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Original article

Grifola Frondosa Polysaccharide (GFP) improves neutrophils immune function of heavy load exercising rats

Jian Huang, Yali Shi, Minjun Sun

College of Physical Education LuDong University Yantai, P. R. China - E-mail: jian_huang38@yahoo.com

Summary. *Grifola frondosa* polysaccharide (GFP) is the extract of *Grifola frondosa.* In this study, 50 male wistar rats were separated into S + C, S + T, LG + T, MG + T and HG + T groups to study the effect of GFP on the immune function of heavy load exercising rats. In S + T group, blood neutrophil number, neutrophil phagocytic index and bactericidal capacity decreased significantly, and adhesion function showed remarkable increase after 8 weeks excessive exercise. Low, medium and high doses of GFP were administered to different groups based on gavage. After gavaging GFP during the eight weeks of excessive exercise, blood neutrophils in the MG + T group and HG + T group were 15.3% and 7.9% higher than in the S + T group. The blood neutrophil phagocytic indices in the MG + T and HG + T groups increased to 1.19 and 1.20, respectively, from 1.02 in the S + T group. The neutrophil bactericidal ability of blood cells in the MG + T and HG + T groups was also observed to be 46.6% and 45.6%, respectively, in comparison with 39.7% for S + T and 39.2% for LG + T group. Blood neutrophil adhesion function in the MG + T and HG + T groups was 57.4% and 57.6%, respectively. This is significantly lower than 65.6% in the S+T group. We demonstrate that feeding a medium or high dose of GFP can improve the neutrophil immune function of excessively exercising rats. A medium dose of GFP shows the most significant effect.

Key words: *Grifola frondosa* polysaccharide (GFP), overtraining, neutrophil, polymorphonuclear neutrophil, PMN, rat immunomodulation

Introduction

Grifola frondosa (GF) belongs to the Polyporaceae tree genus (*Grifola*). *Grifola frondosa*, known commonly as maitake mushroom, is a well-known edible and medicinal fungi, nutritious, delicious with a reasonable nutrition ratio. It is rich in protein, carbohydrates, fiber, vitamins, trace elements and biotin. *Grifola frondosa* is known as the "King of Immunization" against tumors. *Grifola frondosa* polysaccharide (GFP) is the extract of *Grifola frondosa*, which is composed of different polysaccharide dextran, glucose, xylose, fucose, xylose , mannose, galactose and a small amount of protein complex components. The *frondosa* polysaccharide contains a proteoglycan (maitake D-fraction). The proportion of its protein and polysaccharide is 7:3, and the average molecular weight is 1 million g/mol. The polysaccharide moiety is composed of β-(1–6) glucan with β-(1–3) side chains and β-(1–3) glucan with β-(1–6) side chains. The D-fraction in polysaccharide can greatly activate cellular immune function, thereby enhancing the body›s immune system.

Studies of immunocompetent cells show that GF's D-fraction can control the Th-1/ Th-2 proportion of T lymphocyte, inhibit B cell activity, strengthen and help T cell activity, and induce spleen and lymph cells to secrete Γ-IFN, and IL-8 (1). *Nanba* (2) studied the activation of various immune cells by GF's D-fraction. After feeding the mice with a daily dose of 0.5 mg/kg or 1.0 mg/kg GF D-fraction for 10 days, natural killer (NK) cells and cytotoxic T cells increased by 1.5-–2.2 times. Interleukins and superoxide anion content also

increased. A study by Okazaki (3) also confirmed that polysaccharides can stimulate macrophages to release cytokines.

Excessive acute exercise and inappropriate chronic training can cause physiological and psychological fatigue which can lead to decreased exercise capacity or training-associated syndromes. Moderate activity may enhance immune function, whereas prolonged, high-intensity exercise temporarily impairs immune competence (4, 5). Animal experiments showed that body weight, blood indicators, neuroendocrine and immune function could have significant changes after overtraining. Upper respiratory tract infections (URTI) symptoms often occur in the athletes during excessive training periods. Exhaustion after long exercise training period or an important game can lead to URTI incidence. Nieman et al. (6) and Heath et al. (7, 8) reported that URTI incidence for a large amount of exercise training is two to four times higher than URTI incidence for a small amount of exercise training (6-8).

