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## Study of *Grifola frondosa polysaccharide* (GFP) on Peritoneal Macrophage Activities of the Long-Term Heavy Load Exercising Mouse

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**Abstract:** Long-term heavy-load exercise can decrease macrophage activity. In this study, we separated 50 Kunming (KM) mice into comparison groups to investigate the immune-regulatory effects of *Grifola frondosa polysaccharide* (GFP) on long-term heavy-load exercising mice. We evaluate NO content, macrophage survival and growth, intracellular GSH and macrophage phagocytosis 4 weeks after gavaging long-term heavy-load exercising mice with GFP. After exercise, NO content, macrophage survival and growth, phagocytic index significantly reduced in the mice not fed GFP. Both medium and high doses of GFP drastically increased NO, phagocytic index and decreased intracellular GSH. High doses of GFP also increased macrophage survival and growth. With this study, we demonstrate that four weeks of heavy-load exercise can weaken peritoneal macrophage activity. A supplement of GFP fed to these mice improved their peritoneal macrophage activity. The effect for the high-dose GFP treatment is especially significant.

**Key words:** *Grifola frondosa polysaccharide* (GFP), long-term, heavy-load exercise, mice, peritoneal macrophage activity

### INTRODUCTION

*Grifola frondosa polysaccharide* (GFP) is a bioactive polysaccharide extracted from *fungi frondosa*. It is rich in protein, carbohydrates, fiber, vitamins, trace elements and biotin. *Grifola frondosa polysaccharide* (GFP) is the extract of *Grifola frondosa*, composed of different polysaccharide dextran, glucose, xylose, fucose, xylose, mannose, galactose and a small number of protein complexes. The *Froncosa* 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. The polysaccharide moiety is composed of  $\beta$ -(1-6) glucan with  $\beta$ -(1-3) side chains and  $\beta$ -(1-3) glucan with  $\beta$ -(1-6) side chains. The D-fraction in polysaccharide can greatly activate cellular immune function; enhancing the body's immune system. Studies have shown that GFP D-component can activate various immune cells. After feeding mice a daily dose of 0.5 or 1.0 mg/kg GF D-components for 10 days, natural killer (NK) cells and cytotoxic T cells increased by a factor of 1.5-2.2. Interleukins and superoxide anion content also increased. Thus GFP can fight against tumor and HIV, regulate cardiovascular function and perform other functions (Nanba *et al.*, 1987; Adachi *et al.*, 1987).

Adachi's studies (Adachi *et al.*, 1994; Adachi *et al.*, 1998) showed cytokines and NO, the product of mouse Kupffer cells significantly improved after 4 to 7 days mice were injected intravenously GRN,  $\beta$ -1,3-glucan. *In-vitro* experiments also confirmed that GRN induces macrophage to release cytokines IL-1, IL-6 and  $\alpha$ -TNF etc. Okazaki *et al.* (1995) and Yao and Zhang (1994) also

have confirmed grifola can stimulate macrophages to release cytokines.

High-load exercise can decrease immune function and consequently more occurrences of infections. Upper respiratory tract infection (URTI) symptoms often appear in the athletes during training. Higher endurance after heavy exercise training or an important game can lead to URTI. Nieman *et al.* (1990) and Heath *et al.* (1991, 1992) reported that URTI incidence for a large amount of exercise training is two to four times higher than for a small amount of exercise training (Nieman *et al.*, 1990; Heath *et al.*, 1991, 1992). Mackinnon and Hooper reported that URTI symptoms appear for 42% of swimmers after 4-week high intensity training (Mackinnon and Hooper, 1996).

Macrophages are biologically active cells, widely distributed in the body. They play an important role in starting and coordinating immune response (Lin *et al.*, 2009). They help control the infection process and are the body's the first line of defense against germ invasion. Macrophages are widely distributed in the lung, liver, kidney, abdominal cavity, muscle, blood and other tissues and organs. It has functions such as phagocytosis, bactericidal, inhibiting tumor and secretion of inflammatory cytokines (Murphy *et al.*, 2004). Long-term or short-term low-intensity exercise can enhance macrophage phagocytosis (Ferrandez and De la Fuente, 1999). However, there is no relevant research on how long-term heavy load training can affect the activity of macrophages. There is also no report of whether GFP can modulate the immune activity of

macrophages after excessive exercise. Accordingly, we designed an experiment to study the impact of long-term heavy load training on macrophage, immune cell function and chances of infection and whether GFP is capable of regulating the activity of macrophages for long-term heavy load exercising mice.

