Research Article

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Immunoregulatory function of *Dictyophora echinovolvata* spore polysaccharides in immunocompromised mice induced by cyclophosphamide

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**Abstract:** The purpose of this study was to investigate whether the *Dictyophora echinovolvata* spore polysaccharides (DESP) affect the immunity in immunocompromised mice induced by cyclophosphamide (CTX). The healthy female Kunming mice were randomly divided into six groups, including a normal control (NC) group, a positive control group, a model control (MC) group, and three groups treated with low-, intermediate-, and high-dose polysaccharide, respectively. A series of immunoregulatory properties were determined, including humoral and cellular immunity, immune function, and immune factors of mononuclear macrophages. Compared with NC and MC groups, treatment with DESP significantly increased the spleen index and decreased the thymus index; increased the serum concentrations of immunoglobulin (Ig)A, IgG, IgM, hemolysin, IL-1β, and IL-2; delayed the allergic reaction; and improved the splenic lymphocyte transformation ability; and enhanced the phagocytosis of macrophages and the ability to secrete IL-6, TNF-α, caspase-1, and NO with DESP supplementation. These results indicated that DESP might have a good regulatory effect on CTX-induced immunodeficiency in mice, adjust the body’s immune imbalance, and improve the symptoms of low immunity.

**Keywords:** immunomodulatory activity, *Dictyophora echinovolvata*, spore polysaccharides

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**1 Introduction**

*Dictyophora indusiata* is a stinkhorn fungus that belongs to Eumycota, Basidiomycotina, Basidiomycetes, Phallales, and Phallaceae families [1,2]. It is edible as a delicacy in China, has a long history [3], and is called “queen of the mushrooms” due to its beautiful appearance and delicious taste [4]. This mushroom is also rich in vitamins and micro minerals, has a lot of health benefits to the eyes, acts as a tonic to cardiovascular systems, and shows partially medicinal effects like mental tranquilization and antitumor activity [5–8]. Polysaccharides are a kind of natural polymer linked by aldose or ketose through glycosidic bonds. As one of the fundamental substances to maintain the normal function of life, polysaccharides are essential biological macromolecules in vivo [1,9].

The pileus of *D. indusiata* is covered with olive green mucus, which contains the basidiospore that includes all germplasm of *D. indusiata*. The polysaccharides from the volva of *Dictyophora rubrovolvata* were extracted by hot water, and one of them had an inhibitory effect on the tumor cells of S180 to some extent [6]. As in the previous study, *D. indusiata* polysaccharide could inhibit the immunosuppressive function of cancer-associated fibroblasts [10]. *Dictyophora echinovolvata* Zang is one of the species of *D. indusiata*. At present, it has been the most important commercialized species that occupy most of the market shares of *D. indusiata* in China due to its easy cultivation, high nutritional value, and biological function [11]. The mature fruiting body of *D. indusiata* can be divided into three parts: the pileus covered with spores, the volva, and the edible part, which consists of the stipe and white net-like veil [2]. The edible part of the fruiting body of *D. indusiata* is very delicious and expensive. The volva and pileus that account for 65% of the whole mushroom (by fresh weight) are dumped without use and may cause environmental pollution and resource
wastage [12]. The new study has reported that water extractable polysaccharides by *D. indusiata* play a vital role in the process of antioxidation, hepatic-, and renal protection on obese mice [13]. In addition, previous research also provided the similar evidences associated with the polysaccharide such as *Lonicera japonica* and *Ganoderma lucidum* [14–18]. *L. japonica* polysaccharide was used to analyze the immune regulation function in immunosuppressed mice induced by cyclophosphamide (CTX) in order to better develop this Chinese herbal medicine plant [15]. Other studies have reported that the spores of *G. lucidum* were confirmed to have various biological functions [16–18]. These results indicated that the nutritional and biological functions of this abundant waste needed to be thoroughly evaluated to realize the highest possible economical profit [19,20].

However, the spores of *D. indusiata* polysaccharides associated with the immunity regulated function are not clean, especially *D. echinovolvata*. Previous results have shown that the crude polysaccharide fraction of edible *D. echinovolvata* might be a potential function for preventing neurodegenerative diseases in which oxidative stress and apoptosis were involved [21]. In our lab, we isolated and characterized polysaccharides from *D. echinovolvata* spore. The objective of this study was to conduct a qualitative analysis of *D. echinovolvata* spore polysaccharides (DESP) and explore and investigate its immunomodulatory function in CTX-induced immunosuppressed mice models. The ameliorative effects of DESP were estimated by organ index, splenic lymphocyte proliferation, macrophage phagocytosis, and serum cytokines.

