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Ganoderma atrum polysaccharide attenuates oxidative stress induced by d-galactose in mouse brain

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ABSTRACT

Aims: Ganoderma atrum polysaccharide (PSG-1), the main constituent of *G. atrum*, has been reported to attenuate oxidative stress in vitro. The aim of this study was to investigate whether PSG-1 has a protective effect on the brain against oxidative stress induced by D-galactose (D-gal) in vivo.
Main methods: Mice were intraperitoneally (i.p.) injected with D-gal (100 mg/kg body weight) once daily for 10 weeks. From the seventh week, D-gal-treated mice received PSG-1 (50, 100, or 150 mg/kg body weight) once daily for the last 4 weeks. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GSH-Rd), and the contents of glutathione (GSH), glutathione disulfide (GSSG) and malondialdehyde (MDA) in the brain were measured using different biochemical methods to evaluate the changes of the antioxidant ability in the PSG-1 treated mice. Apoptosis, reactive oxygen species (ROS) and calcium levels were determined by flow cytometry.
Key findings: Administration of PSG-1 significantly reduced apoptosis in the mouse brain in a dose-dependent manner. PSG-1-evoked reduction of apoptosis was associated with the decrease of MDA and GSSG contents, and the increase of SOD, CAT, GPx and GSH-Rd activities, and GSH contents. PSG-1 treatment was also found to attenuate ROS production and calcium accumulation.
Significance: PSG-1 has a potential to be used as a novel therapeutic agent for the protection of aging brain tissue against oxidative damage by modifying the redox system and maintaining calcium homeostasis.

Introduction

Normal aging is characterized by a gradual loss of cognitive performance exhibited by decline in reasoning, mental processing, memory and spatial ability. Brain senescence plays an important role in cognitive dysfunction, which has been implicated in neurodegenerative disorders (Keller, 2006; Middei et al., 2006; Opii et al., 2008).

Accumulating evidence suggest that oxidative stress resulting from reactive oxygen species (ROS) generation has been largely implicated in the pathogenesis of age-related degenerative changes in the brain (Head et al., 2009). ROS is thought to play a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems. The excess of ROS can damage cellular lipids, proteins, or DNA inhibiting their normal functions (Valko et al., 2007). Brain is particularly susceptible to be attacked by ROS because it was found to generate more toxicants per gram of tissue than any other organ (Anbarasi et al., 2006). Numerous in vivo data suggest that a diet rich in antioxidants supports the maintenance of animal health (Lai et al., 2004; Willis et al., 2009). Considerable experimental evidence suggest that naturally occurring polysaccharides affect the function of animal and human brains by directly lowering oxidative stress (Chang and So, 2008; Lu et al., 2007; Sudheesh et al., 2009). Therefore, the focus has recently moved to screen the potential of natural dietary components, particularly of polysaccharides for the prevention of brain damage during aging. *Ganoderma atrum*, one of the most popular medicinal/nutritional fungi, belongs to the polyporaceae family of Basidiomycota. Evidence for the protective effect of *G. atrum* came from epidemiological and experimental studies, in which positive associations between consumption of *G. atrum* and vigority were reported (Chen and Li, 1993; Gao et al., 2004, 2005). *G. atrum* polysaccharide (PSG-1), a major bioactive component, was purified from *G. atrum* with a purity of >99.8%, and its primary structural features and molecular weight were characterized (Chen et al., 2008). A previous study has reported that PSG-1 exhibited a potent antioxidant activity, as assessed by a radical scavenging test in vitro (Li et al., 2009).

D-galactose (D-gal) is a naturally occurring substance in the body. Numerous studies have demonstrated that ROS could be generated in the course of D-gal metabolism. Evidence has shown that D-gal caused aging-related changes including the behavioral and neurochemical changes. It is thus widely used as an animal model for studying age-

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related damage in the brain (Chiu et al., 2009; Luo et al., 2009; Tian et al., 2005; Zhong et al., 2009). Therefore, the present study was designed to investigate whether administration of PSG-1 has protective effects on D-gal-induced impairment in the mouse brain, and further examine the molecular mechanisms of its action.

