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Immunomodulatory effect of *Ganoderma lucidum* polysaccharide extract on peritoneal macrophage function of BALB/c mice

Yanling Hu[1,2], Zhongxiang Lin[3], Hairong Fu[1,2], Jing Xia[1,2], Wei Xiong[1,2], Guoli Li[1,2*]

[1]*Basic Medicine Department, Chongqing Three Gorges Medical College, 404120, China*

[2]*Chongqing Key Laboratory, Development and Utilization of Genuine Medicinal Materials, Three Gorges Reservoir Area, 404120, China*

[3]*Pharmacy Department, Chongqing Three Gorges Medical College, 404120, China*

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ABSTRACT

Ganoderma lucidum has been suggested as a natural immunomodulator. It is not yet clear exactly which combination of this extract is responsible for its immunomodulatory effects. Still, it appears that the 3-complement (CR3) receptor on the surface of immune cells acts as a receptor for beta-glucans (glucan-8), which is the main component of this extract. Since glucose-6-phosphate dehydrogenase (G6PD) plays a vital role in regulating macrophage functions, including nitric oxide production, we considered the effect of this extract on viability, G6PD enzyme activity, and nitric oxide (NO) production in peritoneal BALB/c macrophages. First, peritoneal macrophages of BALB/c mice were isolated and treated with concentrations (0.001, 0.01, 0.1, 1, 10, and 100 g/ml) of *Ganoderma lucidum* polysaccharide extract (GL-PS). After 24 hours of incubation by MTT test, we evaluated the viability of macrophages, and the effective dose was determined to be 0.1g/ml. To determine the specific activity of glucose-6-phosphates, they were incubated with GI-PS for 24 hours at a 0.1 mg/ml dose. Determination of protein concentration was obtained by the Bradford method in cell supernatant extract. Also, after 18 hours of incubation, the amount of nitric oxide (NO) production was measured using Grace colorimetric method. According to the results, a dose of 0.1µg/ml of Ganoderma lucidum polysaccharide extract had the most significant effect on the viability (stimulation coefficient) of peritoneal macrophages compared to other amounts ($p < 0.05$). It was also found that a dose of 0.1µg/ml GL-PS increases NO production and the specific activity of the G6PD enzyme ($p < 0.05$). *Ganoderma lucidum*, a medicinal fungus, is widely used in East Asian countries, especially in China, to increase the quality of life and longevity. After this study, we concluded that GL-PS extract has an immunomodulatory effect on macrophage activity. Therefore, the polysaccharide extract of this fungus can be used as a strengthening agent of the phagocytic system against infectious agents and pathogens such as the Leishmania parasite because of the production of nitric oxide by macrophages plays an essential role in defense against them.

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Introduction

Ganoderma lucidum (GL-PS) is one of the basidiomycetes and belongs to the Ganodermataceae subspecies of the aphilophore species (1). Its aqueous polysaccharide extract is widely used in East Asian countries, especially in China, (as traditional medicine) to prevent diseases such as hypertension, bronchitis, arthritis, nephritis, gastric ulcer, genetic tumor disease and scleroderma (2).

Ganoderma lucidum also has effects such as fibrotic, lowering cholesterol, and lowering blood sugar (3). The major carbohydrate epitope responsible for its antitumor activity and its cell surface receptor has not yet been fully elucidated. *Ganoderma lucidum* seems very safe because oral administration of its extract has no effect. Macrophages are immune cells that play an essential role in defense against various infectious agents (2). The bactericidal mechanisms of macrophages mainly involve the production of reactive oxygen radicals and the production of nitric oxide (NO) (4, 5). However, various studies have shown that nitric oxide production is the most important leishmaniasis mechanism of infected macrophages (6). The metabolic pathway of pentose phosphate, which is present in most cells, is the most important source of NADPH production within cells. NADPH produced by coenzyme A is the activity of many essential enzymes, including the enzyme complex nitric oxide synthase (NOS) within macrophages (7).