To carry out this study, we separated mice into several comparison groups to investigate the impact of GFP feeding on peritoneal macrophage activities in the exercise-induced immunosuppressed mice. We fed excessive exercise mice GFP to observe the polysaccharide modulating effects on macrophage activities. We evaluated and assessed the means of using nutritional science to prevent declining macrophage activity caused by excessive exercise and drew our conclusions.

## MATERIALS AND METHODS

**Experimental animals and grouping:** Fifty male specific pathogen-free (SPF) grade Kunming (KM) mice, weighing 18-22 g, underwent this experiment. We divided the mice into cages with five per cage and fed a diet that was in accordance with the national standard for routine feeding of rodents. The investigation took place in an animal husbandry environment of  $23\pm 25^{\circ}\text{C}$  with humidity kept at 40-60%, illumination time was about 12 h. The rodents followed the natural circadian rhythm.

After the 50 mice underwent adaptive feeding for 3 days, they were randomly divided into a saline group (S+C), a saline+exercise group (S+T), a high dose GFP+exercise group (HG+T), a medium-dose GFP+exercise group (MG+T) and a low dose GFP+exercise group (LG+T). Each group of 10 animals had grown up in two cages, five per cage, following the national standard for second-grade animals.

**Experimental model design:** Figure 1 shows the experiment design and data analysis procedures for this study. The entire experiment lasted four weeks. Similar to the experimental design for immunomodulatory studies in Shi *et al.* (2012) and Zhu *et al.* (2007), the experimental design in this study followed the protocols and procedures as described in Hohl *et al.* (2009), Xu and Bian (2005) and Si (2005). These protocols and procedures have seen use in a variety of studies (Jin and Feng, 2005; Jia and Yuan, 2007).

**Dose of GFP:** We administered GFP was administered by gavage. The standard human dosage of GFP is 3 g/day. Surface coefficient is a comprehensive parameter for comparing the conversion of the GFP between humans and animals according to body surface area. This coefficient took into a variety of factors, including metabolism. According to body surface areas of humans and mice, we calculate the equivalent dose in the mice as follows:

- 1: Animal dose (per kg) = the known daily animal dose x body surface coefficient/unknown animal body weight
- 2: The body surface coefficient was assigned to be 0.0026 (Xu and Bian, 2005), thus the dose for mouse (per kg) =  $3000 \text{ mg} \times 0.0026 / 0.02 \text{ kg} = 390 \text{ mg/kg}$
- 3: Gavage dose: the HG+T group received a dose at 800 mg/kg/day; the MG+T group was dosed at 400 mg/kg/day and the LG+T group was dosed at 200 mg/kg/day

GFP solutions were prepared with normal saline to concentrations of 800, 400 and 200 mg/10 ml, respectively. We intragastrically administered GFP aqueous solutions at 0.2 ml/day and the S+C and S+T groups were given the same volumes of saline. Intragastric administration took place daily between 8:00 am and 9:00 am for 4 weeks.

**Exercise conditions and training designs:** We put the mice in a plastic swimming pool with dimensions of  $100 \times 60 \times 50 \text{ cm}^3$ . Water depth was set at 40 cm. The water depth was more than twice the mouse body length and water temperature was  $27\text{-}30^{\circ}\text{C}$ . The water temperature was similar to that used in other studies (Shi *et al.*, 2012; Si, 2005; Xia *et al.*, 1989).

