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Ganoderma atrum Polysaccharide Improves Age-Related Oxidative Stress and Immune Impairment in Mice

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ABSTRACT: The aim of the present study was to investigate whether oxidative stress and immune dysfunction could be attenuated by *Ganoderma atrum* polysaccharide (PSG-1) in D-galactose (D-gal)-induced aging mice, and provide evidence for its effects. The results showed that PSG-1 significantly decreased lipid peroxidation in liver, brain, and spleen, but concomitantly increased the activities of superoxide dismutase, catalase, and glutathione peroxidase compared with the D-gal group. Elevation of glutathione contents and attenuation of glutathione disulfide contents were also found in PSG-1-treated animals. Furthermore, the results showed that PSG-1 treatment increased basal lymphocyte proliferation as well as T cell and B cell proliferation and enhanced interleukin-2 production. Taken together, the results suggested that PSG-1 had potential as a novel agent to promote health and improve aging-associated pathologies, at least in part, via modification of the redox system and improvement of immune function.

KEYWORDS: *Ganoderma atrum polysaccharide, aging, oxidative stress, immune function, lymphocyte proliferation*

INTRODUCTION

Aging has become one of the biggest challenges around the world. It is a physiological process with a progressive, endogenous, and irreversible accumulation of adverse changes that increase vulnerability to disease and, finally, death.[1] Although the precise cellular mechanism responsible for aging remains enigmatic, there is increasing evidence that oxidative stress and immunological decline may play a critical role in the pathogenesis of aging, as well as age-related morbidity and mortality.[2−4]

Oxidative stress occurs when there is an imbalance between relative shortages in antioxidant defenses and increased production of reactive oxygen species (ROS). The excess ROS could interact degeneratively with cellular components, including proteins, lipids, and nucleic acids, causing cellular dysfunction. Because of this, oxidative stress has been implicated in a variety of human diseases as well as in the aging process.[5,6] In addition to oxidative stress, aging is also associated with impaired ability of the immune system both structurally and functionally.[7,8] These positive effects of dysfunction in immunity could result in lowered susceptibility and vulnerability to bacterial and viral infections during old age, which stand out as the most common causes of disease and aging. In this regard, using naturally occurring substances and dietary compounds endowed with antioxidant and immunopotentiating properties may promote health and lower the risk of developing age-related diseases.[9,10]

Ganoderma atrum has been extensively used as functional food and medicine to promote health and maintain the body's resistance against infection by potentiating immunity.[11,12] *G. atrum* polysaccharide (PSG-1) is believed to be the main component for the biological activity of *G. atrum*, and its primary structural features and molecular weight have been characterized. PSG-1 was a homogeneous protein-bound polysaccharide, with a weight-average molecular weight of 1013 kDa based on gel permeation chromatography (GPC) analysis. The predominant monosaccharides in PSG-1 were glucose, galactose, and mannose in a molar ratio of 1:1.28:4.91, detected by gas chromatography (GC). A β-elimination reaction demonstrated that the protein and carbohydrate were linked by an O-linkage.[13] Despite much evidence of the beneficial effects of *G. atrum*,[14] it has not been fully characterized whether PSG-1 is able to improve aging-associated oxidative stress and immune dysfunction. Thus, the aim of this study was to examine whether PSG-1 has potential in decreasing oxidative stress and improving immune function involved in aging.

D-Galactose (D-gal) is a reducing sugar that could form advanced glycation end products in the body. ROS can be generated in the course of D-gal metabolism and subsequently cause oxidative stress. In addition, studies have demonstrated that D-gal leads to a mimic regression change of aging in the immune system in vivo. Immune dysfunction and oxidative stress are similar to those observed in normal aging mice.[15−17] In this study, we investigated whether PSG-1 may attenuate oxidative stress and improve immune dysfunction induced by D-gal in mice and further examined the possible mechanisms for its effects.