Neutrophils (also known as polymorphonuclear cells or Poly Morphonuclear Neutrophil, [PMN]) are the most abundant peripheral blood leukocytes, accounting for 60 to 70 percent of the outer peripheral leukocytes, and are directly involved in the first defense of the body's immune system. Neutrophils have various functions such as chemotaxis, phagocytosis and bactericidal activity. Excessive exercise activates the burst of neutrophil oxidatives, leading to the drop in the phagocytic activity and a decline of neutrophils immune function (9).

It has already been known that some fungus such as *Ganoderma lucidum* has a potent immunomodulatory effect (10, 11). Even though GF traditionally has been regarded as the "King of Immunization", and GFP D-fraction can directly kill cancer cells and activate the immune system, there is no report of whether GF can modulate the change of neutrophil function of the body after excessive exercise. In this study, we divided the rats into several comparison groups and investigated the impact of GFP feeding on the immune function in exercise-induced immunosuppressed rats. Excessive exercise rats were fed with GF in order to observe the polysaccharide modulating effect on neutrophil function. The means of using nutritional science to prevent the neutrophil immune function decline caused by excessive exercise are evaluated and assessed.

Materials and Methods

Animals

Fifty male wistar rats (weighing 246 ± 6.9 g), were purchased from Shandong Green Leaf Pharmaceutical Co. After adaptive feeding, they were randomly divided into groups S + C (Saline + Normal Activity), S + T (Saline + Training), HG + T (High dose GFP + Training), MG + T (Medium dose GFP + Training), and LG + T (Low dose GFP + Training), depending on the exercise load and GFP gavage dose. Each group of 10 animals was raised in two cages, five per cage, following the national standard for 2nd grade animal. Each cage was plastic, and equipped with a stainless steel cover, a glass bottle and a stainless steel water suction pipe. Broken disinfection wood chips were used at the cage bottom. They were replaced 2 or 3 times a week. The temperature was kept at 21–24°C and illumination time was about 12 hours. Rats were fed with the national standardized solid mixed food and free diet.

Experimental model design

Figure 1 shows the experiment design and data analysis procedures for this study. The entire experiment lasted eight weeks. Similar to the experimental design for immunomodulatory studies in Shi et al. (12) and (10), the experimental design in this study followed the protocols and procedures as described in Hohl et al. (13), Xu and Bian (14) and Si (15). These protocols and procedures have been used in a variety of studies (16, 17).

Exercise conditions

Rats were set to exercise in a fiberglass swimming pool, 150 × 60 × 70 cm^3, with the water depth of 60 cm, which was more than twice the length of each rat. Water temperature was set at 32–34°C.

Dose of GFP

GFP capsules were provided by the Jiangsu Yancheng Shennong Health Food Company. Gavage was used to feed GFP. The surface coefficient is a comp-