Glucose-6-phosphate dehydrogenase (G6PD) is a rate-limiting enzyme and the most critical enzyme in

*Corresponding author. E-mail: liliguo2022w@yeah.net

this metabolic pathway (8). Previous studies have also shown that people with G6PD deficiency have an increased risk of various infections, including Toxoplasma, Rickettsia, and Helicobacter pylori, as well as the severity of the disease (9). In this study, the immunomodulatory effect of a polysaccharide extract of *Ganoderma lucidum* on viability, the specific activity of the G6PD enzyme and nitric oxide production by BALB/c mouse macrophages were investigated.

Materials and methods

Isolation of peritoneal macrophages of BALB/c mice

Female BALB/c mice (8-9 weeks old) were used to isolate peritoneal macrophages. RPMI was first injected into the mouse peritoneum, and then the mouse peritoneal fluid was aspirated under sterile conditions. The aspirated cell suspension was washed twice with PBS, and after centrifuging the cells at 300 g for 10 minutes, the ruby fluid was discarded, and the cell mass at the bottom of the tube was suspended in 1ml RPMI containing FCS. Macrophages were counted to 1×10^6, and their viability was determined and used in various tests. All tests were performed in triplicate (n = 3) and repeated at least three times.

MTT test

In the early 1980s, Mosmann described this method (10). In each 96-well microplate well, 100µl of cell suspension obtained from mouse peritoneum was added, and macrophages were in separate groups with different concentrations (0.001, 0.01, 0.1, 1, 10, and 100 g/ml) of *Ganoderma lucidum* polysaccharide extract (GL-PS) were treated.

Then microplate was placed for 24 hours at 37°C and 5% CO_2. After this period, the percentage of viability (stimulation coefficient) of macrophages was determined by the MTT test. A solution containing MTT was prepared at 5 mg/ml concentration, and 25µl was added to each well. After 4 hours of incubation at 37°C, 100µl of dimethyl sulfoxide (DMSO) was added to all wells and mixed thoroughly to dissolve all formed aqueous crystals. Not read) was read by Multiscan MS ELISA reader at 570 nm and was reported as the excitation coefficient. The excitation index for the group treated with activating substances and the cytotoxicity percentage for the treated group was calculated from the following formulas:

$$SI = \frac{Absorption\ rate\ of\ stimulated\ cells}{Absorption\ rate\ of\ unstimulated\ cells\ (control\ group)}$$

Nitric oxide (NO) concentration measurement

For this test, macrophages were treated with an adequate concentration of 0.1 g/ml polysaccharide extract of *Ganoderma lucidum* (GL-PS). SNAP-treated macrophages boosting nitric oxide + IFN-ɣ and IFN-ɣ + LPS pathways were used as positive controls because these substances have been shown to stimulate NO production by macrophages. NMMA-treated macrophages were also used as a negative control because it inhibits NO production. The amount of NO_2 accumulation as an indicator of NO production in the supernatant of cultured cells was determined by the "Grace" colorimetric method and using the standard curve of sodium nitrite. 50µl of supernatant of macrophages cultured with 50µl of the solution containing ethylene amide Dihydrochloride (0.1 mg/ml), sulfanilamide (1 mg/ml), 5% phosphoric acid, and distilled water were incubated at room temperature for 10 minutes. The sample absorbance was read at 540 nm.

Determination of G6PD enzyme activity

To measure the specific activity of the G6PD enzyme (specific activity in terms of U/mg), mouse peritoneal macrophages were poured into 5×10^5 cells in each 96-well microplate well for 24 hours at an effective concentration of 0.1 g/ml of *Ganoderma lucidum* polysaccharide extract. The microplate was then exposed to 37°C and 5% CO_2 for 24 hours.

After this period, the macrophages were isolated from the bottom plate, and the activity of the G6PD enzyme in the cell extract of the lysed macrophages was determined. For this purpose, peritoneal macrophages isolated from the bottom of each well were centrifuged (200 g for 10 minutes at 4°C) and then suspended in PBS and intervals for lubrication under sonication (1-min times, 10-s burst). The cell extract was centrifuged for 12 minutes at 12,000 g at 20°C for 20 minutes and kept on ice until assayed. 200µl of cell extract with 0.75 ml of Tris-5 M hydrochloric acid buffer containing 3mM $MgCl_2$ (pH 8.7), 25µl of 4.7µM glucose-6-phosphate, and 7.8µM NADPH were mixed and increased NADPH uptake

after 5 minutes of incubation at 37°C was read at 339nm. The concentration of cellular protein in 50µl of macrophage cell extract was determined by the Bradford method, and the specific activity of the enzyme was obtained (11). The test results were analyzed using SPSS software and ANOVA statistical analysis.