MATERIALS AND METHODS

Reagents. PSG-1 was extracted and purified following our previously published method.[13] D-gal, bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

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concanavalin A (Con A), and lipopolysaccharides (LPS) were purchased from Sigma (St. Louis, MO). Cell culture products were obtained from Life Technologies (Paisley, Scotland). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malonaldehyde (MDA), glutathione (GSH), and glutathione disulfide (GSSG) assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sen Xiong Biotech (Shanghai, China).

Animals. Mice (Kunming strain, 8 weeks old), weighing 22.0 ± 2.0 g, were purchased from Jiangxi College of Chinese Medicine, Jiangxi, China. Mice were housed at nine per cage and maintained at a constant temperature (25 °C), on a 12/12 h reversed light/dark cycle. These mice were acclimated to our laboratory environment for 1 week before the experiment. 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 Institutes of Health, Bethesda, MD (NIH Publication 85-23, 1996). All procedures were approved by the Animal Care Review Committee, Nanchang University, China.

Animal Treatment. At the beginning of the experiment, mice were randomly divided into six groups (n = 9 per group). The experimental groups were divided as follows: (i) control group: mice were injected intraperitoneally (ip) once daily with the same volume of 0.9% sodium chloride for 10 weeks; (ii) D-gal group: mice received a daily ip injection of D-gal at a dose of 100 mg/kg body weight for 10 weeks; (iii) D-gal + PSG-1−50 group, (iv) D-gal + PSG-1−100 group, and (v) D-gal + PSG-1−150 group: mice received a daily ip injection of D-gal at a dose of 100 mg/kg body weight for the first 6 weeks; from the seventh week, mice were ip injected with PSG-1 at doses of 50, 100, and 150 mg/kg body weight for 4 weeks, respectively (meanwhile, also ip injection of D-gal at a dose of 100 mg/kg body weight); (vi) PSG-1−150 group: mice without D-gal treatment received the same volume of sodium chloride 0.9% injection for 6 weeks; from the seventh week, mice were ip injected with PSG-1 at a dose of 150 mg/kg body weight for 4 weeks.

Preparation of Samples. Twenty-four hours after the last PSG-1 administration, blood was collected by retroorbital venous plexus and mice were sacrificed by cervical dislocation. Blood samples were allowed to clot for 1−2 h; the serum was separated by centrifugation at 2000g for 5 min and stored at −20 °C. Organs and tissues were immediately collected for experiments or stored at −80 °C for later experiments.

Biochemical Analysis. Liver, brain, and spleen were weighed, minced, and then homogenized in cold phosphate-buffered saline (PBS). After centrifugation at 2000g at 4 °C for 5 min, supernatant was collected for biochemical measurements. Protein contents in the supernatants were determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. Levels of GSH, GSSG, and MDA and activities of SOD, CAT, and GPx in the homogenate supernatant were assayed using the commercially available colorimetric assay kits.

Preparation of Lymphocyte Cells. Primary cultures of spleen lymphocytes were prepared from the extirpated spleens according to the method described by Song et al.[15] with some modifications. Briefly, the spleens were removed aseptically after the mice were sacrificed. Single-cell spleen suspensions were prepared by rubbing the tissue and then filtered and centrifuged (800g, 4 °C) for 8 min. Contaminating erythrocytes were lysed by treating the pellet with 5 mL of Tris−ammonium chloride lysis buffer (0.18 M of NH_4Cl in 0.17 M Tris, pH 7.2) for 3 min. Samples were washed twice in PBS/0.1% BSA. After centrifugation (300g, 5 min), the lymphocytes were resuspended in a complete medium containing RPMI 1640, 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Measurement of Lymphocyte Proliferation. Splenocytes were seeded in 96-well flat-bottom microplates in triplicate at 5 × 10^4 cells/well. The cells were cultured with 2.5 μg/well Con A or 10 μg/well LPS as stimulated samples or in complete medium alone as nonstimulated samples. After incubation for 48 h, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 h in a humidified incubator (5% CO_2, 37 °C). The plates were read on a Mmicroplate reader at 570 nm. The absorbance (A) was transformed into lymphocyte proliferation ratio for comparison: lymphocyte proliferation ratio = test A/normal control A × 100%.

