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Original Research Article

Effect of JUNCAO-cultivated *Ganoderma lucidum* spent mushroom substrate-hot water extract on immune function in mice

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Abstract

Purpose: *To evaluate the effect of JUNCAO-cultivated Ganoderma lucidum spent mushroom substrate-hot water extract (SMSG-HWE) on murine immunity.*
Methods: *Five groups of mice (n = 10) received water with 0.00, 0.14, 0.28, 0.84, or 1.68 g/kg of SWSG-HWE, respectively, orally for 30 days. Various biochemical parameters of serum and tissues, including spleen and thymus indices, were determined were determined for the mice*
Results: *The following markers were significantly higher in the 0.84 g/kg SMSG-HWE group than in the control group (all $p < 0.05$): splenic lymphocyte proliferation, a marker of cell-mediate immunity; dinitrofluorobenzene-induced delayed hypersensitivity; and the number of haemolytic plaque-forming cells, as a marker of humoral immunity. Phagocytic rate, which evaluates mononuclear-macrophage function as a marker of innate immunity, was significantly higher in both the 0.84 g/kg HWE and 1.68 g/kg SWSG-HWE groups, while phagocytic index was significantly higher in the 0.28 g/kg SWSG-HWE group, compared to the control group (all $p < 0.05$). Natural killer cell activity also was significantly enhanced in the 0.84 g/kg and 1.68 g/kg SWSG-HWE groups ($p < 0.05$).*
Conclusion: *These findings indicate that SWSG-HWE enhances murine immune function, and may be suitable as a potential additive in animal feed.*

Keywords: *Hot water extract, Cell-mediated immune function, Concanavalin A, Mononuclear-macrophage function, Haemolytic plaque, Humoral immunity, Innate immunity, Animal feed additive*

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INTRODUCTION

Ganoderma lucidum, which is known in China as Lingzhi, is highly regarded valued for its medicinal properties. *G. lucidum* has several pharmacological properties, including modulation of immune function, prevention of oxidative damage [1,2], protection of the liver, and reduction in serum glucose levels [3,4]. *G. lucidum* also exhibits anti-tumour activities [5], is associated with cell cycle arrest [6,7], induces apoptosis [8], inhibits motility and tumour growth [3,4,9], and is associated with anti-angiogenesis [10] and anti-mutagenesis [11].

Traditional cultivation of *G. lucidum* involves the

use of wood, an ecologically important and valuable resource, as a substrate. To avoid the depletion of this resource, a new method, JUNCAO technology, has been developed to cultivate *G. lucidum*. JUNCAO (in Chinese: JUN, fungus; CAO, grass) technology uses herbaceous plants, such as *Dicranopteris dichotoma* and *Pennisetum purpureum,* instead of wood to cultivate *G. lucidum* and other fungi for nutritional and medicinal purposes [12].

Lin reported that the spent mushroom substrate of *G. lucidum* (SMSG) cultivated by JUNCAO prevented and controlled piglet diarrhoea and enhanced pig immune function [13]. Here, we investigated the effect of *G. lucidum* spent mushroom substrate-hot water extract (SMSG-HWE) on murine immunity by examining differences in concanavalin A (conA)-induced splenic lymphocyte proliferation, carbon clearance, number of plaque-forming cells, serum haemolysin levels, and NK cell activity in groups of mice receiving SMSG-HWE compared to control mice. We also examined the optimal dose of SMSG-HWE for enhancing immune function.

EXPERIMENTAL

Animals

Pathogen-free BALB/c mice (females, 18 to 20 g) were procured from Shanghai Silaike Experimental Animal Company, Ltd. (Shanghai, China; animal license no. SCXK Shanghai 2007-0005). The mice underwent 1 week of acclimation to the laboratory conditions prior to treatment. Room temperature was maintained at 25 °C with 60 – 70 % relative humidity. The mice were provided with standard pellet feed and water *ad libitum*. All the animal care and protocols were approved by the Animal Care Committee of Peking University Health Center (approval no. LA2015055）. All studies were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals [14]. All procedures described below were conducted in accordance with the Inspection and Evaluation of Health Food [15].

Animal groupings

The mice were divided into four groups (Groups I - IV, respectively) containing 50 mice each. Each group was then randomly divided into five subgroups (n = 10): control (CK), Group 1 (G1), Group 2 (G2), Group 3 (G3), and Group 4 (G4). These groups received SWSG-HWE doses of 0.00, 0.14, 0.28, 0.84, and 1.68 g/kg, respectively.

Group I specimens were examined for plaque-forming cells and macrophage phagocytosis of chicken red blood cells (RBCs). Serum haemolysin assays also were performed on these specimens, and spleen and thymus indices were calculated from the extracted organs. Group II specimens were used to measure NK cell activity and conA-induced splenic lymphocyte proliferation. Group III specimens were evaluated for delayed hypersensitivity. Group IV specimens were tested for carbon clearance.