Mice in the S group without exercise underwent normal physiological activities and did not swim. Mice in the S+T, HG+T, MG+T and LG+T groups swam with loads of 5% of body weight (a fine copper wire, equivalent to 5% of each mouse's weight, tied to the root of its tail). Flowing water forced the mice to swim. GFP administration was conducted between 9 and 10 am and exercise training started at 3 pm. The swimming training lasted for 4 weeks, 6 times per week, with no training on Sundays. From the first week, the loaded swimming training started at 20 min with daily increases of 5 min reaching 45 min/day. Exhausted mice that exercised less than 45 min were quickly picked up, dried off and allowed to continue swimming. Exhaustion accompanied increased serum-free radical levels and decreased hypoxia tolerance time. The cumulative 45 min/day training time was required. At the end of the training, mice were quickly dried off with a hair dryer.

A total of five mice died due to improper gavage, untimely judgment of mice exhaustion and failure to pick the mice up from drowning. Two died in the S+T group, one died in the HG+T group, one died in the MG+T group and one died in the LG+T group.

## Experiment measurements

**Preparation and culture of mouse peritoneal macrophages:** After four weeks of sports and gavage, the mice received intraperitoneal injection of 4 to 6% starch broth. We harvested cells after 4 days, killing 6-8 week-old Kunming mice in order to harvest the

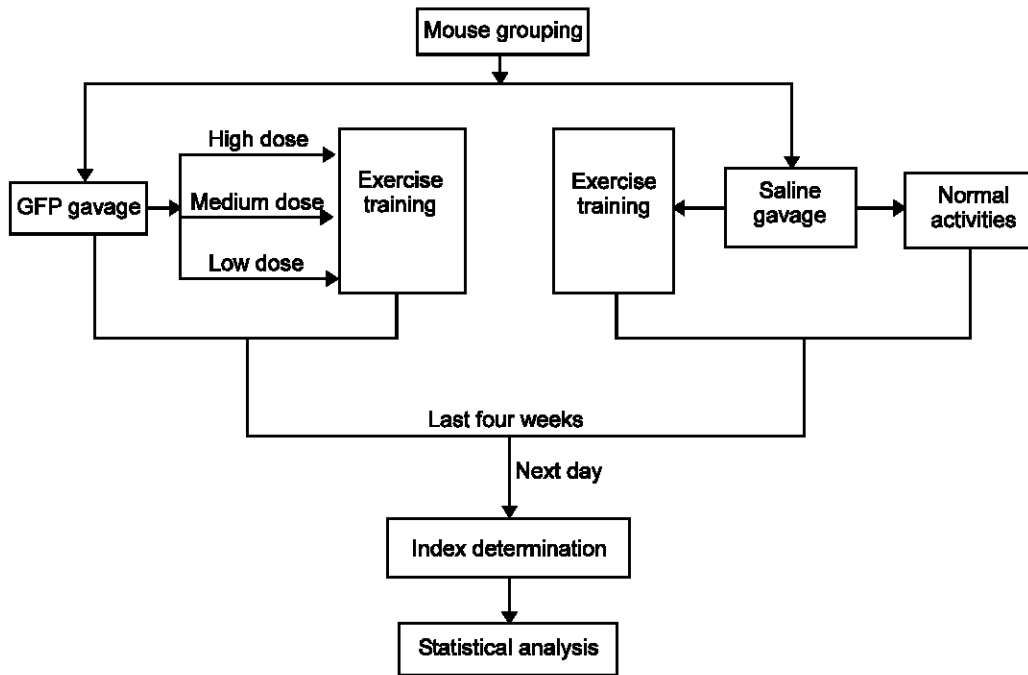


Fig. 1: Schematic chart of the experiment design and data analysis procedures

peritoneal macrophages. The mice were soaked with 75 degree alcohol for 5 min, intraperitoneally injected 5 ml of serum-free RPMI (Roswell Park Memorial Institute) solution. We lay the mice flat and rubbed the abdomen for 2-3 min, then waited for another 5-7 min, opened the abdominal cavity of mice under sterile conditions. When the observed intestine became light yellow, we extracted the peritoneal fluid with a syringe, then used 1000 *r/min* centrifugation for 10 min to centrifuge the serum. After washing twice with PBS (phosphate buffer saline), we re-suspended cells with serum-free culture medium RPMI1640 and then transferred to 6-well plates and set in a 5% CO<sub>2</sub>, 37°C incubator for adherent culture for 2 h. After decanting in adherent cells, adherent cells are macrophages. An optical microscope further inspected the adherent cells. They were consistent with the morphology of macrophages. We gently added PBS (PH 7.4) and then carefully pipetted the cells into a centrifuge tube, re-suspended the macrophage cells with RPMI1640 solution and counted the cells with cell count plate, adjusted the concentration to 2 x 10<sup>6</sup>/ml.