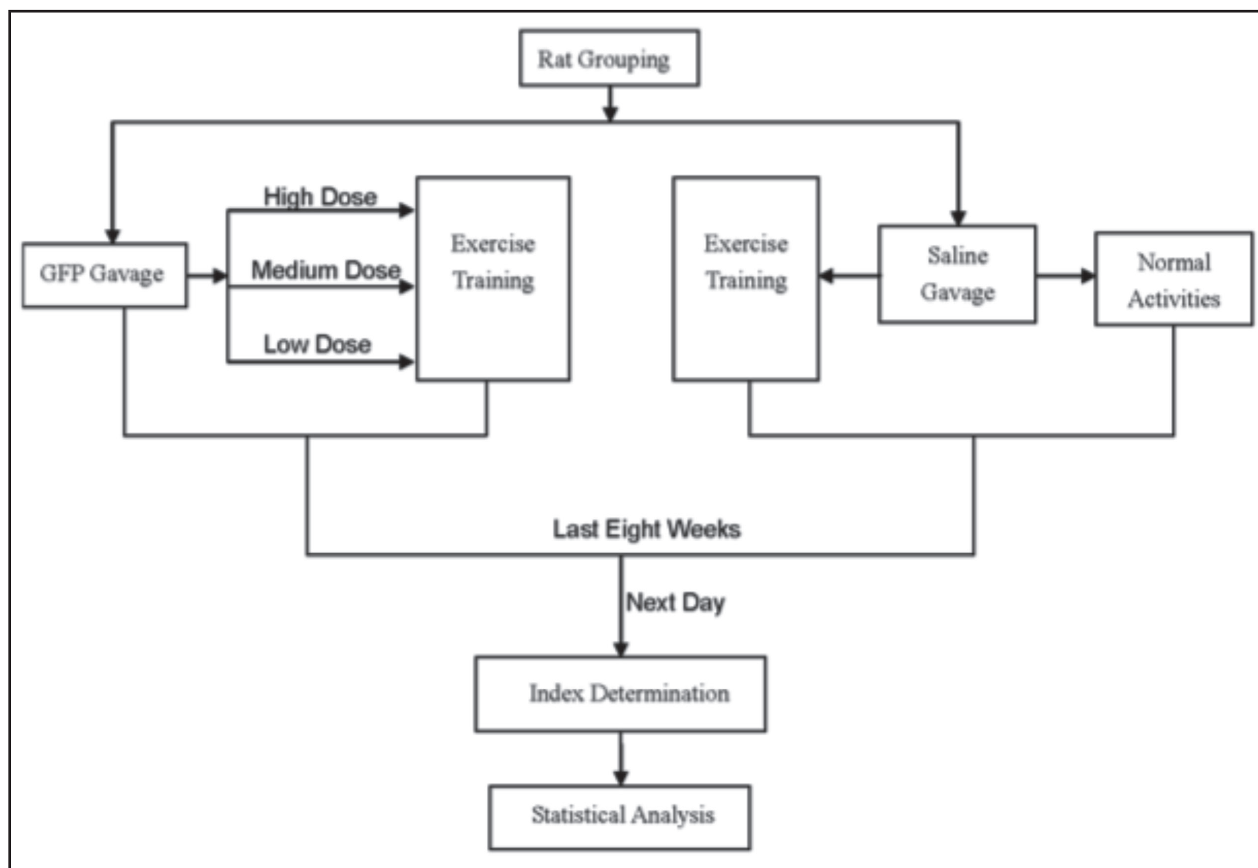

Figure 1. Schematic chart of the experiment design and data analysis procedures.

rehensive parameter for the conversion of the GFP between humans and animals according to the body surface area (14). This coefficient took into account of a variety of factors, including metabolism.

The known human dosage of GFP is 3 g/day. According to body surface areas of humans and the animal, the equivalent dose in the mice was calculated as follows:

Animal dose (per kg) = known daily animal dose ´ body surface coefficient / unknown animal body weight.

The body surface coefficient was assigned as 0.018 (14). Thus the dose for rat (per kg) = 3000 mg ´ 0.018 / 0.25 kg = 216 mg / kg.

Training Design

The total training time is 8 weeks. We conducted adaptive swimming for 60 min per day for the first one week, then gradually increased the exercise time to 150 min per day for the other 7 weeks. When exhaustion occurred in the 150 min training session for some rats, we picked them up and dried them with absorbent paper for the rest of 10 min, then put them into the water to swim, trying to make the training time to reach the stipulated exercise time. After each training session, we administered intragastrically different doses of GFP (or saline) for different rat groups. Training was conducted six days a week, and lasted eight weeks. At the end of the experiment, three rats died. Since the number of the death is insignificant in comparison to the total 50 experiment samples, we did not repeat the experiment to replace the three rats.