G. lucidum strain and hot water extraction

The *G. lucidum* strain used in this study was obtained from the National Engineering Research Center of JUNCAO Technology (Fuzhou, China). *G. lucidum* was cultivated using *Dicranopteris dichotoma, Miscanthus floridulus*, wheat bran, and gypsum culture media in a ratio of 38, 40, 20, and 2%, respectively. The method of hot water extraction used in this study was conducted as previously described [16]. The moisture content of the medium was approximately 60 %. The SWSG-HWE was composed of 23.58 % crude protein, 17.60 % ash, 15.79 % polysaccharide, 4.95 % amino acid, and 2.0 g/kg of crude fat, as determined by high-performance liquid chromatography.

Determination of spleen and thymus indices

Mice were sacrificed, after which the spleen and thymus were removed immediately. Spleen and thymus indices were calculated by dividing the wet weights of each organ by the body weight.

Assessment of ConA-induced splenic lymphocyte proliferation

In a sterile environment, the spleen was excised and placed into petri dishes containing sterile Hank's buffered salt solution (HBSS) (Thermo Fisher Scientific, Waltham, USA). Spleens were each separated using sterile forceps and then pressed through 200 mesh (Puluoshi, Fuzhou, China). HBSS was used to wash the resulting splenocytes, which were then centrifuged three times for 10 min each at 1,000 r/min, and assayed using an MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) Cell Proliferation and Cytotoxicity Assay Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The optical density (OD) values of the samples were measured in a microplate reader (Molecular Devices, San Francisco, USA) at 570 nm. The result was considered positive if the OD values of the samples from any of the experimental groups

G1-G4 were significantly higher than those from CK.

Delayed hypersensitivity test

On day 26 of the experiment, a 3 × 3 cm region of abdominal hair was removed from each mouse using an electric razor. The bare skin was then daubed evenly with 50 μL of dinitrofluorobenzene (DNFB) (Aladdin® China) for sensitization. On day 30, two sides of each mouse's right ear were daubed with 10 μL of DNFB. Twenty-four hours following sensitization of the right ear, each mouse was sacrificed by cervical dislocation, and both ears were cut to obtain 8-mm diameter pieces for evaluation. The difference in weight between the right and left ear was calculated. The result was considered positive if the right and left ear differences were significantly higher in any of the experimental groups G1 - G4 compared to CK.

Evaluation of serum haemolysin levels

On day 26 of the experiment, mice were inoculated with sheep red blood cells (SRBCs). On day 30, the mice were sacrificed and blood was collected. The blood was centrifuged for 10 min at 2,000 r/min, from which serum was collected and diluted with normal saline in a 1:1 ratio. One-hundred microliters of diluted serum was placed in a 96-well plate with 100 μL of 10 % SRBCs. Normal saline was used instead of diluted serum for control group specimens. The plate was incubated at 37 °C for 3 h. The result was considered positive if the amount of agglutination was significantly higher in any of the experimental groups G1-G4 compared to CK.

Splenocyte antibody production

On day 26 of the experiment, mice were inoculated with SRBCs. Mice were sacrificed by cervical dislocation on day 30, and splenocyte suspensions (3×10^6 cells/mL) were prepared as described above.

Carbon clearance test

Diluted India ink (10 mL/kg) was injected into a vein in the tail of each mouse. Then, 2 and 10 min following injection, 20 μL of blood was collected from a vein in the inner canthus, and immediately added to 2 mL of 0.1 % Na_2CO_3 solution. The OD at 600 nm was measured by a 722-Vis spectrophotometer (Jinghua, Shanghai, China) using 0.1 % Na_2CO_3 solution as the blank control. The phagocytic index was calculated after the mice were sacrificed and the spleen and thymus were removed.

Measurement of NK cell activity

A lactate dehydrogenase (LDH) release assay was used to measure NK cell activity. After washing three times with HBSS, 60 mL of routinely subcultured target cells was diluted with 6 mL of cell-counting buffer. An automatic cell counter (Thermo Fisher Scientific, Waltham, USA) was used to count the number of NK cells. NK cell activity was calculated as in Eq 1.

$$P = \{(A_1 – A_2/A_3)\}100 \text{ ……………………. } (1)$$

where A_1, A_2 and A_3 are the absorbance of the sample, target cell spontaneous release and target cell maximum release, respectively.