### 2 Materials and methods

#### 2.1 Polysaccharides and reagents

DESP were prepared in the laboratory. The fruiting body of *D. echinovolvata* was purchased from a local commercial market in Shunchang, Fujian, China. The homogenate of *D. echinovolvata* gelatin was boiled in distilled water for 2 h at a designed temperature. After centrifugation to remove debris fragments, the supernatant was concentrated by rotary evaporation. Protein was removed and collected as the crude polysaccharide fraction of *D. echinovolvata* gelatin. The gelatin was obtained through precipitation with four volumes of 95% ethanol, centrifugation, and freeze-drying.

CTX and levamisole hydrochloride were obtained from Baxter International Co., Ltd. and 20% sheep red blood cell (SRBC) was obtained from Nanjing Senbega Biotechnology Co., Ltd. The fetal bovine serum was obtained from Zhejiang Tianhang Biotechnology Co., Ltd.; RPMI-1640 medium is the product of GE Medical Life Science Co., Ltd.; NP-40, knife bean globulin (ConA) is a Sigma product; Hank’s solution, MTT kit, and neutral gum are products of Beijing Solebo Technology Co., Ltd., whereas Penicillin (10,000 U/mL) and streptomycin (10,000 μg/L), 1:1 (v/v) acetone–methanol fixing solution, 4% (v/v) Giemsa-phosphate buffer, 0.1% sodium carbonate solution, heparin, and ink were prepared by us. Methanol, acetone, sodium carbonate, Giemsa dye solution, and heparin were obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd.

#### 2.2 Experimental design and samples

Female, 7-week-old Kunming mice were obtained from SLAC Laboratory Animal Company (Shanghai, China, certificate no.: CXK [Shanghai] 2003-0003). After 1 week of acclimatization to the animal laboratory with drinking and feeding freely, all mice were randomly assigned to six groups, including a normal control (NC) group, a positive control (PC) group, a model control (MC) group, and three groups treated with DESP (low-, intermediate-, and high-dose; 400, 800, and 1,600 mg/kg day, respectively; LP, MP, and HP) for 4 months. The PC group was given levamisole hydrochloric acid with 25 mg/kg day intragastrically. The NC and MC groups were given saline intragastrically. The gavage volume of mice in each group was 0.1 mL (10 g/bw). On the 21st day, except for the NC group, all mice were intraperitoneally injected with 50 mg/kg day CTX for 1 week to develop low immunity. The mice in the NC group were injected with the same amount of saline. The mice were fed under controlled environmental conditions (temperature: 21–23°C; humidity: 40–60%) and a 12 h light/dark cycle.

**Ethical approval:** The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals and were approved by the Fujian Provincial Animal Care and Use Committee and the Fujian province Zoological Society.

#### 2.3 Organ index

At the end of the experimental period, the mice, fasted without water for 12 h, were weighed and sacrificed after
cervical dislocation. The spleen, thymus, and liver samples were collected, and excess fascia and adipose tissue were removed simultaneously. Then, the organs were weighed, and the immune organ index was calculated according to the following formula:

\[
\text{organ index} \% = \frac{\text{organ weight (spleen, thymus, liver)}}{\text{body weight}} \times 100\%.
\]

### 2.4 Delayed allergic reaction

After lavage 24 days in mice, they received intraperitoneal injections of 0.2 mL of 5% (v/v) SRBC. The thickness of the metatarsal part of the left posterior foot of mice was measured by a thickness gauge 4 days after immunization with 5% (v/v) SRBC. The average value was obtained by measuring the thickness of the metatarsal part of the left posterior foot of mice three times. Then, 20% of 20 μL (v/v) SRBC was subcutaneously injected at the measurement site. The thickness of the left posterior metatarsal was measured 24 h after injection. The same measurement was made three times, and the average was taken. Mice vola pedis thickening is equal to the average thickness of the second vola pedis minus the average thickness of the first vola pedis.