Materials and methods

Animals

Eight week-old female mice (Kunming strain), weighing 22.0 ± 2.0 g [Grade II, Certificate Number SCXK (gan) 2006-0001] were purchased from Jiangxi College of Chinese Medicine, Jiangxi, China. These animals were acclimated to our laboratory environment for 1 week before the experiment. They were housed under standard conditions (25 °C, 12 h light, 12 h dark), and were allowed free access to food and water during the experiment period. All animals used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health, USA (NIH Publication No. 85-23, 1996). All procedures were approved by the Animal Care Review Committee, Nanchang University.

Chemicals and reagents

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Fluo-3/AM was obtained from Biotium Company (Hayward, CA, USA). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (San Jose, CA, USA). D-gal and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), glutathione reductase (GSH-Rd), glutathione (GSH) and glutathione disulfide (GSSG) assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Extraction of polysaccharides from G. atrum

Standard procedure was followed for the isolation of polysaccharides from *G. atrum* (PSG-1) with a purity of >99.8%. Briefly, the polysaccharide fractions were prepared from the fruiting bodies of *G. atrum*, which were collected from Ganzhou, Jiangxi Province, China. All extracts were pooled, and the polysaccharide-enriched fractions were precipitated by the addition of 80% (v/v) ethanol. The polysaccharide fraction was further purified by gel filtration chromatography. Its primary structural features and molecular weight were characterized by infrared spectrometry, gas chromatography, size exclusion chromatography, amino acid analyzer and high-performance liquid chromatography (HPLC) (Chen et al., 2008). PSG-1 solution was freshly made by dissolving PSG-1 in 0.9% NaCl. It was then diluted to appropriate concentrations before use in each experiment.

Animal treatment

The mice were randomly divided into 3 groups: control group (n = 9), D-gal group (n = 9) and PSG-1 group (n = 27). Except control group, mice were intraperitoneally (i.p.) injected with D-gal at a dose of 100 mg/kg body weight once daily for 10 weeks while those of control group were treated with the same volume of 0.9% NaCl. From the seventh week, PSG-1 group mice were i.p. injected with PSG-1 at a dose of 50, 100 or 150 mg/kg body weight respectively once daily for 4 weeks (n = 9 for each dose). Control group and D-gal group mice were injected with the same volume of 0.9% NaCl.

Preparation of brain samples

Twenty-four hours after the last PSG-1 administration, animals were sacrificed. Brains were carefully and quickly removed and put into cold saline, and the right hemispheres were immediately dissected for cerebral cortex on a cold plate, weighed and homogenized with ice-cold saline and stored at −80 °C for biochemical analysis. The left hemispheres were immediately dissected for cerebral cortex and minced. The tissue fragments were dissociated by treatment with 0.125% trypsin 3 to 4 times at 37 °C, then filtered and centrifuged at 1000 rpm for 8 min for further evaluation.

Biochemical assays

The supernatants were immediately assayed for levels of GSH, GSSG and MDA, and activities of SOD, CAT, GPx and GSH-Rd, using the commercially available colorimetric assay kits. The protein contents in the supernatants were determined by the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as a standard.

Flow cytometric analysis of apoptosis

The percentage of apoptotic cells was determined using an Annexin V-FITC kit according to the manufacture's instruction. Briefly, the resulting pellet was resuspended in phosphate buffered saline (PBS), and stained with Annexin V and propidium iodide (PI) in binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACStar Plus flow cytometer.

Measurement of ROS

Determination of intracellular ROS was based on the oxidation of DCFH-DA to the fluorescent product, 2′,7′-dichlorofluorescein (DCF). The resulting pellet was incubated with 10 μM of DCFH-DA at 37 °C for 20 min. The resuspension was pelleted, washed, and resuspended in PBS. Then, DCF fluorescence was monitored by flow cytometry with FACSort cell sorter (Becton Dickinson) at wavelengths of 480 ± 30 nm (excitation) and 535 ± 40 nm (emission).

Measurement of calcium concentration

The change of calcium concentration in the brain was determined by the fluorescence of the calcium-sensitive dye fluo-3/AM as described (Sedova and Blatter, 2000). The resulting pellet was loaded with 20 μM membrane-permeant acetoxymethyl ester of the dye (fluo-3/AM; Molecular Probes) for 50 min at 37 °C in the standard Tyrode solution containing 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.3). Detached pellet was harvested by low-speed centrifugation (1000 *g*), resuspended in 10 mM HEPES and analyzed on a flow cytometer at wavelengths of 488 nm (excitation) and 530 ± 5 nm (emission). Data were expressed in arbitrary units.