S + C group: We did not conduct exercise training, only maintained normal physiological activities, i.e. normal life activities, metabolism, stress, growth and development, and genetic characteristics unchanged. We supplemented with a daily dose of saline.

S + T group: After each training, we supplemented with doses of saline.

MG + T, HG + T, LG + T groups: Training methods were the same as for the S + T groups, but after each training gavaged with freshly prepared solutions of GFP. The MG + T group was dosed at 200 mg/kg/day, the HG + T group was dosed at 400 mg/kg/day, and the LG + T dosage was 100 mg/kg/day. Each dose was prepared with a physiological saline to a GFP concentration of 1333 mg/100 ml, 2666 mg/100 ml, and 667 mg/100 ml. GFP saline solution was administered at 3.75 ml/day for an average rat weight 250 g. Such a dosage is equivalent to 50 mg/day for the MG + T group, 100 mg/day for the HG + T group, and 25 mg/day for the LG + T. This is in line with the equivalent dose in other studies (14). The S + C and the S + T groups were given same volumes of saline. Intragastric administration was done daily between 8:00 am and 9:00 am for eight weeks.

Index determination

One day after the last training, rats were anesthetized with pentobarbital sodium. 5 ~ 6 ml of blood was taken and placed in a sterile heparin vacuum tubes.

Neutrophil count

We took approximately 0.1 ml of blood first. Blood cell counters were used for blood analysis following the procedure as described in Xu et al. (14).

Nitroblue tetrazolium reduction test

Neutrophils were activated after they engulfed bacteria or particles, thus leading to increased energy and oxygen consumption. The hydrogen from oxidative dehydrogenation was ingested in the NBT (nitroblue tetrazolium) of cytoplasm. Thus the yellow NBT was changed to blue formazan, which was refractive and became punctual particles deposited in the cells. Microscopic examination was conducted for the formazan percentage of positive cells which were representative for the bactericidal capacity.

A 20 μl heparin solution was added on a clean glass slide, then 40 μl of blood was drawn and gently mixed with the heparin solution on the slide. We added one drop of NBT solution and mixed gently. We set the wet box into an incubator at 37°C for 15 min, then gently shook at room temperature for 15 min. Smear slides were made with samples to test blood, then fixed for 3 min with methanol after the blood dried. We instilled Swiss-Giemsa dye for 3 min, gently rinsed with water, naturally dried it, and examined it under microscope. A neutrophil cytoplasm containing blue formazan is a NBT positive cell. 100 neutrophils were counted to calculate the percentage of NBT-positive neutrophils (15).

Neutrophil phagocytosis (assay for phagocytosis of polymorphonuclear leukocyte)

Following the procedures in Wu et al. (18), the Staphylococcus were inoculated in a 5 ml broth, cultured 12 h at 37°C in the incubator, then 0.1 ml was taken out for bacterial count. We put it in a 100°C water bath for 10 min to kill bacteria and calculated the number of bacteria per ml. We adjusted the saline solution for 6×10^8 bacteria/ml and set it at 4°C for further use. We used a hemoglobin pipette to take 40 μl of blood and immediately filled it into the concave recess hole of the clean slide containing 20 μl heparin (concentration of 20 U/ml). We gently stirred the mix, added 20 μl of prepared broth and mixed well. We placed it at a 37°C preheated container covered with wet gauze and put the container in the 37°C incubator for 30 min and shook it at 10 min intervals. We took a drop of the mixture into a clean glass slide, and pushed it into thin slices. After it dried, we used methanol to fix for 4~5 min and then dyed the staining for 2~3 min with alkaline methylene blue dye. We examined it under an oil microscope, counted 100 neutrophil randomly and wrote down the number of neutrophils for which phagocytosis occurred and did not occur respectively. For the occurrence of neutrophil phagocytosis, we recorded the number of bacteria it swallowed.

Phagocytes% = Number of neutrophils which swallowed bacteria in 100 neutrophils

Phagocytic index = Total bacteria number in 100 neutrophils/ 100

Determination neutrophil adhesion function

To determine the neutrophil adhesion function, the experiment steps of Wu at al. (18) were followed.