### 2.5 Splenic lymphocyte transformation experiment

The mice were sacrificed through cervical dislocation, and they soaked in 75% alcohol for 5 min. The spleen was exposed through an abdominal incision and then isolated, collected, and put into a petri dish containing 5 mL Hank’s solution. Subsequently, the spleen was placed on a 200-mesh stainless steel net and placed in a small dish containing the proper amount of aseptic Hank’s solution to make a single-cell suspension. After filtration with a 200-mesh screen, the suspension was washed with Hank’s solution three times. The target cell concentration was achieved by diluting to 1 \times 10^6 cell/mL in RPMI-1640 medium containing 10% fetal bovine serum. The cell suspension 200 μL/well was added to the 96-well cell culture plate. Each splenic cell suspension was divided into experimental group and control group. The ConA solution (final concentration 5 μg/mL) was used in the experimental group. They were cultured in an incubator at 37°C with 5% CO₂ for 48 h. The 110 μL of DMSO was added into the cell to terminate reaction. The OD value was measured by an enzyme labeling instrument at 570 nm. The proliferation capacity of lymphocyte A is equal to the average OD value of the experimental group minus the average OD value of the control group.

### 2.6 Serum hemolysin and cytokine determination

The concentration of serum hemolysin, immunoglobulin (Ig) A, G, and M, interleukin-1β, and interleukin-2 was determined by ELISA kit (Langton, Shanghai, China) according to the manufacturer’s instruction.

### 2.7 Macrophage phagocytosis

The blood was collected from the inferior pterygoid vein of a chicken with an aseptic blood collection needle for obtaining chicken red blood cells (CRBCs). Also, 2% (v/v) CRBC suspension was prepared with normal saline after centrifuging at 1,000 rpm for 10 min. Mice were intraperitoneally injected with 2% CRBC suspension. After 30 min, the mice were sacrificed by cervical dislocation, and the peritoneal macrophages after intraperitoneal injection of 2 mL of normal saline were collected. Peritoneal macrophages were prepared as described in previous studies [22,23]. After dying in Giemsa-phosphate buffer for 3 min, the number of semi-swallowed red blood cells, total phagocytes, and total macrophages were counted using an optical microscope. The phagocytosis index and phagocytosis rate were calculated based on the formula. The percentage of phagocytosis (%) and tosis index were analyzed by the following formula: the percentage of phagocytosis = \( \frac{N_1}{N_2} \) and the tosis index = \( \frac{N_1}{N_2} \), where \( N_1 \) is the number of macrophages that engulf CRBCs, \( N_2 \) is the number of macrophages and total CRBCs consumed, and \( N_2 \) is the number of macrophages.

### 2.8 Carbon clearance test

A total of 0.2 mL ink (diluted with saline at 1:4) was injected intravenously into the tail vein of mice. After inoculating ink, blood was sampled and put into a heparin tube from the eyeball of mice at 5 and 18 min, respectively. Then, 20 μL of blood samples was added into 2 mL of 0.1% sodium carbonate solution with a tube for accuracy and analyzed in a spectrophotometer at 600 nm. Finally, the mice were killed by cervical dislocation, and the spleen and liver samples were collected. They were weighed.
and recorded accurately. The carbon clearance index $\alpha$ was calculated according to the following formula:

$$K = \frac{\lg OD_1 - \lg OD_2}{t_1 - t_2},$$

$$\text{Carbon clearance index } \alpha = \frac{W_1}{W_2} \times \sqrt[3]{K},$$

where $W_1$ is the body weight and $W_2$ is the weight of liver and spleen.

### 2.9 Data analysis

The experimental data were analyzed by the least significant difference model of ANOVA with SPSS 20.0 software. Each repetition was used as a statistical unit of the experiment, and the results were expressed as mean ± SEM.

### 3 Results and discussion

#### 3.1 Effects on body weight and organ index in immunocompromised mice

As shown in Figure 1, compared with the NC group, the bodyweight of mice was significantly increased ($P < 0.05$) in the PC group and the MP group ($P < 0.05$). The results showed that the treatment of the middle-dose DESP and levamisole hydrochloric acid could achieve the same effect, which further indicated that the treatment of the middle-dose DESP had the effect of promoting immunity similar to that of levamisole hydrochloric acid.

As shown in Figure 2, compared with the NC group, the liver index in the MP group was significantly higher than that of the other groups ($P < 0.05$); the spleen index was significantly decreased in the MC, PC, LP, MP, and HP groups ($P < 0.001$ or $P < 0.001$). These results indicated that the immunocompromised mice model was successfully constructed. Compared with MC and PC groups, the spleen index in the LP, MP, and HP groups was significantly increased ($P < 0.05$ or $P < 0.01$). Meanwhile, the thymus index was also significantly increased in the group treated with the middle-dose DESP ($P < 0.05$). These results suggested that DESP intragastrically could increase the weight of important immune organs like the spleen and thymus gland in mice, thereby affecting the immune function. Although DESP treatment increased the index of the spleen and thymus, the organ atrophy caused by low immunity was irreversible. Thymus and spleen are the main immunization organs. The spleen is the largest peripheral immune organ innervated with sympathetic nerves and controlled by the adrenomedullary system in the body [24]. A bigger immunity index indicates a stronger immune
capability [25]. The results of the thymus index and spleen index indicated that DESP could enhance the cell-mediated immunity and stimulate T cell formation [26].

