Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2015, **13**(10): 0760−0766
Chinese Journal of Natural Medicines

Pleurotus nebrodensis polysaccharide (PN-S) enhances the immunity of immunosuppressed mice

CUI Hai-Yan, WANG Chang-Lu*, WANG Yu-Rong, LI Zhen-Jing, CHEN Mian-Hua, LI Feng-Juan, SUN Yan-Ping

Key Laboratory of Food Nutrition and Safety, Ministry of Education, School of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China

Available online 20 Oct., 2015

[ABSTRACT] In the present study, the effects of *Pleurotus nebrodensis* polysaccharide (PN-S) on the immune functions of immunosuppressed mice were determined. The immunosuppressed mouse model was established by treating the mice with cyclophosphamide (40 mg/kg/2d, CY) through intraperitoneal injection. The results showed that PN-S administration significantly reversed the CY-induced weight loss, increased the thymic and splenic indices, and promoted proliferation of T lymphocyte, B lymphocyte, and macrophages. PN-S also enhanced the activity of natural killer cells and increased the immunoglobulin M (IgM) and immunoglobulin G (IgG) levels in the serum. In addition, PN-S treatment significantly increased the phagocytic activity of mouse peritoneal macrophages. PN-S also increased the levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), and nitric oxide (NOS) in splenocytes. qRT-PCR results also indicated that PN-S increased the mRNA expression of IL-6, TNF-α, INF-γ, and nitric oxide synthase (iNOS) in the splenocytes. These results suggest that PN-S treatment enhances the immune function of immunosuppressed mice. This study may provide a basis for the application of this fungus in adjacent immunopotentiating therapy against cancer and in the treatment of chemotherapy-induced immunosuppression.

[KEY WORDS] Immunosuppressed mice; *Pleurotus nebrodensis* polysaccharide; Cyclophosphamide

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2015)10-0760-07

Introduction

The value of polysaccharides and derivatives in food, agriculture, and medicine has been well documented [1-2]. Polysaccharides offer health benefits, such as lowering blood cholesterol and blood pressure and protecting against infections and inflammation [3]. Dietary polysaccharides elicit diverse immunomodulatory effects in animal tissues, including blood, gastrointestinal tract, and spleen [4-5]. Polysaccharide intake stimulates the immune system and improves survival in cancer patients [6]. *Pleurotus nebrodensis* (Inzenga) Quél. (Pleurotaceae), known as Funcia di basilicu, is a critically endangered fungus native to China, Southern Europe, and Central Asia. The hot water extract and dry powder of the fruitsof cultured *P. nebrodensis* have been shown to prevent hypertension [7]. However, few studies have focused on *P. nebrodensis* polysaccharides (PN-S) and their bioactivities.

The immunomodulatory effects of PN-S in *in vitro* systems have been investigated [8]. PN-S can induce the functional activation of macrophages through remarkable changes in the morphology and by inducing nitric oxide (NO) release, increasing mRNA expression and cytokine secretion of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS), and stimulating the phagocytosis of macrophages *in vitro*. These results confirm the vital role of PN-S in triggering immune responses. However, the *in vivo* efficacy of PN-S in immunological effector cells, which have a key function against tumor growth under immunosuppression, is poorly understood. Most of the *in vivo* results come from studies with a polysaccharide injection, rather than oral administration [9-10]. Polysaccharides that elicit effects *in vitro*, or by injection, may be ineffective or may exhibit different effects when taken orally [11]. Moreover, well-characterized PN-S products must be developed based on a complete understanding of the immunomodulatory

[Received on] 04-Sep.-2014

[Research funding] This work was supported by the Ph.D. Training Foundation of Tianjin University of Science and Technology (No. 201402).

[*Corresponding author] E-mail: clwl23@tust.edu.cn

These authors have no conflict of interest to declare.

effects and specific applications of oral polysaccharides. Thus, the present study was designed to determine the immune activation effects of PN-S *in vivo*.

Materials and Methods

Materials

Sixty-four male BALB/c mice (4 weeks old, weighing 18 to 22 g) were purchased from the Animal Center of Peking University Health Science Center [SCXK(jing)2011-0012, Beijing, China]. IgG and IgM kits were obtained from Jiancheng Biotech (Nanjing, China). The 1640 medium was purchased from Thermo (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco GRL (Grand Island, NY, USA). Phosphate buffered saline (PBS), 100 $U \cdot mL^{-1}$ streptomycin, and 100 $U \cdot mL^{-1}$ penicillin, and neutral red were purchased from Solarbio (Beijing, China). Cyclophosphamide (CY), hematoxylin-eosin (HE) reagent, concanavalin A (ConA), and lipopolysaccharide(LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CCK-8 assay kit was purchased from Xijiemei (Beijing, China). The RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). The PrimeScript 1st Strand cDNA Synthesis kit was obtained from Takara Bio (Dalian, China). The SYBR Green PCR master mix was purchased from Invitrogen (Shanghai, China).