NK cell activity was transformed as in Eq 2.

$$X = \sin^{-1}(P)^{\frac{1}{2}} \text{ ………………………….. } (2)$$

Macrophage phagocytosis of chicken RBCs

One millilitre of 20 % chicken RBC suspension was injected intraperitoneally into each mouse. The mice were sacrificed 30 min after injection. The percentage of macrophage phagocytosis was calculated as:

$$P = \frac{N1}{N2} * 100 \text{ ……………………….} (3)$$

where N1 represents the number of macrophage phagocytosing chicken erythrocytes, and N2 represents the number of total macrophages. The percentage of phagocytosis was transformed via the following equation:

$$X=\sin^{-1}(p)^{\frac{1}{2}} \text{ ……………………….. } (4)$$

Phagocytic index (I) was computed as in Eq 3.

$$I = \frac{N3}{N4} \text{ ……………………………… } (5)$$

where N3 and N4 are the number of chicken erythrocytes and macrophages phagocytosized by chicken erythrocytes and macrophages.

Statistical analysis

The data were analysed by one-way analysis of variance using SPSS 20 (IBM, USA). Groups with equal variance were analysed by LSD, whereas groups with unequal variance were analysed by Game-Howell method. $P < 0.05$ was considered significant for all statistical comparisons.

RESULTS

Effect of SMSG-HWE on body and organ parameters

No significant differences in body weight were observed between any of the treatment groups G1-G4 and CK (data not shown). Visual inspection revealed no pathological changes (e.g., atrophy, bleeding, or swelling) in any group.

There were no significant differences in the spleen or thymus indices between any of the experimental groups G1-G4 and CK (all $p > 0.05$, Table 1). These results suggest no significant effect of SWSG-HWE on immune organ size in mice.

Table 1: Effect of SMSG-HWE on organ indices in mice

Group	Thymus index (%)	Spleen index (%)
CK	0.26±0.05	0.35±0.04
G1	0.25±0.06	0.38±0.04
G2	0.29±0.05	0.35±0.06
G3	0.29±0.03	0.35±0.04
G4	0.27±0.05	0.35±0.05

Data are reported as mean ± SD (n = 10)

Effect of SMSG-HWE on cell-mediated immune function

ConA-induced splenic lymphocyte proliferation and delayed hypersensitivity were evaluated to explore the effect of SMSG-HWE on cell-mediated immune function. A significant difference in ConA-induced splenic lymphocyte proliferation was observed between G3 (0.84 g/kg) and CK (Table 2) such that the OD was significantly higher in G3 compared to CK ($p < 0.05$). In addition, the difference in swelling between the left and right ear was significantly higher in G3 (0.84 g/kg) and G4 (1.68 g/kg) compared to CK ($p < 0.05$, Table 2), suggesting that SWSG-HWE enhances delayed hypersensitivity.

Effect of SMSG-HWE on humoral immunity function

The haemolytic plaque number was significantly higher in G3 compared to CK ($p < 0.05$, Table 3). There were no significant differences in serum haemolysin antibody levels between any of the experimental groups G1-G4 and CK ($p > 0.05$, Table 3).

Table 2: Effect of SMSG-HWE on proliferation ability of splenic lymphocytes and delayed hypersensitivity in mice

Group	Absorbance	Difference in swelling left-right ear/mg
CK	0.030±0.014	19.5±1.6
G1	0.031±0.009	20.8±1.5
G2	0.030±0.009	20.7±2.0
G3	0.045±0.026*	23.2±3.0*
G4	0.038±0.010	22.3±2.8*

Data are reported as mean ± SD (n = 10); * $p < 0.05$ compared to CK

Table 3: Effect of SMSG-HWE on haemolytic plaque number and serum haemolysin antibody levels in mice

Group	Haemolytic plaque number/10^3	Serum haemolysin antibody levels(%)
CK	3.17±0.41	60.1 ± 14.8
G1	3.52±0.43	55.3 ± 15.0
G2	3.78±0.69	61.5 ± 17.5
G3	3.84±0.40*	71.7 ± 27.2
G4	3.81±0.75	69.8 ± 26.3

Data are reported as mean ± SD (n = 10); * $p < 0.05$ compared to CK

Effect of SMSG-HWE on innate immunity

Mononuclear-macrophage function.

The phagocytic rate was calculated to evaluate macrophage function in each group. The transformed phagocytic rate was significantly higher in groups G3 and G4 compared to CK ($p < 0.05$, Table 4). In addition, the phagocytic index was significantly higher in G2, and the carbon clearance index was significantly higher in G3, compared to CK ($p < 0.05$, Table 4).