Statistical analyses

Values are expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was applied to determine the statistical significance between various groups. A value of $P<0.05$ was considered to be statistically significant.

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Results

Changes of general appearance in D-gal-treated mice with or without PSG-1

In the present investigation, mice were subjected to i.p. injection with D-gal once daily at a dose of 100 mg/kg body weight for 10 weeks. From the seventh week, D-gal-induced damage mice were treated with PSG-1 (50, 100, or 150 mg/kg body weight) once daily for the last 4 weeks. During the entire experiment process, there were no inflammations in injection sites of mice and significant differences in general appearance among the mice that received 2 i.p. injections daily at the start of the study or at the time of killing.

Effects of PSG-1 on apoptosis in the brain of D-gal-treated mice

Apoptosis is a basic biological phenomenon with a wide range of implications in human disease including brain injury. To examine whether PSG-1 could protect against aging-induced apoptosis in the brain, flow cytometric analysis was used to quantify the rate of apoptosis by using double staining of Annexin V and PI (Fig. 1). A significant increase of apoptosis was observed in mice treated with D-gal alone, compared with control mice. However, treatment with PSG-1 showed a significant resistance in apoptosis in mice undergoing D-gal treatment. These results suggest that PSG-1 is a potent protective agent against brain injury induced by D-gal.

Effects of PSG-1 on MDA contents in the brain of D-gal-treated mice

The amount of MDA, a product of lipid peroxidation, is a measure of oxidative stress status of a cell or tissue. In this study, after the administration of D-gal at a dose of 100 mg/kg body weight once daily for 6 weeks, the contents of MDA in the D-gal-treated mice were significantly increased compared with those of control (Shi et al., 2009). From the seventh week, the D-gal-treated group was continuatively injected with the same dose of D-gal once daily for the last 4 weeks. After the treatment of D-gal for 10 weeks, the contents of MDA were also determined, and the data showed that MDA was significantly enhanced in the D-gal-treated group (Table 1), compared with the control group. In contrast, this increase of MDA levels in the brain of D-gal treated mice was attenuated by PSG-1 administration in a dose-dependent manner. The results above suggest that PSG-1 could protect against age-related oxidative stress.

Effects of PSG-1 on ROS production in the brain of D-gal-treated mice

Oxidative stress occurs mainly due to increased production of ROS during the pathogenesis of brain aging. As shown in Fig. 2, D-gal caused a rapid and significant increase in DCF fluorescence (measured in arbitrary units) from 4.17 ± 1.95 in control mice to 57.25 ± 4.35 in D-gal-treated mice ($P<0.01$). However, PSG-1 caused a dose-dependent attenuation in DCF fluorescence to 18.98 ± 3.01, 8.28 ± 2.83, and 4.59 ± 2.21, respectively (all $P<0.01$ vs. D-gal group). These findings suggest that PSG-1 may have a direct ability to scavenge ROS generation in the brain of D-gal-treated mice.

Effects of PSG-1 on activities of cellular antioxidant enzymes in the brain of D-gal-treated mice

SOD, CAT and GPx are key enzymes in the detoxification of ROS. The effect of PSG-1 on activities of SOD, CAT and GPx in the brain of D-gal-treated mice was examined in this study. As shown in Table 2, D-gal treated mice possessed significantly low activities of SOD, CAT and GPx compared with the control group, whereas PSG-1 (50, 100 or 150 mg/kg) treatment inhibited the reduction induced by D-gal in a dose-dependent manner. These data indicate that PSG-1 could