Isolation and purification of the PN-S polysaccharide

The PN-S was extracted from *P. nebrodensis* as described in a previous study from this laboratory [8]. The molecular mass of PN-S was determined to be 200 kDa by gel filtration. The percentage of total protein and carbohydrate constituents were determined to be 2.6% ± 0.9% and 92.4% ± 6.1% (*W*/*W*), respectively. The component sugars of PN-S were determined by gas chromatography (GC). The coixans were hydrolyzed with acid, reduced, and acetylated, indicating that the neutral sugar components were xylose, mannose, glucose, and galactose, in the molar ratio of 1 : 2.7 : 34.4 : 1.5, with a trace of arabinose. Using Fourier transform infrared spectroscopy (FTIR) (Bruker, Ettlingen, Germany), the structure of PN50G was determined to possess a backbone composed of α-D-glucopyranosyl (Glcp) residues. Ultraviolet spectrophotometry (Agilent Santa Clara, CA, USA) confirmed the absence of nucleic acids.

Immunosuppressed mice and drug administration

The animals were provided with water and mouse chow *ad libitum* and were housed in a rodent facility at (22 ± 1) °C with a 12 h/12 h light-dark cycle for acclimatization. All animals were allowed one week to adapt to their environment before treatment. All procedures involving animals and their care were approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences. Sixteen mice were used for the toxicity experiment. The mice were weighed before the experiment and randomly divided into six groups (n = 8/group). Four mice from each group were used for viscera separation, and the remaining four were used for experimental cell studies. The normal control mice were treated once daily with physiological saline solution for 15 days. The experimental groups included the model group and four PN-S groups (20, 40, 80, and 160 $mg \cdot kg^{-1} \cdot d^{-1}$ in 0.2 mL solutions). In the model group, 0.2 mL of CY (40 $mg \cdot kg^{-1}$) was administered through intraperitoneal injection every other day. At 24 h after the last drug administration, the animals were weighed and sacrificed; the liver, spleen, and thymus were excised and weighed immediately. The visceral index indices were calculated according to the formula, index ($mg \cdot g^{-1}$) = weight of visceral/body weight.

Assay of natural killer (NK) cell activity

The NK cell activity was determined using a CCK-8 assay kit. The mouse spleens were aseptically removed from the sacrificed mice and gently homogenized 0.1 $mol \cdot L^{-1}$ of cold PBS, and passed through a 200-mesh sieve to obtain single-cell suspensions in accordance with the method of Yuan *et al* [12]. The splenocytes (2×10^5 cells/mL) and S180 cells (1×10^4 cells/mL) were plated onto 96-well plates with the ratio 50 : 1 of effectors to target. The plates were then incubated at 37 °C for 20 h, and CCK-8 (10 μL) was added. Following another 4 h of co-culture, the optical density (OD) of each well was measured at 450 nm using a plate reader (ASYS Hitech GmbH, Eugendorf, Austria). In addition, the absorbance measurements were also recorded for the target cell control, blank control, and effector cell control. The percentage of NK cell activity was determined by the following equation: % NK cell activity = 1 − [(optical density value of test samples − optical density value of effector cell control)/optical density value of target cell control] × 100.

Determination of IgM and IgG levels

The blood samples (1 mL) were collected and serum samples were obtained by centrifuging (3 000 × *g*) at 4 °C for 10 min. The effects of PN-S on IgM and IgG production in the sera of CY-treated mice and controls were determined with a mouse IgM and IgG ELISA kit, according to the manufacturer's instructions.

Mouse peritoneal macrophage proliferation assay

Macrophages were collected by peritoneal lavage. After washing the isolated peritoneal cells twice with cold PBS, they were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 $U \cdot mL^{-1}$ streptomycin, and 100 $U \cdot mL^{-1}$ penicillin and plated (1×10^4) on 96-well plates [13]. After incubation for 48 h, the cell viabilities of mouse peritoneal macrophage treated with PN-S at different concentrations and controls were determined by the MTT assay [14].

Neutral red uptake assay

Macrophages were collected and plated on 96-well plates. After 24 h of incubation at 37 °C in 5% CO_2, the medium was removed, and 0.1% neutral red in PBS (100 μL) was added to each well and incubated for additional 4 h. The cells were washed with PBS thrice; 1% acetic acid solution (*V*/*V*) in 50% ethanol (*V*/*V*) (100 μL) was then