Table 4: Effect of SMSG-HWE on the ability of peritoneal macrophages to phagocytose chicken red blood cells in mice

Group	Phagocytic rate %	Transformed phagocytic rate	Phagocytic index	Carbon clearance index
CK	9.40±1.60	0.09±0.02	0.132±0.013	4.04±0.72
G1	10.05±1.21	0.10±0.01	0.142±0.018	3.82±1.21
G2	10.60±1.85	0.11±0.02	0.150±0.012*	4.27±0.69
G3	11.15±1.43	0.11±0.01*	0.147±0.026	4.79±0.75*
G4	10.85±1.56	0.11±0.02*	0.144±0.021	4.76±0.58

Data are reported as mean ± SD (n = 10); * $p < 0.05$ compared to CK

Natural killer cell activity

An LDH releasing assay was used to evaluate NK cell activity. NK cell activity was significantly higher in G3 and G4 compared to CK ($p < 0.05$, Table 5). These results suggest that 0.84 g/kg

and 1.68 g/kg SMSG-HWE have an effect on NK cell activity in mice.

Table 5: Effect of SMSG-HWE on NK cell activity in mice

Group	NK cell activity (%)	Transformed NK cell activity
CK	15.64±4.49	0.16±0.05
G1	20.30±8.37	0.21±0.09
G2	21.66±8.80	0.22±009
G3	23.18±8.21*	0.23±0.09*
G4	22.80±7.89*	0.23±0.08*

Data are reported as mean ± SD (n = 10); * $p < 0.05$ compared to CK

DISCUSSION

The immune system, which includes both the innate (non-specific) immune system and the adaptive immune system, protects an organism from pathogenic microbes and toxins. Cell-mediated immunity is typically associated with the adaptive immune system, but its function is also intertwined with that of the innate system. Cells involved in the murine immune system include macrophages, B cells, T cells, and dendritic cells. These cells can be found in the mouse spleen, and therefore the in vitro mouse spleen cell culture is a method commonly employed to study murine immune system response.

In this study, we investigated the response of cell-mediated immunity in mice following administration of SWSG-HWE. ConA is a plant mitogen known to induce T cells in mice, and is often used in murine models to evaluate T-cell proliferation. Our results showed that SWSG-HWE enhanced ConA-induced splenic proliferation in mice. We also investigated the strength of cell-mediated immunity following SWSG-HWE administration by measuring the degree of ear swelling as a measure of delayed hypersensitivity induced by DNFB. Our results indicated that cell-mediated immune function was stronger in experimental groups treated with certain doses of SWSG-HWE than in the control group.

The reticuloendothelial system, also known as the mononuclear phagocyte system, is another important part of the immune system. The degree of phagocytosis is one indicator of innate immune strength. Carbon particle clearance is commonly used to measure mononuclear phagocytosis, and the phagocytic index reflects the phagocytic function of the reticuloendothelial system. In this study, SMSG-HWE was associated with an increased phagocytic index and improved macrophage phagocytic function. SMSG-HWE also enhanced NK cell activity, the carbon clearance index, and peritoneal macrophage phagocytosis in the presence of chicken RBCs.

Like cell-mediated immunity, humoral immunity is also associated with the adaptive immune system but intertwined with the innate system. One marker of humoral immunity is serum haemolysin, which is associated with the production of antibodies. Both serum haemolysin levels and haemolytic plaque counts can be used to evaluate humoral immune function. In this study, the haemolytic plaque count was significantly higher in the group that received 0.84 g/kg SWSG-HWE, suggesting that some doses of SWSG-HWE enhance humoral immunity.

Many studies have investigated the use of spent mushroom substrate (SMS) as a feed additive [16,17]. For example, a previous study reported that the spent substrate of *Agaricus bisporus* is a potential source of roughage for ruminants [18]. SMS may be acceptable as animal feed for several ruminant species (e.g., sheep [19] and Holstein steers [20]) because it is easy to digest due to an enzymatic conversion process that occurs during cultivation. In dairy cows, SWSG-HWE may enhance immunity and antioxidant capacity, leading to improvements in milk quality and yield as well as haematological values [21,22]. In addition, previous studies have shown that polysaccharides isolated from *G. lucidum* can stimulate the immune system [23,24]. The SWSG-HWE used in this study contaied 15.79 % polysaccharides, suggesting that polysaccharides are the component within SWSG-HWE that affected murine immune function in our study.

A previous study reported increases in mouse spleen and thymus indices; serum concentrations of superoxide dismutase and catalase; and total anti-oxidative capacity in mice treated with SWSG-HWE following cyclophosphamide immunosuppression, suggesting that SWSG-HWE improves the recovery of immune function [25], which is consistent with the findings from our study. Other results from that study indicated that SWSG-HWE also can inhibit interleukin-1β, interferon-γ, and tumour necrosis factor-α concentrations in the serum of immune-deficient mice [26].

CONCLUSION

The results obtained in this study indicate that 0.84 g/kg dose of SWSG-HWE has optimal effect on all aspects of murine immune function. These

and previous findings, therefore, suggest that this optimal dose of SMSG-HWE can potential be used as a feed additive but further studies are required confirm this.

DECLARATIONS

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Conflict of interest

The authors declare no conflict of interests associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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