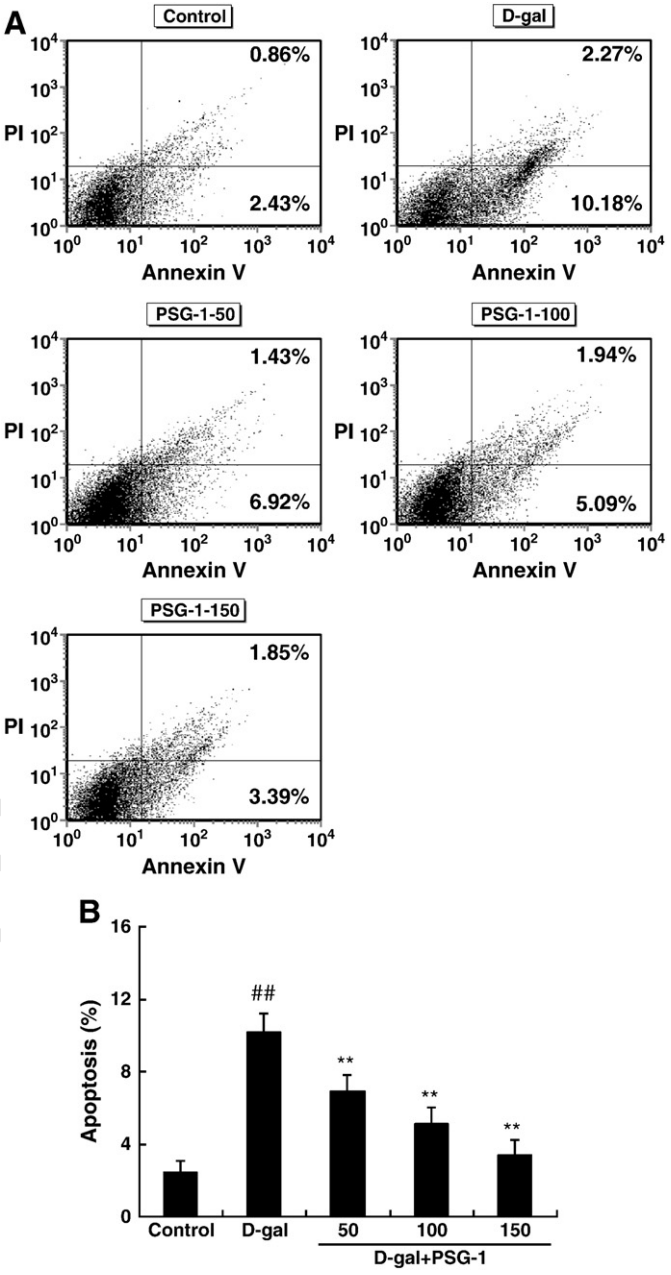

Fig. 1. Effects of PSG-1 on apoptosis in the brain of D-gal-treated mice. (A) Detection of apoptotic cells by Annexin V and PI double staining. Impaired mice induced by D-gal were treated with or without PSG-1. The cerebral cortex was stained with Annexin V and PI labeling and analyzed by flow cytometry. (B) Column bar graph of apoptosis. Eight independent experiments were performed and all gave similar results. Values were expressed as means ± SEM of 9 mice. $^{\#\#}P<0.01$ vs. control group and $^{**}P<0.01$ vs. D-gal group.

Table 1
Effects of PSG-1 on MDA contents in the brain of D-gal-treated mice.

Group	N	D-gal (mg/kg/day)	PSG-1 (mg/kg/day)	MDA (μmol/l)
Control	9	Vehicle	Vehicle	13.89 ± 0.58
D-gal	9	100	Vehicle	$28.27 \pm 1.08^{\#\#}$
PSG-1-50	9	100	50	$19.43 \pm 0.78^{**}$
PSG-1-100	9	100	100	$16.12 \pm 0.71^{**}$
PSG-1-150	9	100	150	$15.43 \pm 0.63^{**}$

Values were expressed as means ± SEM of 9 mice.
$^{\#\#}$ $P<0.01$ vs. control group.
** $P<0.01$ vs. D-gal group.

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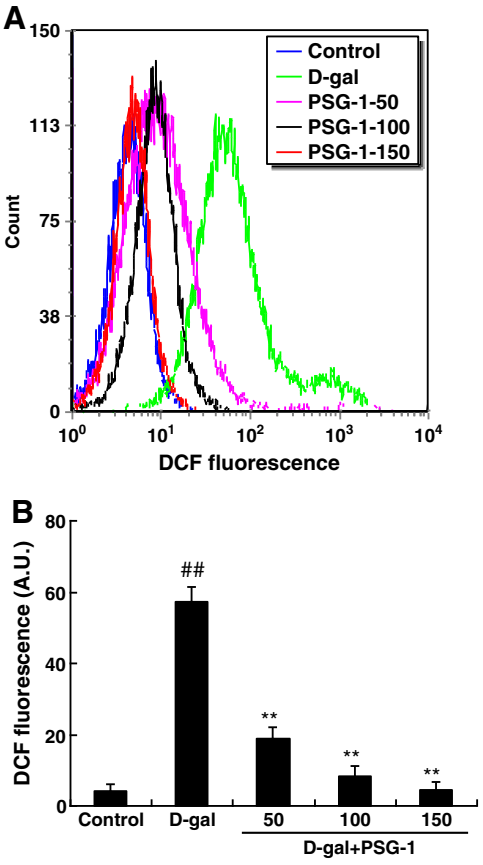