**Macrophage survival and growth:** MTT (Methylthiazolyldiphenyl-tetrazolium) bromide colorimetric analysis helped measure macrophage survival and growth of macrophage cells. We took 100 µl macrophage solution with the concentration of 2 x 10<sup>6</sup>/ml, added it to each well of 96-well plates and then added serum-free 1640RPMI (Roswell Park Memorial Institute) medium. After adding 20 µl of MTT solution per well and culturing for 4 h, we discarded supernatant,

added 150 µl of dimethyl sulfoxide in each well, set the well plate on the shaker for 10 min and made the resulting purple crystals dissolve. We measured absorbance at 570 nm with enzyme immunoassay instrument. It indirectly reflected the quantity of the live macrophage cells.

**Measurement of NO content:** We took 100 µl of solution with a concentration of 2 x 10<sup>6</sup>/ml of suspended macrophage and added it to 96-well plate containing 100 µl medium containing 1 mg/L LPSipo polysaccharide in each well, cultured it for 24 h. NO itself is a free radical gas. After NO was produced, it was rapidly released into the supernatant and became stable NO<sub>2</sub><sup>-</sup>. Thus measuring the amount of NO<sub>2</sub><sup>-</sup> can indirectly determine NO production. 50 µl Griess reagent was added to 50 µl supernatant, the reaction between Griess reagent and sample produced nitrite. Measuring absorbance at 570 nm and calibration with the standard concentration of sodium nitrite standard can determine the nitrite content (in unit of µ mol/10<sup>6</sup> cells/ml).

**Intracellular GSH determination:** The first step is breaking macrophages. We first put 100 µl solution with the macrophage concentration of 2 x 10<sup>6</sup>/ml in the 96-well plate and gently washed adherent macrophage cells with Hanks solution once, added double distilled water in each well, repeatedly froze at 30°C and thawed at 30°C five times for 30 min each time to make the broken macrophage suspension solution, took this 100-µl macrophage suspension solution and added distilled water to make 2 ml of the sample.

Following our first step, DTNB colorimetry determined the intracellular GSH. We took 0, 0.02, 0.04, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 0.2, 1 mmol/L GSH, diluted with distilled water to a concentration of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10  $\mu\text{mol/L}$  GSH. Each standard solution is 2 ml. For reference, we also added 2 ml of double-distilled water to a blank tub to create a reference for calibration of DTNB colorimetry.

At the 423-nm wavelength, we read absorbance of the standard solution sample. Using GSH concentration in the standard as abscissa, we drew the curve of absorbance as a function of GSH concentration. Then we used the curve as the standard to obtain the concentration of the sample GSH with the measurements of the absorption measurements of the samples.

#### **Determination of macrophage phagocytosis (*in vitro*):**

After gently washing the adherent macrophages as mentioned above with Hanks, we mixed them with chicken erythrocytes at 1:4 to 1:8 and incubated for 30 min. We shook the mixed solution every 10 min during incubation, then centrifuged it. We fixed the cell pellet suspension on the smear with 1:1 acetone- methanol for 5 min, then stained it with Giemsa. We counted the macrophages under the microscope:

Phagocytic index = Total chicken erythrocytes in the 100 macrophages/100

**Data processing:** We presented the data as mean $\pm$ standard deviation (X $\pm$ SD). SPSS11.5 statistical software aided data processing. S+C, S+T, HG+T, MG+T and LG+T group data were processed with one-way analysis of Variance (ANOVA) first. Considering the sample size of this experiment was not large, the Least Significant Difference (LSD) method was used for multiple comparisons. The criterion of significance level ( $p$ ) was set to be 0.05 and that of a very significant level was 0.01.