Step 1: Neutrophil cell count before nylon fibers.

1 ml of heparin anticoagulant was first pushed into thin slices. After it dried, we used methanol to fix it for 4~5 min, then used alkaline methylene blue dye staining for 2~3 min. We used an oil microscope to count the number of neutrophils before nylon fibers.

Step 2: Neutrophil cell count after nylon fibers. We followed the same procedure to count the number of neutrophils after nylon fibers.

Adhesion rate (%) =100 ´ (100 - number of neutrophils through a nylon fiber)/neutrophil cell count before the nylon fiber

Data processing

The data was presented as mean ± standard deviation (X ± SD). SPSS11.5 statistical software was used for data processing. S + C and S + T groups were compared using the paired T test. The S + T, HG + T, MG + T, and LG + T sets of data were processed with one-way ANalysis Of VAriance (ANOVA) first. Considering that the sample size of this experiment was not large, the least significant difference (LSD) method was used for multiple comparisons. The criteria of significance level (P) was defined to be 0.05, while a very significant level was defined to be 0.01.

Results

Excessive exercise and effect of gavage on the number of blood neutrophils

Figure 2a shows that the number of neutrophils in rat blood after 8 weeks of overtraining decreased significantly. The number of neutrophils in the S + T group was significantly lower when compared to the number of neutrophils in the S + C group. The numbers of neutrophils for the S + C and S + T groups were 6,400/ml and 5544/ml respectively. In comparison, the blood neutrophils in MG + T group and HG + T group were significantly higher than S + T group. The neutrophils in the MG + T group and HG + T group were 5,870/ml and 6,270/ml respectively. For the LG + T group, the numbers of neutrophils for the LG + T group were 5570/ml, and we did not observe increased neutrophils in comparison with the S + T group.

Excessive exercise and impact of feeding GFP on neutrophil phagocytosis

Figure 2b shows the neutrophil phagocytic index in the overtraining rats for different groups. The phagocytic index dropped to 1.02 for the S + T group in comparison with 1.23 for S + C group.

After 8 weeks of overtraining, the blood neutrophil phagocytic index in the MG + T and HG + T groups were significantly higher than the S + T group. The neutrophil phagocytic index for the HG + T and MG + T groups were 1.20 and 1.18, respectively. Similar to the neutrophils in Figure 2a, the neutrophil phagocytic index for the LG + T group was 1.04, and we did not observe increased neutrophil phagocytic index for the LG + T group in comparison with the S + T group.

Effect of excessive exercise and feeding GFP on neutrophil bactericidal capacity

Figure 2c shows neutrophil bactericidal capacity for different experiment groups reached a significant level. Bactericidal capacity was 48 for the S + C group. It significantly dropped to 39.7 for the S + T group. After feeding GFP, neutrophil bactericidal capacity was significantly higher in the HG + T and MG + T groups with bactericidal capacity at 45.5 and 46.7 respectively. The neutrophil bactericidal capacity was 39.2 for the LG + T group, and showed no significant changes from 39.7 of the S+T group.

Impact of excessive exercise and GFP on neutrophil adhesion function

After overtraining, rat blood neutrophil adhesion function in the S + T group increased significantly from the reference S + C group. Neutrophil adhesion function for the S + C group and the S + T group were 57.8 and 65.6 (Figure 2d). Also in Figure 2d, neutrophil adhesion function for the MG + T and the HG + T groups dropped to similar levels as the S + C group, with values of neutrophil adhesion function at 57.6 and 57.4 respectively. For the LG + T group, neutrophil adhesion function was 65.0 and did not show any significant change from neutrophil adhesion function in the S + T group.