Fig. 2. Effects of PSG-1 on ROS generation in the brain of D-gal-treated mice. (A) Flow cytometric histograms of fluorescence of 2′–7′-dichlorofluorescein (DCF) in the cerebral cortex. (B) Column bar graph of fluorescence for DCF. Eight independent experiments were conducted and all gave similar results. Values were expressed as means ± SEM of 9 mice. $^{\#\#}P<0.01$ vs. control group and $^{**}P<0.01$ vs. D-gal group.

attenuate D-gal-induced changes in activities of cellular antioxidant enzymes in the brain of D-gal-treated mice.

Effects of PSG-1 on GSH and GSSG levels, and GSH-Rd activity in the brain of D-gal-treated mice

The GSH and GSSG levels are also considered to be important markers of cellular oxidative stress. We thus tested whether GSH and GSSG levels, were affected by PSG-1 treatment. As shown in Table 3, GSH levels in the brain of D-gal-treated mice were significantly lower than those in controls. Consistent with this, the levels of GSSG in the D-gal group were significantly higher than the control group. GSH-Rd is an important NADPH-dependent enzyme that maintains GSH in a reduced state and thereby causes an increase in GSH level and decrease in GSSG level. Our data showed that GSH-Rd activities in the D-gal group were significantly decreased compared with those in vehicle controls (Table 3). In contrast, PSG-1 administration suppressed the increase of GSSG and the decrease of GSH and GSH-Rd in D-gal-treated mice in a dose-dependent manner. Taken together, these results suggest that PSG-1 could attenuate oxidative stress via modification of the GSH and GSSG levels, and GSH-Rd activity in the brain of mice.

Table 2
Effects of PSG-1 on activities of antioxidant enzymes in the brain of D-gal-treated mice.

Group	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Control	61.71 ± 3.74	23.20 ± 0.94	287.57 ± 8.15
D-gal	34.57 ± 1.74$^{\#\#}$	15.80 ± 0.62$^{\#\#}$	170.74 ± 7.87$^{\#\#}$
PSG-1-50	40.62 ± 2.18*	18.30 ± 0.76**	215.22 ± 10.36**
PSG-1-100	51.55 ± 2.40**	21.17 ± 0.98**	254.36 ± 11.86**
PSG-1-150	59.54 ± 3.03**	23.43 ± 1.07**	270.33 ± 9.46**

Values were expressed as means ± SEM of 9 mice.
$^{\#\#}$ $P<0.01$ vs. control group.
* $P<0.05$ vs. D-gal group.
** $P<0.01$ vs. D-gal group.

Table 3
Effects of PSG-1 on GSH and GSSG contents, and GSH-Rd activity in the brain of D-gal-treated mice.

Group	GSH (μmol/l)	GSSG (μmol/l)	GSH-Rd (U/mg protein)
Control	124.60 ± 4.28	77.10 ± 4.01	225.48 ± 19.21
D-gal	65.80 ± 3.77$^{\#\#}$	111.22 ± 4.68$^{\#\#}$	155.17 ± 6.21$^{\#\#}$
PSG-1-50	86.11 ± 3.91**	98.01 ± 4.49*	217.44 ± 18.98*
PSG-1-100	115.48 ± 5.52**	86.01 ± 4.47**	232.61 ± 15.10**
PSG-1-150	121.80 ± 4.32**	83.57 ± 4.15**	238.00 ± 18.31**

Values were expressed as means ± SEM of 9 mice.
$^{\#\#}$ $P<0.01$ vs. control group.
* $P<0.05$ vs. D-gal group.
** $P<0.01$ vs. D-gal group.