## **RESULTS**

**Long-term heavy load training exercise on the survival of macrophages:** In comparison of groups HG+T, MG+T, LG+T with S+T group, feeding high doses of GFP can significantly improve macrophage cell survival. Feeding low doses GFP had no effect on the survival of macrophages. Comparison between group S+C and group S+T shows that macrophage cell survival decreased significantly after long-term heavy load exercise, reaching significant levels (Fig. 2a). The value dropped from 0.182 for group S+C to 0.136. The value of the macrophage cell survival increased to 0.165 for group HG+T, reaching very significant levels in comparison to group S+T. For groups MG+T and LG+T, the values for macrophage cell survival were 0.152 and 0.149, respectively.

**Effect of GFP on the GSH in macrophages:** In group S+C and group S+T, long-term heavy load exercise did not lead to significant change in the macrophage GSH levels (Fig. 2b). The GSH values for the groups S+C and S+T were 4.91 and 4.80  $\mu\text{mol/L}$ . For group HG+T and group MG+T, feeding high dose of GFP can significantly reduce the GSH of mouse macrophage after long-term heavy load exercise in comparison with group S+T. Figure 2b shows that macrophages produced significantly less GSH in the groups HG+T and MG+T in comparison to the group S+T. The GSH values for group HG+T and MG+T were 3.52 and 3.83  $\mu\text{mol/L}$ . For the group LG+T, the GSH value was 4.51  $\mu\text{mol/L}$  suggesting low-dose GFP in group LG+T had no significant effect on the mouse macrophages after heavy-load exercise.

#### **Effect of GFP on the NO from macrophages of heavy-load exercising mice:**

In comparison with S+T group, feeding high dose GFP in groups HG+T, MG+T can significantly improve the production of NO from macrophages after heavy-load exercise. The NO value for S+T was 4.77  $\mu\text{mol}/10^6$  cells/ml. It increased to 6.44  $\mu\text{mol}/10^6$  cells/ml for the group HG+T and 6.13  $\mu\text{mol}/10^6$  cells/ml for the group MG+T. Feeding low dose of GFP in the group LG+T had less effect on the production of NO in comparison with S+T group. The NO value for LG+T was 5.39  $\mu\text{mol}/10^6$  cells/ml.

#### **Comparison of macrophage phagocytic function in different groups:**

The phagocytosis rate in the S+T group dropped to 23.4% from 28% in the S+C group. In comparison with S+T group, feeding the low dose of GFP in the LG+T group had no significant effect on murine macrophage phagocytosis (Fig. 2d). The phagocytosis rate increased from 23.4 to 27%. On the other hand, the rates for the groups HG+T and MG+T only reached 28.2 and 27.1% and did not reach significant levels.

In the experiment for the phagocytic index, phagocytic index for the S+C group was 4.39 and it dropped to 3.8 in the S+T group. High and medium doses of GFP in the HG+T group and MG+T group can significantly improve macrophage phagocytic index after long-term, heavy-load exercise. Phagocytic index in the MG+T group was 4.4, and reached significant level. Phagocytic index in the MG+T group was 4.87, reached very significant level in comparison with group S+T. On the other hand, the phagocytosis index in the group LG+T was 4.0, slightly higher than 3.8 for the group S+T. Macrophage phagocytosis indexes in the group HG+T and group MG+T were significantly higher than that in the low-dose exercising group (LG+T) (Fig. 2e).