Measurement of Interleukin-2 (IL-2). The level of IL-2 in serum was measured by a sandwich ELISA kit (Senxiong Biotech, Shanghai, China). The assay was carried out according to the instructions provided in the kits. A standard curve was constructed using standards provided in the kits, and the cytokine concentration was determined from the standard curve using linear regression analysis.

Statistical Analyses. Statistical analyses were carried out using SPSS for Windows (version 11.5). The results were expressed as the mean ± SEM. One-way analysis of variance followed by the Student−Newman−Keuls test was applied to calculate the statistical significance between various groups. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Effect of PSG-1 on Body Weight in D-Gal-Treated Mice. In the present study, mice were injected with D-gal (ip, 100 mg/kg body weight) once daily for 10 weeks. From the seventh week, D-gal-induced 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 mice received 2 ip injections daily at the start of the study or at the time of killing. After the last administration, mice were sacrificed at the indicated time, and body weight was observed. Results showed that body weights of D-gal-treated mice were significantly decreased as compared with that of control mice. When D-gal-treated mice were administered PSG-1, their body weights were increased significantly compared with the D-gal group (Figure 1). The data indicate that PSG-1 may have the potential to protect aging mice against losing weight induced by D-gal.

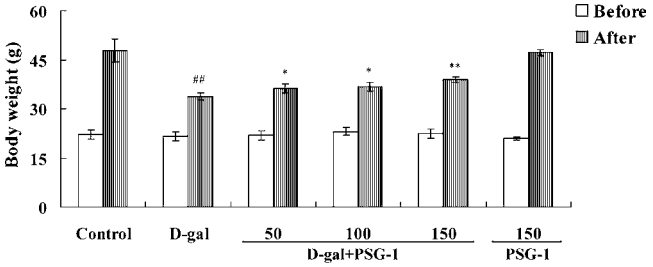

Figure 1. Effects of PSG-1 on body weight in mice. Body weights in all groups were measured at the start of the study or at the time of killing. "Before" indicates that body weights were determined in all mice before the first administration on day 1. "After" indicates that body weights were determined in all mice 24 h after the last administration. Values are expressed as the mean ± SEM of nine mice: (##) P < 0.01 compared to the control group; (*) P < 0.05 and (**) P < 0.01 compared to the D-gal group.

Effect of PSG-1 on Oxidative Stress in D-Gal-Treated Mice. MDA, a breakdown product of lipid peroxidation, is widely used as a measure of oxidative stress.[18] The data (Figure 2) showed that D-gal induced a significant increase in MDA contents in the liver, brain, and spleen, compared with the control group. Interestingly, the increase of MDA contents induced by D-gal in these tissues was attenuated by PSG-1 administration. These results suggest that PSG-1 may attenuate oxidative stress induced by D-gal.

Effect of PSG-1 on Activities of Cellular Antioxidant Enzymes in D-Gal-Treated Mice. Antioxidant enzymes

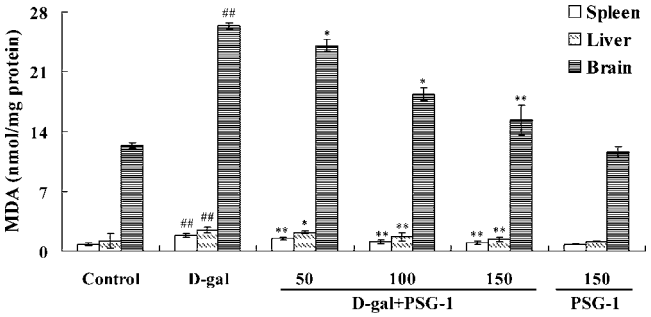

Figure 2. Effects of PSG-1 on the contents of MDA in tissues. The contents of MDA were significantly increased in the liver, brain, and spleen of the D-gal group. After the administration of PSG-1, the contents of MDA were decreased compared with the D-gal group. Values are expressed as the mean ± SEM of nine mice: (#) $P < 0.05$ and (##)$P < 0.01$ compared to control group; (*) $P < 0.05$ and (**) $P < 0.01$ compared to D-gal group.