Discussion

Neutrophils account for 60% to 70% of the total white blood cells in the blood, and many receptors were expressed on the surface, including pattern recognition receptors, complement receptors and various chemokines. Neutrophils also release a variety of inflammatory mediators such as leukotriene, Eotaxin. Neutrophils play an important role in the non-specific immunity of blood, and have strong chemotaxis and phagocytosis. As the first line of defending bacterial invasion and pathogens in the local infection, neutrophils can quickly penetrate the capillary wall into the infected site, identify and play a phagocytic role in swallowing the pathogens with the pattern recognition receptor. After killing pathogens, the neutrophils become pus cells. Neutrophil disintegration can release a variety of lysosomal dissolution of the surrounding tissue abscess to prevent the spread of local pathogens in the body, thus it also play an anti-infective role (15).

There are few reports about the mechanism of Grifola frondosa polysaccharide on neutrophils. The xylene-induced mouse ear swelling test and turpentine-induced rat airway granulation hyperplasia model were used to observe the anti-inflammatory effect of Grifola frondosa B-glucan (19). The results show that Grifola frondosa B-glucan has obvious anti-inflammatory effects on acute and chronic inflammation of

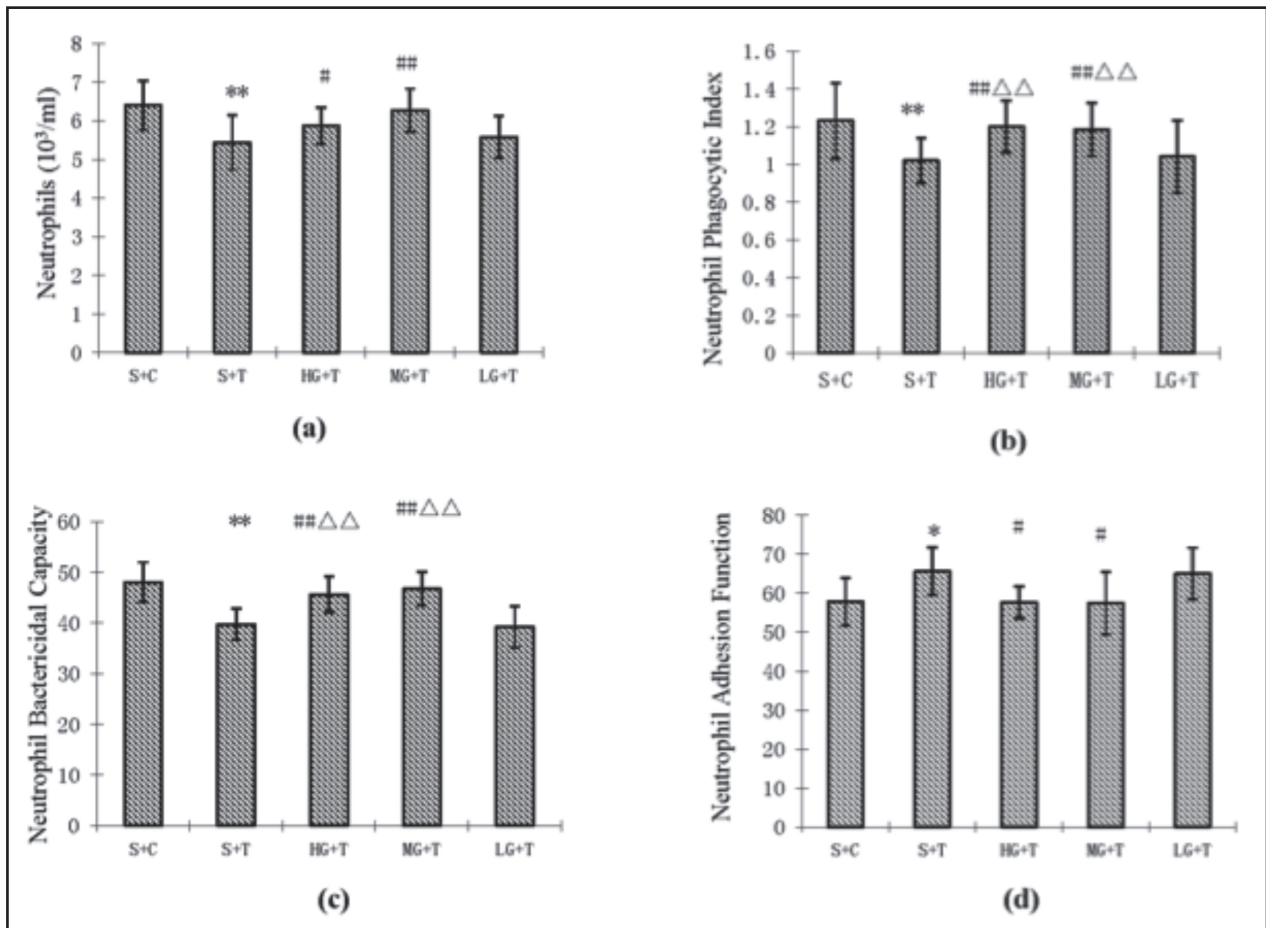

Figure 2. Comparisons between groups S + C, S + T, LG + T, MG + T and HG + T for (a) number of neutrophils, (b) neutrophil phagocytic index, (c) neutrophil bactericidal capacity, and (d) neutrophil adhesion rate. Each value is represented with mean ± standard deviation.

* and ** denotes $P < 0.05$ and $P < 0.01$ in comparison with S + C group, # and ## denote $P < 0.05$ and $P < 0.01$ in comparison with the S + T group respectively. Δ and ΔΔ denote $P < 0.05$ and $P < 0.01$ in comparison with the LG + T group.

experimental animals, and has analgesic effect on pain response caused by acetic acid. However, the cells involved in the inflammatory process could be neutrophils, monocytes, and lymphocytes. Above study did not provide an in-depth analysis of the mechanism. Our study confirmed that excessive exercise can cause a variety of neutrophil immune dysfunctions, revealed the mechanism of over-exercise-induced sudden increase in the incidence of infectious diseases. We found that Grifola frondosa polysaccharide could resist the decrease of neutrophil function in rats caused by excessive exercise. The effect of GFP on neutrophil function was demonstrated