## **DISCUSSION**

**Impact of long-term heavy-load exercise on mouse peritoneal macrophages:** This study shows that long-term heavy load exercise can have significant effect on

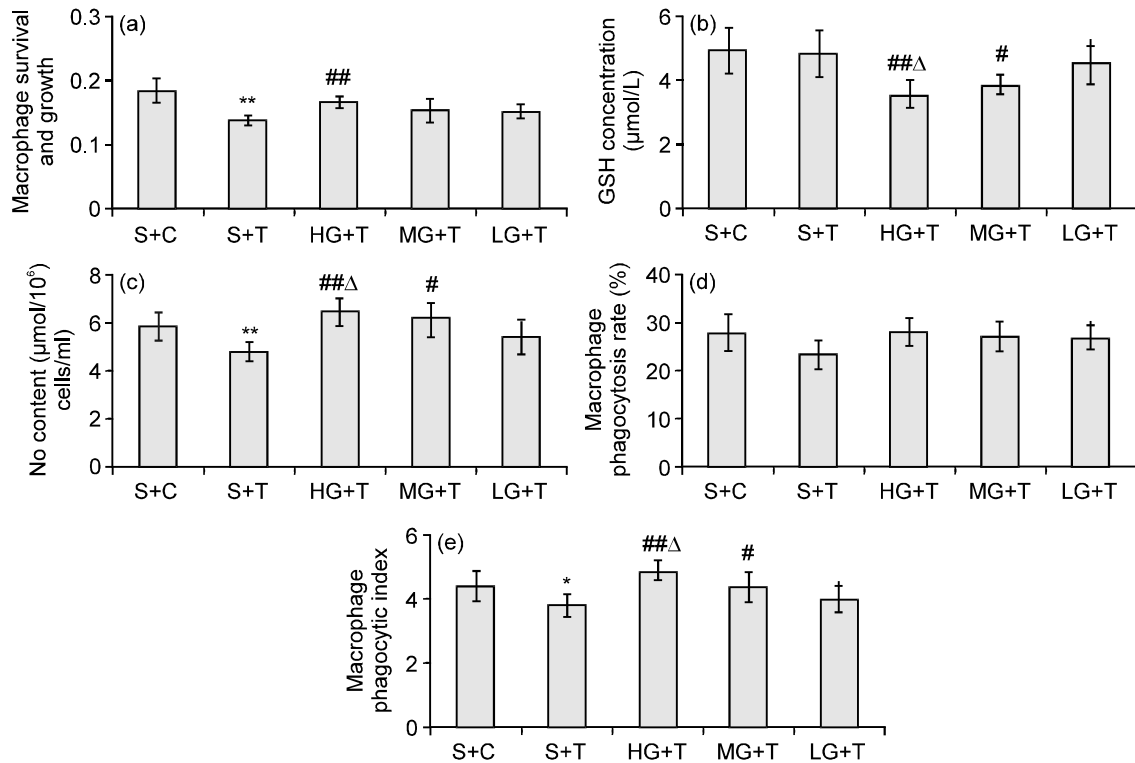


Fig. 2: Comparisons between groups S+C, S+T, LG+T, MG+T and HG+T for (a) macrophage survival and growth, (b) GSH concentration, (c) NO content, (d) macrophage phagocytosis rate and (e) macrophage phagocytosis index. Each value is represented with mean±standard deviation. \* and \*\* denote p<0.05 and p<0.01, respectively, in comparison with S+C group. # and ## denote p<0.05 and p<0.01, respectively, in comparison with the S+T group. Δ and ΔΔ denote p<0.05 and p<0.01, respectively, in comparison with the LG+T group

mouse peritoneal macrophages. This is consistent with other studies. Prolonged endurance exercise training suppresses immune function (Nieman, 2000; Pedersen and Nieman, 1998). Exhausted exercise inhibits the MHC II expression of peritoneal macrophages, thereby affecting the antigen-presenting of the macrophages, inhibiting the function of macrophages (Cecilia and Woods, 1999; Cecilia *et al.*, 2000). Yao and Zhang (1994) found that murine macrophages secrete IL-1 levels and phagocytosis decreased significantly after 6 weeks of 120 min/day of swimming training. Macrophage phagocytosis after overtraining was significantly lower than the reference control group with a decrease of approximately 27% (p<0.05) (Yao and Zhang, 1994).

Phagocytosis is the most important function of macrophage. Specific and non-specific immune processes can activate macrophages to produce a series of chemokines, cytokines and NO to protect the host against infection (Takizawa *et al.*, 1996; Sorokin *et al.*, 1997). Overtraining leads to decreasing phagocytosis and inhibit its ability to remove bacteria. This might be one of the mechanisms for increasing the risk of infection after overtraining. Our results show

macrophage phagocytosis rate and phagocytic index decreased significantly after long-term heavy-load exercise. This is consistent with above studies.