Effects of PSG-1 on concentration of calcium in the brain of D-gal-treated mice

To further investigate whether the protective effect of PSG-1 is related to a suppression of intracellular calcium accumulation, fluo-3 fluorescence intensity was examined. A calibration procedure was used in each experiment to convert fluo-3 fluorescence values into absolute concentration of calcium. A significant increase in concentration of calcium was observed in the D-gal group, compared with that of the control group (Fig. 3). However, treatment with PSG-1 inhibited the D-gal-induced increase in calcium concentration in a dose-dependent manner. These results suggest that PSG-1 could attenuate the elevation of calcium concentration in the brain undergoing D-gal treatment.

Discussion

Aging is a fundamental biological phenomenon with a wide range of medical and social importance. It is well known that many age-related behavioral changes in motor and cognitive performance occur even in the absence of specific, age-related, neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease (Butterfield et al., 1999; Kenyon, 2010). Research suggests that the aged brain may provide a sensitive environment for the development of these diseases, leading to even more severe deficits in memory and/or motor function (Wang et al., 2009). In both financial and human terms, it is extremely important to explore methods to retard or reverse brain aging and their subsequent behavioral manifestations.

The D-gal-lesioned rodents have been used for brain aging studies, as D-gal induced behavioral and neurochemical changes can mimic many characteristics of the natural brain aging process in human (Wei et al., 2008). Apoptosis has been implicated in various pathological conditions involving in aging and was commonly used as an index of brain injury (Zhang and Bazan, 2010). In the present study, we found that chronic treatment of D-gal induced a significant increase of apoptosis in the brain, suggesting brain damage induced by D-gal. It is well established that ROS is both a key regulator of apoptotic signaling and an initiator of oxidative damage to DNA, lipids and proteins under different circumstances. The brain, with high oxygen demand, and relatively deficient in anti-oxidative defense, is the most susceptible organ to oxidative damage. Studies with D-gal-lesioned mice have demonstrated that D-gal-induced oxidative stress is a potent inducer of apoptosis (García-Matas et al., 2010; Recuero et al., 2009; Ritchie, 2009). In the present study, we also found that D-gal induced an increase in ROS generation and lipid peroxidation, and concomitantly

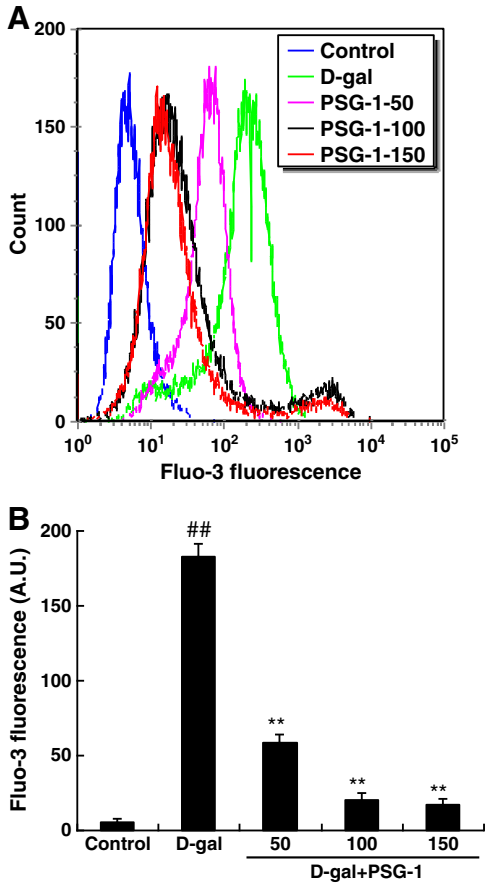

Fig. 3. Effect of PSG-1 on concentration of calcium in the brain of D-gal-treated mice. Concentration of calcium was measured by fluorescent fluo-3 AM staining. (A) Flow cytometric histograms of fluorescence of fluo-3 in the cerebral cortex. (B) Column bar graph of fluorescence for fluo-3. Values were expressed as means ± SEM of 9 mice. $^{\#\#}P<0.01$ vs. control group and $^{**}P<0.01$ vs. D-gal group.

increase of apoptosis, further suggesting that there is a close relationship between oxidative stress and brain injury.