Our results show that neutrophil bactericidal capacity decreased significantly after overtraining, which is consistent with previous studies. Exercise can cause respiratory burst of phagocytic cells and phagocytosis-related changes (5, 7). Research shows excessive exercise can activate the burst of peripheral blood neutrophil NADPH oxidase, resulting in excessive ROS (reactive oxygen species) and peroxide level increase in serum. ROS can cause death of neutrophil cells (20).

Our results also show that phagocytic neutrophil and phagocytic index decreased and the neutrophil adhesion rate increased significantly in the S + T group after overtraining. It was reported that the measured PMN-EC (polymorphonuclear leukocytes-endothelial cells) adhesion rate and surface adhesion molecules increased significantly in an experiment on rabbit burns (21). Cell adhesion molecules of neutrophils in lung injury of rats also increased. Hayakawa at al. (22) demonstrated that epithelial cell damage or increased expression of adhesion molecules can enhance the adhesion of neutrophils and PMN-EC, thus clogging the capillaries and releasing free radicals to cause tissue damage . In our experiment, neutrophil adhesion significantly increased after overtraining in the S + T group. Overtraining may cause inflammation and lead to exercise-induced immunosuppression. This observation and corresponding mechanism should be similar to the experiment results from *Chen et al.* (21) and *Xie et al*. (23). Further in-depth study is still needed.

Fungal polysaccharides are now recognized as a highly effective immune enhancer. Many fungi polysaccharide preparations have been widely used in clinics. The results in treating autoimmune diseases, immune dysfunction syndrome and cancer are encouraging. Therefore, use of fungi polysaccharides to prevent immune suppression is a scientific and effective method. Natural polysaccharides are difficult to be absorbed by the body due to their insolubility, large molecular weights and high viscosity. The maitake polysaccharide capsules used in this study are produced after identifying the structure of biological polysaccharide and consequently reducing its molecular weight, restructuring, and modifying it to get a new maitake polysaccharide (D- fraction), which is the most effective ingredient and easily to be absorbed by the animal body.

