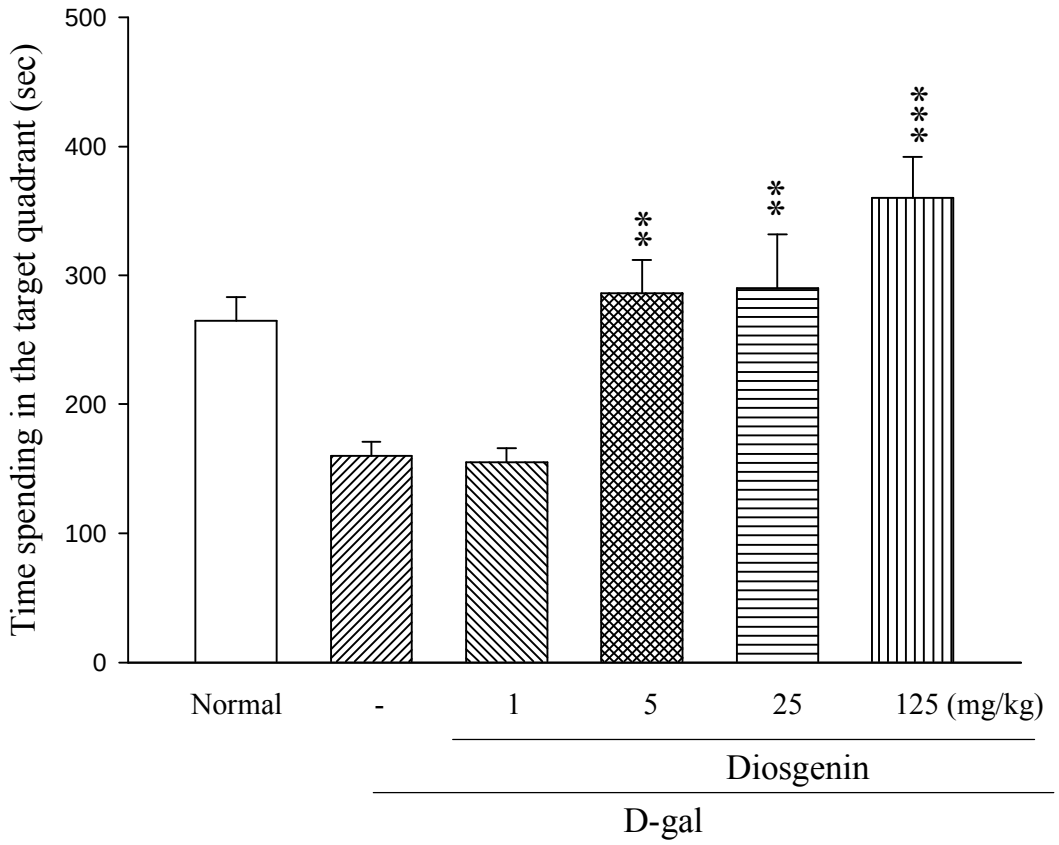
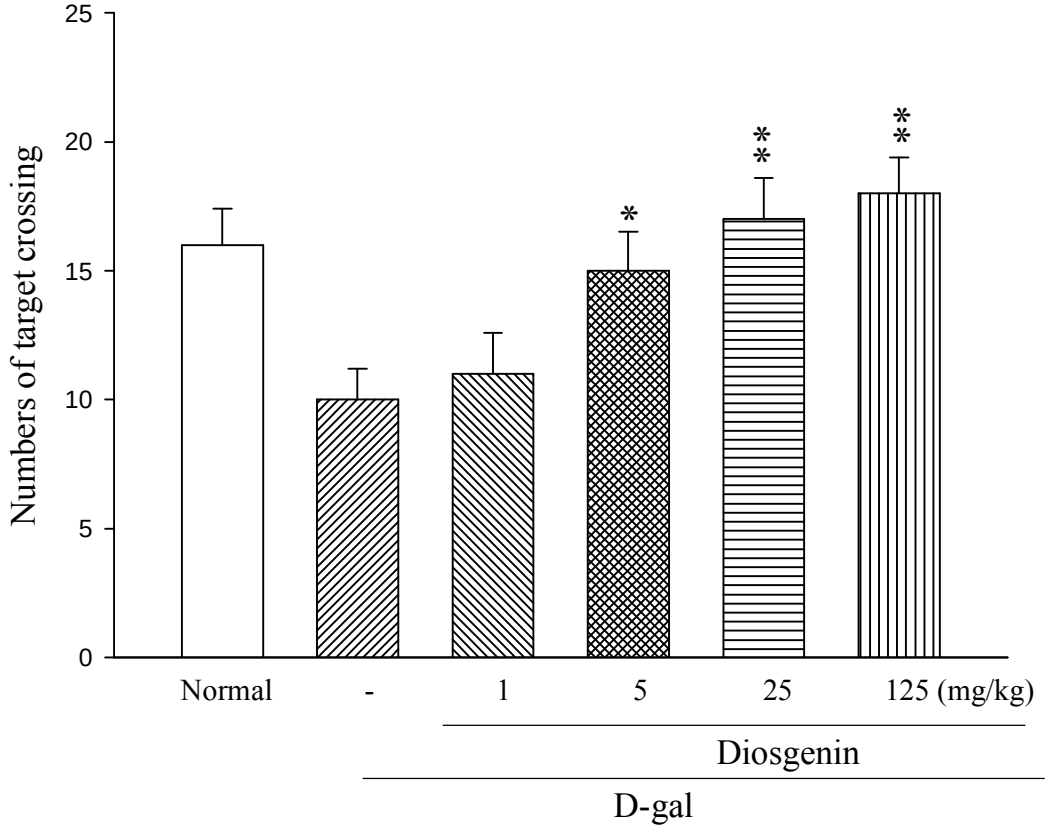