(SOD, CAT, and GPx) are the first line of protection for tissues or cells against oxidative stress.[19] As shown in Figure 3, D-gal-treated mice showed significantly lower activities of SOD, CAT, and GPx compared with the control group, whereas PSG-1 (50, 100, or 150 mg/kg) treatment alleviated the reduction induced by D-gal. These data indicate that PSG-1 may attenuate oxidative stress, at least in part, by regulating activities of these antioxidant enzymes in the D-gal-treated mice.

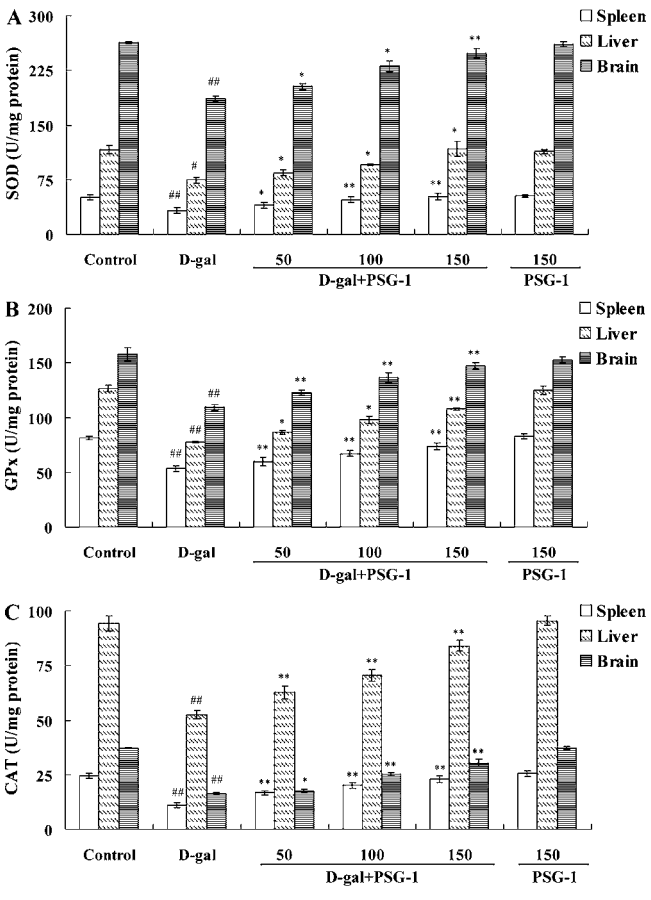

Figure 3. Effects of PSG-1 on activities of (A) SOD, (B) CAT, and (C) GPx in tissues. After administration of D-gal for 10 weeks, there was a decrease of enzymatic activities of SOD, CAT, and GPx. From the seventh week, D-gal-treated mice received PSG-1 (50, 100, or 150 mg/kg body weight) once daily for the last 4 weeks. PSG-1 evoked an increase of SOD, CAT, and GPx activities. Values are expressed as the mean ± SEM of nine mice: (#) $P < 0.05$ and (##) $P < 0.01$ compared to the control group; (*) $P < 0.05$ and (**) $P < 0.01$ compared to the D-gal group.

Effect of PSG-1 on GSH and GSSG Levels in D-Gal-Treated Mice. GSH defends cells or tissues against oxidative stress, which reacts nonenzymatically with hydroxyl radicals to produce GSSG.[20] As shown in Figure 4, GSH levels in tissues were significantly lower than those in controls. Correspondingly, the levels of GSSG in the D-gal group were significantly higher than in the control group. Interestingly, PSG-1 significantly inhibited the changes of GSSG and GSH in D-gal-treated mice. These findings suggest that PSG-1 may protect mice from oxidative stress induced by D-gal via increasing GSH levels and decreasing GSSG levels.