Results and discussion

Results of MTT test

As shown in Table 1, the results of the MTT test on macrophages treated with different concentrations of a polysaccharide extract of *Ganoderma lucidum* (GL-PS) Showed that all these doses significantly increased the viability (stimulation coefficient) of macrophages ($p < 0.05$). But the dose of 0.1 g/ml of a polysaccharide extract of *Ganoderma lucidum* (GL-PS) had the most effect ($p < 0.05$) (Figure 1).

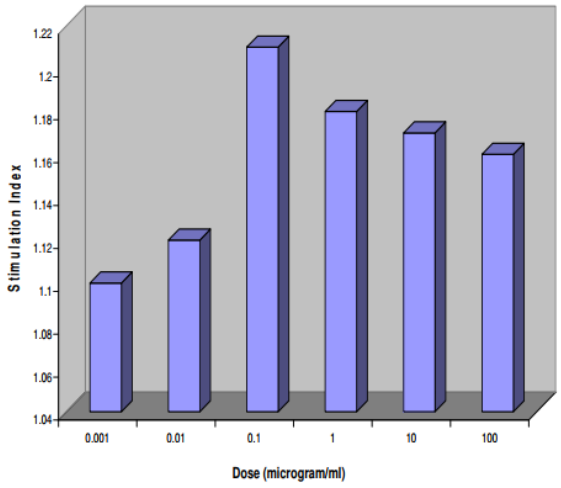

Figure 1. Results of the MTT test on peritoneal macrophages in mice after 24 hours of treatment with a dose of 0.1µg/ml of GL-PS

Results of nitric oxide (NO) concentration measurement

The results of measuring the amount of nitric oxide produced by macrophages treated with 0.1 mg/ml polysaccharide extract of *Ganoderma lucidum* (GL-PS) after 18 hours showed that treatment of macrophages with this extract significantly increased the production of nitric oxide by macrophages compared to the control group (untreated macrophages alone) ($p < 0.05$) (Figure 2).

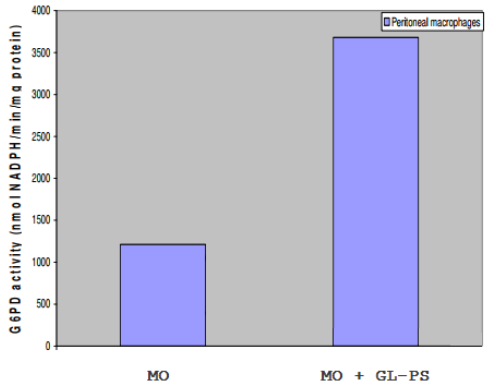

Figure 2. Nitrite production (in micro-molar) by macrophages after 18 hours of incubation with macrophage treatment at a dose of 0.1µg/ml of GL-PS

Results of determining the activity of the G6PD enzyme

Measurement of G6PD enzyme activity in lysis extract of macrophages treated with 0.1 g / ml polysaccharide extract of Ganoderma lucidum showed that the use of this extract significantly increased the activity of G6PD enzyme in treated macrophages compared to the control group ($0.05 > p$) (Figure 3).