The genus *Ganoderma* is one of the most valued fungi. It is well known as "Lingzhi" in Chinese, "Reishi" in Japanese and "Youngzhi" in Korean. This fungus has recently received considerable attention for the health care in the world (Jia et al., 2009; Yue et al., 2006). *G. atrum*, a member of the genus *Ganoderma*, is well-known for its various biological effects and has been used for thousands of years as a functional food for health and longevity purposes (Chen and Li, 1993; Chen et al., 2010; Paterson, 2008). Currently, *G. atrum* has been developed into a variety of foods, such as *G. atrum* tea. PSG-1, the most abundant component isolated from *G. atrum*, has been shown to possess potent antioxidant activity (Li et al., 2010). Consequently, we investigated the protective effect of PSG-1 on the brain toxicity induced by D-gal in the mice. Data obtained from the present study suggested that chronic administration of PSG-1 for a period of 4 weeks attenuated the increase of apoptosis resulted from D-gal in a dose-dependent manner, suggesting protection of PSG-1 against aging-related injury in the brain. Our study further showed that administration of PSG-1 significantly attenuated ROS production and decreased lipid peroxidation. These data suggested that the protective effect of PSG-1 against brain injury may be related to attenuation of oxidative stress.

The major defense mechanisms which the brain uses to reduce free radical injury include enzymatic and non-enzymatic free radical scavenging systems. Enzymatic free radical scavenging systems, such as SOD, CAT and GPx, are major antioxidant enzymes, all of which physiologically reduce ROS levels (Poon et al., 2004; Peng et al., 2009; Sies and Murphy, 1991). GSH is the major cellular defense system against oxidants which reacts non-enzymatically with hydroxyl radicals to produce GSSG (Griffith, 1999; Odat et al., 2007; Pelka et al., 2009). It is normally maintained in a reduced state via NADPH-dependent enzymes, specifically GSH-Rd (Lu et al., 2007). Healthy cells can scavenge free radicals effectively by means of the antioxidant defense system. However, in pathological conditions, when ROS is generated in excess or the cellular antioxidant defense system is defective, ROS may damage cellular components, inhibiting their normal functions (Calabrese et al., 2004). Thus, increase in activities of SOD, CAT, GPx and GSH-Rd, and GSH level, and decrease in GSSG content are considered to be more beneficial in the event of oxidative stress. In accordance with these reports, our results have shown that D-gal-induced brain injury was concomitant with an increase of oxidative stress and depletion of endogenous antioxidants (SOD, CAT and GPx) in the brain. Interestingly, treatment with PSG-1 has been shown to evoke a dose-dependent increase in the activities of these antioxidant enzymes. Our results have also shown that PSG-1 significantly suppressed the increase of GSSG, and the decrease of GSH and GSH-Rd that resulted from D-gal in a dose-dependent manner. From these results we can conclude that PSG-1-mediated protection of the brain may be related to its antioxidant properties.

It is well known that calcium plays a role in tissue injury by activation of calcium-dependent regulatory proteins and degradative enzymes which may irreversibly alter functions of the affected bio-macromolecules (Cusack et al., 1991; Biessels and Gispen, 1996). Previous studies have shown that cells subjected to oxidative stress accumulated calcium, and the alteration of calcium homeostasis could be blocked by antioxidants (Arbogast and Ferreiro, 2010). We thus further evaluate the effect of PSG-1 on concentration of calcium in the brain. Our results demonstrated that D-gal treatment caused a significant increase of calcium accumulation, ROS production and MDA contents. Interestingly, administration of PSG-1 evoked a dose-dependent reduction of calcium concentration, which was associated with an attenuation of ROS generation and decrease of MDA contents in the brain of D-gal-induced senescent mice. These results indicated that alleviating calcium accumulation might be one of the mechanisms for the protective effect of PSG-1 against oxidative stress in the brain.

Conclusions

In summary, our results suggested that PSG-1 significantly attenuated D-gal-induced oxidative stress and apoptosis in the brain. The protective effects of PSG-1 against D-gal-induced oxidative injury in the brain could be attributed to its ability to increase activities of endogenous antioxidants, and alleviate intracellular calcium accumulation. Thus, dietary intervention with foods high in PSG-1 may have positive effects during brain aging.

Conflict of interest statement

All authors have no personal or financial conflict of interest and have not entered into any agreement that could interfere with our access to the data on the research or on our ability to analyze the data independently, to prepare manuscripts and to publish them.

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