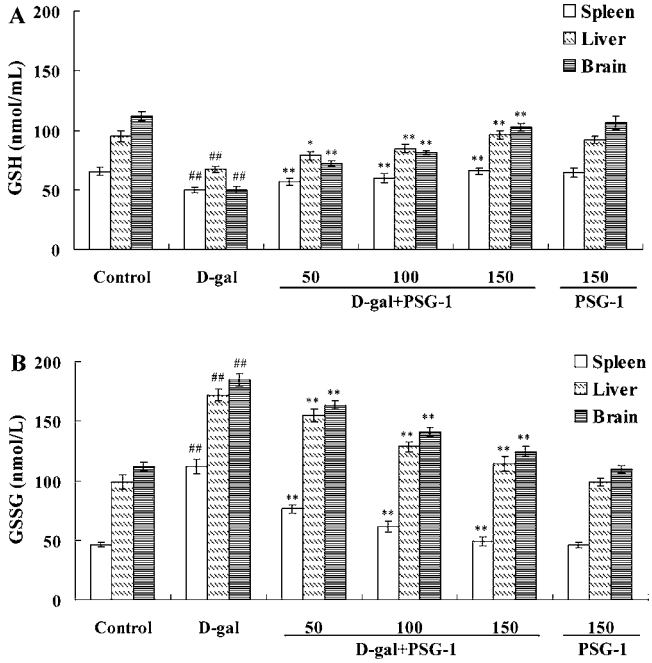

Figure 4. Effects of PSG-1 on levels of (A) GSH and (B) GSSG in tissues. GSH is an important nonenzymatic antioxidant. Treatment with PSG-1 significantly augmented GSH levels compared with the D-gal group. Reduced GSH is rapidly oxidized to GSSG by radicals. Treatment with PSG-1 markedly attenuated GSSG levels compared with the D-gal group. Values are expressed as the mean ± SEM of nine mice: (##) $P < 0.01$ compared to the control group; (*) $P < 0.05$ and (**) $P < 0.01$ compared to the D-gal group.

Effect of PSG-1 on Lymphocytes Proliferation in D-Gal-Treated Mice. It is well-known that the immune system is also involved in age-related process. The capacities of lymphocyte proliferation play a crucial role in immune response.[21] As shown in Figure 5, the basal lymphocyte proliferation appeared to be decreased in the D-gal groups, compared with the control group. Meanwhile, proliferative responses of lymphocytes to both T cell and B cell mitogens (Con A and LPS, respectively) were reduced markedly in D-gal-treated mice, compared with the control group. Interestingly, administration of PSG-1 evoked a significant increase in lymphocyte proliferation activities in the D-gal-treated mice, as compared with the D-gal group. These data suggest that

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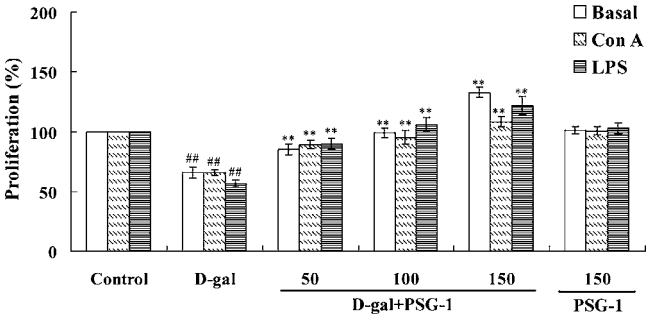

Figure 5. Effect of PSG-1 on lymphocyte proliferation in mice. Basal lymphocyte proliferation and proliferative responses of lymphocytes to both T cell and B cell mitogens (Con A and LPS, respectively) were determined by MTT method. The control murine lymphocyte proliferation ratio was regarded as 100%. Values are expressed as the mean ± SEM of nine mice: (##) $P < 0.01$ compared to the control group; (**) $P < 0.01$ compared to the D-gal group.

PSG-1 may attenuate D-gal-induced immune dysfunction in the mice.

Effect of PSG-1 on IL-2 Production in D-Gal-Treated Mice. Cytokines and soluble glycoproteins exert an effect on immune processes. Manipulation of cytokines is a powerful approach to the regulation of immune functions in aging. IL-2, an important cytokine, exhibits various bioactivities and is involved in immunomodulation.[22] In the present study, effects of PSG-1 on IL-2 level in serum were determined by ELISA. As shown in Table 1, the IL-2 level in the D-gal group was significantly lower than in the control group. Administration of PSG-1 was found to significantly enhance the IL-2 level in the serum of D-gal-treated mice.