Time spending in the target quadrant (sec)
500
400
300
200
100
0
**
**
***
Normal
-
1
5
25
125 (mg/kg)
Diosgenin
D-gal

Fig. 3


Numbers of target crossing
25
20
15
10
5
0
*
**
**
Normal
-
1
5
25
125 (mg/kg)
Diosgenin
D-gal

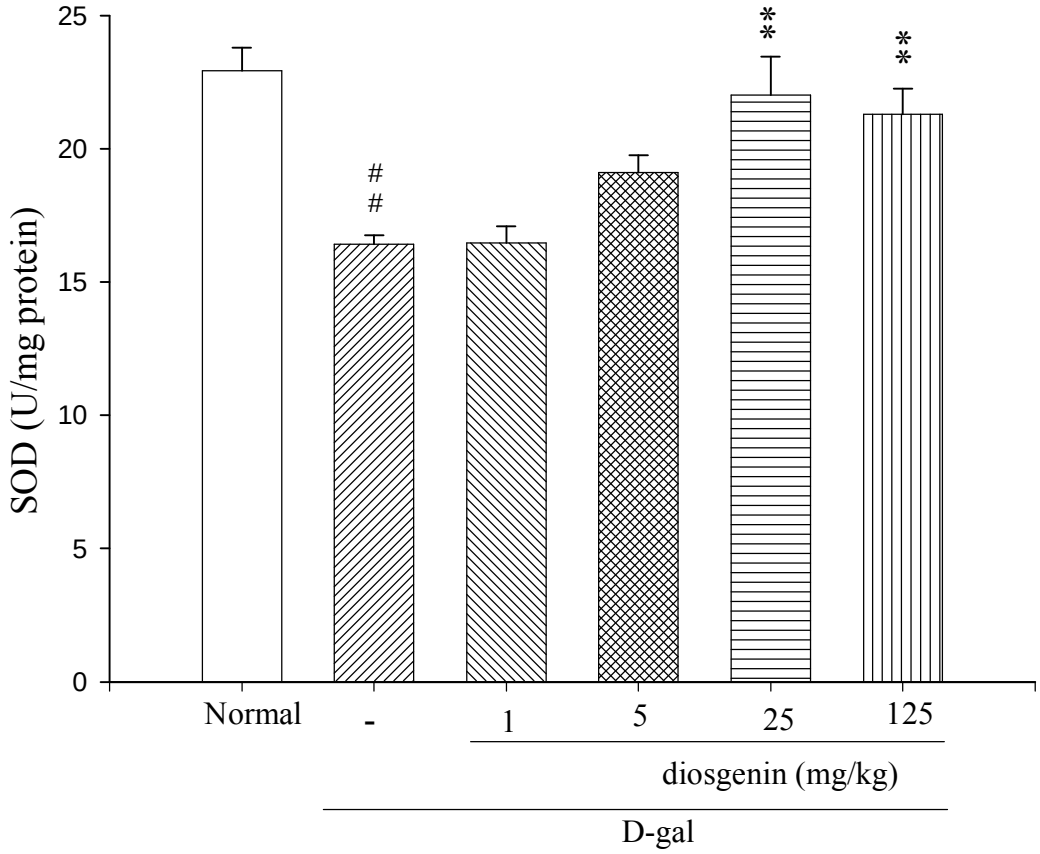

SOD (U/mg protein)
25
20
15
10
5
0
##
**
**
Normal
-
1
5
25
125
diosgenin (mg/kg)
D-gal