There is a lot of evidence that shows that the β-(1→3) glycosidic bond in the polysaccharide is a prerequisite for the activity of the polysaccharide. Monosaccharide structures (α or β) determine the orientation and structure of the glycosidic bond. β-(1→3) glucan helps to curl into a helical structure, and thus can have a higher immune activity and anti-tumor activity. Branches have a greater impact on the immune activity of β-(1→3). High abundance of branches can lead to high activity of β-(1→3). The mechanism for the involvement of β-(1→3)-glucan in immune regulation can be attributed to the identification of β-1,3GR on the surface of monocytes, macrophages, granulocytes and other cells (24).

A lot of research shows that polysaccharides are an effective biological immunomodulatory agent. Its components contain β-1,3-, β-1,6- glucan structures, and thus can greatly activate cellular immune function, consequently enhancing immunity. Nanba (25) extracted and purified D-fraction, and MD-fraction of maitake. The D-fraction with a molecular weight of about 1.4×10^6 is a proteoglycan composed of highly branched β-1,6-glucan. This specific structure makes it have a stronger activity of biological regulation (25). Polysaccharides can activate immune cell populations such as T lymphocytes, macrophages and natural killer cells, and may contribute to secretion of a variety of cytokines, such as IL-2, IL-8, IL-12 (IL: interleuki), TNF (tumor necrosis factors), etc.; thus, it can enhance local tumor immune response and inhibit occurrence of tumor (26).

Among the three LG + T, MG + T and HG + T groups, the MG + T group shows best results in

terms of number of neutrophils (Figure 2a), neutrophil phagocytic index (Figure 2b), neutrophil bactericidal capacity (Figure 2c) and neutrophil adhesion function (Figure 2d). This implies that the regulation of the immune response for the fungal polysaccharides might be dose dependent and two-way adjustable. This phenomenon may be related to homeostatic regulatory mechanisms of the immune system. It is consistent with other studies such as Jeannin et al. (27), which shows that the most appropriate amount of anti-tumor Lentinan fungal polysaccharides is between 15 mg/kg body weight. A low-dose of Lentinan is not effective, and the antitumor activity tends to decline when the dose is greater than 10 mg/kg.

Conclusion

Our results show that after 8 weeks of overtraining, quantities of blood neutrophils in the MG + T group and the HG +T group are significantly higher than that of the S + T group, and no increased neutrophils in the LG + T was observed. Blood neutrophil phagocytosis in the MG + T and the HG + T groups was significantly enhanced relative to the S + T group. The impact of GFP on the neutrophil phagocytosis in the LG + T group blood was not observed after comparing with the S +T group.

Similarly, neutrophil phagocytic indices in the MG + T and the HG + T groups were notably higher than that of the S + T and LG + T groups, reaching a significant level. Bactericidal capacity dropped in the S + T group in comparison with the S + C group. The neutrophil bactericidal ability of blood cells in the MG + T and HG + T groups showed great improvement over the S + T group and reached almost the same level of neutrophil bactericidal capacity as the reference S + C group. On the other hand, no significant change of neutrophil bactericidal capacity was observed for the LG + T group. In the test for neutrophil adhesion function, the performance of MG + T was best, and it was significantly lower than that of the S + T group. The neutrophil adhesion function for the MG + T and HG + T groups dropped to similar levels as the S + C group.

In conclusion, 8 weeks of excessive exercise led to decreased immune function in rats. After supplementing GFP, rat immune function in terms of number of blood neutrophils, neutrophil phagocytosis, neutrophil bactericidal capacity, and neutrophil adhesion function improved. The impacts with medium and high doses of complementary GFP were significant. The medium dose of GFP worked best. We did not observe an effect of the low dose of GFP on improving immune function of overtraining rats.

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Correspondence:
Professor Jian Huang
College of Physical Education
LuDong University
Yantai, 264025, P. R. China
E-mail: jian_huang38@yahoo.com