Effect of PSG-1 on Redox System and Immune Function in Normal Mice. As a naturally occurring polysaccharide, the effects of PSG-1 on normal mice were further investigated in this work. Results showed that body weight in the PSG-1 group (alone at a dose of 150 mg/kg) exhibited a similar pattern to that of the control group (Figure 5). As shown in Figures 2−4, there was no significant difference in lipid peroxidation or enzymatic and nonenzymatic free radical-scavenging systems between the control group and the PSG-1 group. Meanwhile, PSG-1 did not significantly influence lymphoproliferation and IL-2 level compared with the control group in normal mice (Figure 5 and Table 1). These results suggested that administration of PSG-1 alone could not notably affect the free radical-scavenging systems and immune function in normal mice, but it has the ability to improve D-gal aging.

DISCUSSION

The present study describes the effects of PSG-1 on a variety of immune functions and oxidative stress parameters related to aging induced by D-gal. In this work, the most important observations were that PSG-1 markedly suppressed the oxidative stress resulting from D-gal. In addition, PSG-1 protected mice from immune dysfunction induced by D-gal. These effects may be achieved by elevation of SOD, CAT, and GPx activities, GSH contents, lymphocyte proliferation and concentration of IL-2, and decrease of MDA and GSSG contents.

The debilitating consequences of aging are widespread and extremely costly. It is now generally accepted that oxidative stress resulting from ROS generated in aerobic metabolism plays a causative role in aging or age-related destruction.[23] Oxidative stress is shown by increased contents of MDA, a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid. Consistent with previous studies,[24] we also found that mice injected with D-gal showed a significant increase in MDA contents, suggesting that oxidative damage plays an important role in the aging process and associated pathologies. A substantial body of evidence suggests that phytochemicals from diet provide important additional protection against oxidative damage.[25] PSG-1, a naturally occurring polysaccharide from *G. atrum*, has been reported to possess a variety of biological activities, including antioxidant activity.[14,26] In the present study, we also showed that MDA contents in tissues were reduced markedly in the PSG-1-treated mice, compared with the D-gal group. This finding suggested that PSG-1 could attenuate oxidative stress in D-gal-treated mice.

To protect themselves from oxidative stress, cells are equipped with reducing buffer systems including enzymatic (SOD, CAT, and GPx) and nonenzymatic (GSH, α-tocopherol, vitamin C, carotene, and flavonoids) free radical-scavenging systems. SOD catalytically reduces the O_2^- to H_2O_2. H_2O_2 is enzymatically degraded by either GPx or CAT to nontoxic products. GPx, in the process of H_2O_2 metabolism, converts GSH to GSSG.[27,28] Growing evidence supports the view that increasing SOD, CAT, and GPx activities, as well as GSH level, and decreasing GSSG content are beneficial in the prevention of oxidative stress-induced DNA, lipid, and protein damage implicated in disease and aging processes.[29] Consistent with these reports, the data of the present study also showed that administration of PSG-1 significantly improved the activities of antioxidant enzymes (SOD, CAT, and GPx), attenuated the content of GSSG, and increased the level of GSH. These results suggested that the effect of PSG-1 against oxidative stress may be mediated by modification of the redox system in D-gal-treated mice.

Table 1. Effect of PSG-1 on Levels of IL-2 in Serum[a]

group	*N*	D-gal (mg/kg/day)	PSG-1 (mg/kg/day)	IL-2 (pg/mL)
control	9	vehicle	vehicle	113.99 ± 8.05
D-gal	9	100	vehicle	59.82 ± 4.53##
D-gal + PSG-1−50	9	100	50	71.35 ± 3.73*
D-gal + PSG-1−100	9	100	100	75.64 ± 5.01**
D-gal + PSG-1−150	9	100	150	82.46 ± 5.23**
PSG-1−150	9	vehicle	150	111.11 ± 3.38

[a]The levels of IL-2 were determined in serum. Values are expressed as the mean ± SEM of nine mice: (##) $P < 0.01$ compared to the control group; (*) $P < 0.05$ and (**) $P < 0.01$ compared to the D-gal group.