NO is a highly reactive and unstable free radical and one of the major effector molecules that can kill tumor cells and pathogens. NO macrophages produce plays an important role in host defense. It is considered an immune regulatory factor, which affects the broader immune function. Recent studies have shown that the concentration of the GSH in macrophages plays a regulatory role in the synthesis of NO and host-mediated cytotoxicity (Zamora *et al.*, 1997; Romao *et al.*, 1999).

Our results show that the activity of the macrophage cells significantly decreased after the heavy-load exercise, as did NO levels. The intracellular GSH content did not show significant change. Thus, the body of mice was in immunosuppression with long-term heavy load exercise stress.

**Influence of GFP on peritoneal macrophage activity of heavy-load exercising mice:** 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 glycosidic

bond.  $\beta$ -(1 $\rightarrow$ 3) glucan helps polysaccharides 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  $\beta$ -(1 $\rightarrow$ 3). High abundance of branches can lead to high activity of  $\beta$ -(1 $\rightarrow$ 3). The mechanism for the involvement of  $\beta$ -(1 $\rightarrow$ 3)-glucan in immune regulation can be attributed to the identification of  $\beta$ -1, 3GR on the surface of monocytes, macrophages, granulocytes and other cells (Thornton *et al.*, 1996).

A lot of research shows that polysaccharide is an effective biological immunomodulatory agent. Its components contain  $\beta$ -1, 3-,  $\beta$ -1, 6-glucan structures and thus can greatly activate cellular immune function, enhancing immunity. Nanba (Nanba, 1995) extracted and purified D-components and MD-components of Maitake. MD-component is the further extracted and purified component and has the same structure of the  $\beta$ -1,6-glucan of D-component. The D-component with a molecular weight of about  $1.4 \times 10^6$  is a proteoglycan composed of highly branched  $\beta$ -1, 6-glucan. This structure makes it a stronger biological regulator (Nanba and Kub, 1998).

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: interleukin), TNF (tumor necrosis factors), etc., thus it can enhance local tumor immune response and inhibit occurrence of tumor (Kodama *et al.*, 2002). Lei also shows that high doses of Maitake extract can significantly improve the murine peritoneal macrophage phagocytic ability ( $p < 0.05$ ) and the ability to produce NO ( $p < 0.01$ ) (Lei, 2011).

Our results show that the activity of the murine macrophages (MTT), the production of NO and the phagocytic index significantly increased with increasing dose of GFP. The dependent relationship between GFP dose and above biological parameters is obvious. On the other hand, when GFP enhanced the production of NO with murine peritoneal macrophages, the intracellular GSH content decreased with the increase of NO production. This is consistent with Zamora *et al.* (1997) and Romao *et al.* (1999). There was no significant change with the macrophage phagocytosis rate.

**Summary:** Our results show that after overtraining, NO content, macrophage survival and growth, phagocytic index in the MG+T group and the HG+T group are significantly higher than that of the S+T group and we observed no increased NO content, macrophage survival and growth, or phagocytic index in the LG+T. The impact of GFP on the macrophage phagocytosis in the LG+T group blood was not apparent after comparing with the S+T group. On the other hand, intracellular GSH also decreased in MG+T and HG+T groups in this study with the increase of NO content. No significant changes of GSH in S+T groups in comparison with S+C group.

**Conclusion:** In conclusion, 4 weeks of excessive exercise led to decreased peritoneal macrophage activities of the long-term-heavy-load exercising mouse. After supplementing GFP, peritoneal macrophage activities in terms of number of macrophage survival and growth, phagocytic index, NO, macrophage phagocytosis and GSH content all improved even though macrophage phagocytosis rate did not reach significant levels. The impacts of medium and high doses of complementary GFP were significant. We do not observe significant effects of the low dose of GFP on improving peritoneal macrophage activities of overtraining mice.

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