### 3.2 Effect of DESP on cellular immune function in immunocompromised mice

As shown in Figure 3a, compared with the NC group, the rate of swelling in the hind paw was significantly reduced ($P < 0.01$ or $P < 0.001$). Compared with the MC group, the rate of swelling in the hind paw in the PC group and the MP group was significantly increased ($P < 0.05$), which further indicated that the middle-dose DESP treatment could improve the immunity of immunocompromised mice. Figure 3b shows that the DESP treatment could significantly increase the proliferation and transformation of lymphocytes in mice, especially in the MP group ($P < 0.001$). These results suggest that an intermediate dose of DESP can improve the delayed allergic reaction and the splenic lymphocyte transformation ability and improve the immunity of immunocompromised mice. Previous studies have shown that splenic lymphocyte has an important role during the process of cell-mediated immunity based on the CD4$^+$, CD8$^-$, and invariant natural killer T (iNKT) cell [27–29]. The iNKT cells are a distinct population of innate T lymphocytes selected in the thymus [30,31]. They have an important function of rapidly secreting cytokines and an innate T cell population capable of activating and steering adaptive immune responses [28]. Previous reports have shown that CTX suppressed both humoral and cellular immune responses [32]. The present study showed that treatment with DESP could significantly increase the splenic lymphocyte transformation ability (Figure 3b). This result may suggest that DESP may be capable of improving immunity.

### 3.3 Effects of DESP on the humoral and cellular immune factor

As shown in Figure 4, compared with the NC group, the serum concentrations of IgA, IgG, and IgM in the MC, MP, and LP groups were significantly or immensely significantly increased ($P < 0.05$ or $P < 0.001$), but they were significantly decreased ($P < 0.001$) in the PC and HP groups. Compared with the MC group, the serum contents of IgA in MP and LP show a downward trend. Compared with the PC group, the serum contents of IgA, IgG, and IgM in MP and LP groups were significantly decreased ($P < 0.001$). However, the serum contents of IgA in MP and LP show a downward trend. Compared with the PC group, the serum contents of IgA, IgG, and IgM in MP and LP groups were significantly increased ($P < 0.05$ or $P < 0.01$). Based on the present results, they suggested that the intermediate- and low-dose of DESP can stimulate the body to produce higher concentrations of Ig, especially the intermediate-dose of DESP treatment. Immune deficiencies appear to exhibit antibody production deficiencies [33]. In some cases, Ig replacement treatment is the most effective treatment in primary immunodeficiency diseases [34–36]. In the present study, DESP treatment significantly increased IgA, IgG, and IgM concentration, and we speculate that DESP can effectively alleviate the damage of immune deficiency to protect immunocompromised mice.

CTX treatment reduced the concentration of hemolysin, IL-1β, and IL-2 significantly in the MC group,
compared with the NC group, as shown in Figure 4d–f (P < 0.05 or P < 0.01). Compared with NC and MC groups, the contents of hemolysin, IL-1β, and IL-2 were significantly increased (P < 0.01 or P < 0.001) in the MP and LP groups. Compared with the PC group, the concentrations of hemolysin, IL-1β, and IL-2 were significantly increased (P < 0.001) in the MP and LP groups. Hemolysin as an index was used to evaluate the body's immune function by the degree of aggregation of SRBC. IL-1β is produced by activated macrophages, belonging to a cytokine type that can stimulate the proliferation and differentiation of immune response cells and improve its function [8,27]. IL-2 can affect the immune system by affecting T cells and promoting the proliferation of activated B cells, participating in antibody response, detecting tumor and hemato poiesis [29,37]. In addition, the previous results have shown that the D. echinovolvata polysaccharide could inhibit the mitochondria-dependent apoptotic pathway to protect against H2O2-induced neurotoxicity in PC12 cells [21]. Apoptosis and oxidative stress are important features of chronic diseases, including neurodegenerative disorders and immunocompromised individuals [38].

The results showed that the hemolysin in serum of immunocompromised mice induced by CTX was significantly decreased after SRBC injection, indicating that the activity of B cells in mice was inhibited. After intermediate- and low-dose DESP treatment, the hemolysin was significantly increased, which effectively increased the proliferation and antibody production of B cells in mice. In addition, intermediate- and low-dose DESP treatment can significantly increase the contents of IL-1β and IL-2 in immunocompromised mice, suggesting that DESP treatment may promote the activity of macrophages and proliferation of B cells in vivo, improve the immune defense ability, and prevent various chronic diseases.