Fig. 4

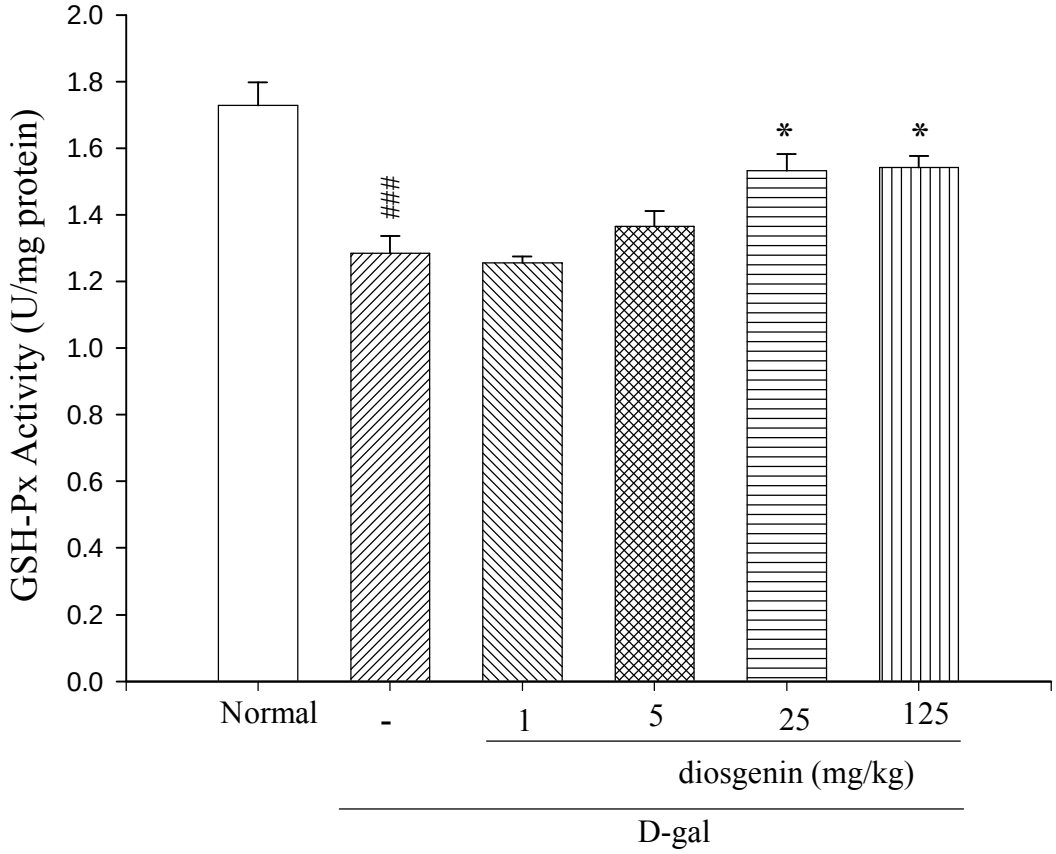
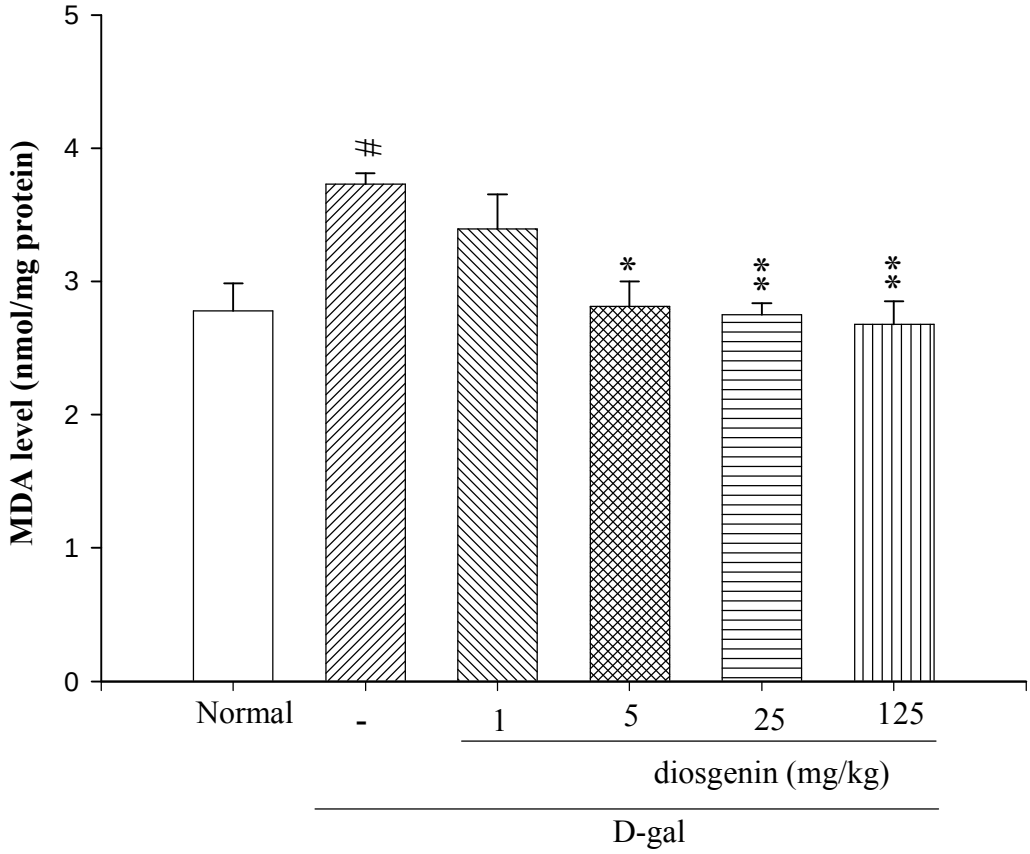

GSH-Px Activity (U/mg protein)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
*
Normal
-
1
5
25
125
diosgenin (mg/kg)
D-gal

Fig. 5


MDA level (nmol/mg protein)
5
4
3
2
1
0
#
*
**
**
Normal
-
1
5
25
125
diosgenin (mg/kg)
D-gal