Several studies have shown that the immune system is also involved in age-related processes. Immune system dysfunction seems to have a key role in senescence, in agreement with the inflammation theory of aging. Recently, as immune function is a marker of health and longevity and a positive relationship has been shown between a good function of lymphocytes and longevity, the immune parameters can be considered appropriate biomarkers of biological aging. One of the most important functions of lymphocytes crucial for their role in immune responses is their proliferative capacity.[21,30,31] In the present study, we also found that D-gal resulted in a decrease of lymphocyte proliferation stimulated with or without T cell mitogen Con A in the D-gal group, compared with the control group. Lymphocyte proliferation in response to LPS, mimicking bacterial infection, was also significantly decreased in the D-gal group. In addition, IL-2 is a cytokine that is very important for lymphocyte reproduction. Decreased IL-2 production reflects a major mechanism by which immune responses are decreased with increasing aging.[32] Our data also showed that D-gal resulted in a decrease of IL-2 production. The decrease of IL-2 production and the loss of proliferative capacity could suggest that the dysfunction in immunity resulted from D-gal treatment.

In the most recent 30 years, numerous polysaccharides have been isolated from mushrooms, fungi, and plants and have been used as a source of therapeutic agents. The most promising biopharmacological activity of these biopolymers is their immunomodulation effects.[33] Our recent studies have shown that PSG-1 possessed immunostimulating activities against immunosuppression induced by cyclophosphamide in mice.[12] However, the effects involved in the PSG-1 improved immune function against immune system dysfunction induced by D-gal are not yet completely understood. In this study, when PSG-1 was ingested in D-gal-treated mice, their lymphoproliferation activities were significantly increased compared to the D-gal group. Moreover, elevation of the IL-2 level in the serum of D-gal-treated mice was also observed upon PSG-1 treatment. These data indicated that alleviating immune dysfunction induced by PSG-1 may be one of the mechanisms of its potential protective effect against aging.

Currently, dietary interventions for alleviating aging with natural dietary components have gained considerable attention because of their health benefits.[34−36] Thus, the present study investigates the effects of PSG-1 on a variety of immune functions and oxidative stress parameters related to aging in normal mice. The data showed that PSG-1 did not significantly affect these parameters compared with the control group in normal mice. As a naturally occurring dietary compound, PSG-1 may have fewer undesirable side effects. On the other hand, these interesting findings lead us to speculate that PSG-1 could maintain the body's resistance against stimuli (stress/damage), and this needs to be further studied. Whether PSG-1 prevents normal mice from aging should be determined through more experiments.

PSG-1 is a major component of *G. atrum*, which has a long history as a functional food and medicine, and can be orally (po) consumed. Although effects of ip administered PSG-1 against aging have been elucidated in the present study, it has not been investigated whether D-gal-induced aging is alleviate by po administered PSG-1 in mice. Therefore, further studies on the effects of po administered PSG-1 against aging are needed.

In conclusion, our current results suggested that PSG-1 may attenuate oxidative stress and immune dysfunction through increasing the activities of enzymatic (SOD, CAT, and GPx) and nonenzymatic (glutathione) free radical-scavenging systems and improving immune function in D-gal-treated mice. Thus, PSG-1 has potential as a novel agent to replace or augment the agent currently used to alleviate aging.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BSA, bovine serum albumin; CAT, catalase; Con A, concanavalin A; D-gal, D-galactose; ELISA, enzyme-linked immunosorbent assay; *G. atrum*, *Ganoderma atrum*; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; LPS, lipopolysaccharides; MDA, malonaldehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide; PBS, phosphate-buffered saline; PSG-1, *G. atrum* polysaccharide; ROS, reactive oxygen species; SOD, superoxide dismutase.

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