### 3.4 Effects of DESP on function of macrophages

As shown in Figure 5a and b, the phagocytic index and phagocytic efficiency of peritoneal macrophages in MC, PC, HP, and LP groups were significantly decreased
but the phagocytic index of peritoneal macrophages in the MP group was significantly increased ($P < 0.05$) compared with the NC group. In the DESP treatment groups, the phagocytic index and phagocytic efficiency of peritoneal macrophages were significantly higher than those in the MC and PC group ($P < 0.001$ or $P < 0.001$), only in the MP group. This indicated that middle-dose DESP could significantly increase the phagocytic activity of peritoneal macrophages in immunocompromised mice. As shown in Figure 5c, compared with the NC group, the
carbon clearance ability was significantly decreased ($P < 0.01$, $P < 0.001$) in the MC and PC groups. DESP treatment can increase the carbon clearance ability of immunocompromised mice as the intermediate-dose DESP treatment significantly increased in particular ($P < 0.01$). Compared with MC and PC groups, the carbon clearance ability of immunocompromised mice was significantly improved with the three doses of DESP treatment ($P < 0.001$ or $P < 0.001$). This result indicated that DESP could significantly improve the phagocytosis of macrophages in immunocompromised mice and suggested that DESP treatment may improve the nonspecific immune ability of immunocompromised mice. Previous immunological assays showed that *D. indusiata* acid-soluble polysaccharides could improve phagocytosis of monocytes [7]. Thus, based on the present results, DESP could be regulating the auxiliary cell ability in the specific immune system.

As shown in Figure 6, treatment with intermediate- and low-dose DESP significantly increased the contents of IL-6, TNF-α, caspase-1, and NO secreted by peritoneal macrophages compared with NC and PC groups ($P < 0.001$). Compared with the MC group, the contents of IL-6, TNF-α, and caspase-1 secreted by peritoneal macrophages in the HP, MP, and LP groups were significantly decreased ($P < 0.001$ or $P < 0.001$). Cytokines as a signal transduction molecule between cells can regulate immune response and participate in immune cell differentiation development [39]. As shown in Figure 4f, treatment with intermediate- and low-dose DESP recovered the amount of IL-2 compared with the MC group ($P < 0.001$), rather than high-dose DESP. IL-2 as a cell growth factor promotes cell proliferation and differentiation, which can induce interferon production, which is involved in the process of autoimmune reactions [40]. TNF-α stimulates the expression of immune mediators and plays a significant role in the host defense [41]. As in previous results, the macrophages are broadly classified into M1 macrophages and M2 macrophages, which have selective anti-inflammatory, pro-fibrotic activities, and induced immunotolerance [42]. Usually, the M1 macrophages have an important ability to secrete cytokines, such as IL-1β, TNF, and IL-6, and activate the inducible nitric oxide synthase generating NO [43]. Notably, the lipopolysaccharide stimulation, indeed, can induce the metabolism of arginine to NO [44]. Based on the present results, DESP had a positive regulatory effect on CTX-induced immunodeficiency in mice. The concentration of hemolysin, IL-2, TNF-α, and NO in the MP and LP groups was higher than the levels in the control group. This result shows that DESP can adjust the body’s immune imbalance and improve the symptoms of low immunity.

### 4 Discussion

The experimental results showed that different doses of DESP could change humoral immunity, cellular immunity, and nonspecific immune function of immunocompromised mice. Among them, treatment with intermediate-dose DESP significantly improved the function of macrophages. The main points are stated as follows. (1) DESP improves liver and thymus organ indexes in immunocompromised mice. (2) DESP significantly improves the delayed allergic reaction and the ability of proliferation and transformation of lymphocytes in immunocompromised mice, indicating that DESP could significantly improve the cellular immune function. (3) Intermediate-dose DESP treatment could significantly increase the phagocytic activity and carbon clearance ability of peritoneal macrophages in immunocompromised mice, increase the speed of blood carbon clearance, and increase the contents of IL-6, TNF-α, caspase-1, and NO secreted by peritoneal macrophages, which indicated that DESP significantly improves the nonspecific immune ability of immunocompromised mice. Overall, the present results suggested that DESP can be developed as a potential health care product that can enhance immunity. However, the specific molecular mechanism of DESP treatment to improve the immune ability of immunocompromised mice needs to be further studied.

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**Data availability statement:** The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.
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