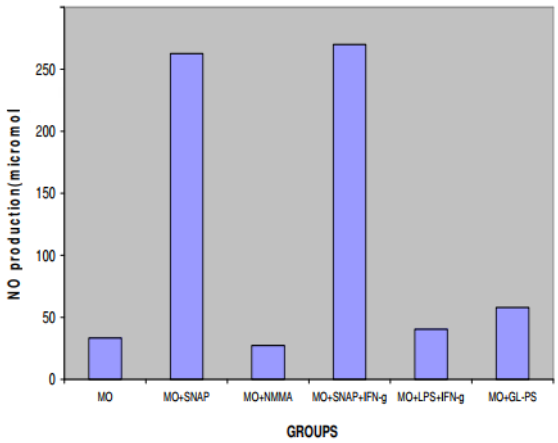

Figure 3. G6PD enzyme activity (specific activity) in peritoneal macrophages of BALB/c mice treated with 0.1 mg/ml of MO GL-PS, macrophage: MO + GL-PS macrophage + 0.1µg/ml of GL-PS

Ganoderma lucidum is a fungus that is widely used as a medicinal fungus in various eastern countries, especially in China, to increase the quality of life and longevity (6). Numerous studies have shown that cultured mycelium and *Ganoderma lucidum* spores are very effective in treating hepatopathy and neoplasia (12-14). One of the reasons that scientists have considered this fungus is that its polysaccharides have anti-tumor effects (15).

Considerable evidence has shown that D-B-glucan obtained from this fungus can induce biological responses in complement immune cells by binding to a complement type 3 membrane receptor (CR3, integrin aMb2, or CD11b/ CD18) (16). The discovery of the specific receptor through which these compounds exert their effects has opened new perspectives for future research (17).

Macrophages are immune cells that play an essential role in defense against various infectious agents. The microbial mechanisms of macrophages mainly involve the production of reactive oxygen radicals and the production of nitric oxide (NO) (18). The metabolic pathway of pentose phosphate (which is present in most cells) is the most important source of extracellular NADPH is intracellular NADPH (19). Coenzyme NADPH is produced by the activity of many vital enzymes, including the nitric oxide synthase (NOS) complex within macrophages. Glucose-6-phosphate dehydrogenase (G6PD) is a rate-limiting enzyme and the most critical enzyme in this metabolic pathway. NADPH is produced in cells (20).

In this study, an attempt was made to investigate the effect of increasing the activity of the pentose phosphate pathway (NADPH production rate), especially glucose-6-phosphate dehydrogenase enzyme, which is the enzyme controlling this metabolic pathway, in the production of nitric oxide by macrophages. In previous studies, the effect of G6PD deficiency has arrived on increasing susceptibility and severity of various infectious diseases, including rickettsiae (9), *Acinetobacter pneumonia* (21, 22), Toxoplasma infection (23), and *Helicobacter pylori* infection (24). It has also been shown that the leukocytes of these individuals have a disorder in the process of killing infectious agents and the production of active oxygen and nitrogen radicals in them is less than average (25). In this study, after macrophage treatment, we investigated the effect of this extract by MTT assay, nitric oxide production, and G6PD activity.

In this study, we investigated the stimulatory effect of different concentrations on macrophages and immune system cells by MTT assay in vitro. As the results of the MTT test showed, all concentrations used GL-PS significantly increased the viability and activity (stimulation coefficient) of mouse peritoneal macrophages and BALB/c, and a concentration of 0.1 g / ml of GL PS (effective dose) has the most significant effect. Examination of 0.1 mg/ml of GL-PS on treated macrophages revealed that this dose increased NADPH production, G6PD enzyme activity, and nitric oxide production in peritoneal macrophages. Therefore, because NADPH is produced, it is an essential coenzyme of enzymes that produce active oxygen and nitrogen radicals, including superoxide and nitric oxide in macrophages, which play a crucial role in defending infected macrophages against pathogens by increasing G6PD enzyme activity. NADPH levels and increased nitric oxide production increase the defense of macrophages against pathogens.

Conclusions

This study showed that GL-PS extract has strong immunomodulatory properties and stimulates the production of nitric oxide and thus the microbial activity of macrophages. This finding is consistent with many of its therapeutic effects. Most studies have reported that GL-PS can induce a strong protective immune response against disease progression. Further research is needed to investigate the impact of GL-PS on cytokine production patterns, lymphocyte subtypes, and intracellular signal transmission to determine this fungus's exact mechanism of action. Our research shows that this medicinal fungus can be beneficial for preventing and treating the disease in patients with defective immune systems.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

Project

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