Original Communication

Immunomodulatory Effect of *Ganoderma Lucidum* Polysaccharides (GLP) on Long-Term Heavy-Load Exercising Mice

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Abstract: Long-term heavy-load exercise can lead to a decrease in the organism's immune response. In this study, we used 100 Kunming (KM) mice to investigate the immune-regulatory effects of *Ganoderma lucidum* polysaccharides (GLP) on long-term heavy-load exercising mice. Peripheral white blood cells (WBC), the absolute value of neutrophils (NEUT), the phagocytic function of macrophages, serum agglutination valence, and the number of plaque-forming cells (PFC) were evaluated 4 weeks after gavaging long-term heavy-load exercising mice with GLP. After exercise, the WBC count in peripheral blood, absolute neutrophil count, macrophage phagocytic index, serum agglutination valence, and the number of plaque-forming cells were significantly reduced in the mice not fed GLP. Both medium and high doses of GLP drastically increased peripheral WBC, absolute neutrophil count, macrophage phagocytic index, serum agglutination valence, and the number of plaque-forming cells in long-term heavy-load exercising mice. High doses of GLP increased peritoneal macrophage phagocytic rate considerably. With this study, we demonstrate that 4 weeks of heavy-load exercise can lead to exercise-induced immunosuppression in mice. A supplement of GLP fed to these mice improves both non-specific and specific immune responses among these mice. The effect for the high-dose GLP treatment is especially significant.

Key words: *ganoderma lucidum,* polysaccharides (GLP), long-term, heavy-load exercise, mice, immunomodulation

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Introduction

Ganoderma lucidum is known as a valuable health product in Chinese medicine. *Ganoderma lucidum* polysaccharides (GLP) are the main active ingredients of *Ganoderma lucidum.* Studies of *Ganoderma* medicinal and nutritional value (mainly in China, Korea, Japan, the United States) show that it contains certain bioactive ingredients (such as polysaccharides, triterpene), and can promote immune function, and prevent and treat various diseases [1].

A study from Mojadadi *et al.* [2] shows that GLP extract has a potent immunomodulatory effect and can be used for potentiation of the immune system against diseases. Zhu *et al.* [3] reported that GLP enhances the immunomodulatory activity of immune effector cells in immunosuppressed mice. Xia *et al.* showed that intraperitoneal injection of GLP BN3 can significantly promote the number of sheep red blood cells (SRBC) and induced plaque-forming cell (PFC) response, indicating that GLP promotes normal humoral immune function in mice [4]. Intraperitoneal injection of GLP showed 83.9 % inhibition on mouse S-180 sarcoma, with half the animals showing complete tumor regression in one study [5]. Kim *et al.* [6] reported that the inhibitory effect of GLP on mouse S-180 sarcoma was 87.6 % and that one third of the animals had complete regression of tumors. Mizuno *et al.* [7] isolated two polysaccharides from *Ganoderma lucidum* fruiting bodies with relative molecular masses of 1,050,000 (II) and 450,000 (V), respectively. When injected intraperitoneally, these polysaccharides inhibited inoculated subcutaneous S-180 growth in ICR-Jcl mice.

Heavy exercise induces marked immunosuppression. Moderate activity may enhance immune function, whereas prolonged, high-intensity exercise temporarily impairs the immune competence [8, 9]. Exhaustive exercise can also inhibit the expression of major histocompatibility complex (MHC II) in peritoneal macrophages, thereby affecting the antigen-presenting role of macrophages and inhibiting macrophage function [10, 11].

Until recently, there were no reports on how *Ganoderma lucidum* modulates immune regulation function. The objective of this study is to investigate the immunomodulatory effect of *Ganoderma lucidum* polysaccharides (GLP) on long-term heavy-load exercising mice. In this study, we divided the mice into several groups and investigated the impact of GLP feeding on the immune function in exercise-induced immunosuppressed mice. We intragastrically administered GLP to the long-term heavy-load exercising mice. Non-specific immune parameters [peripheral blood white blood cells (WBC), number of neutrophils, peritoneal macrophage phagocytic capacity] and specific immune parameters (the relative content of serum agglutination valence and the number of plaque-forming cells) were evaluated in order to conduct this study.

Materials and Methods

Experimental animals and grouping

One hundred male specific pathogen-free (SPF) grade Kunming (KM) mice, weighing 18–22 g, were used for this experiment. One ordinary guinea pig, weighing 300–400 g and one small fat tail sheep, weighing 22 kg, were used as complementary animals in this experiment to extract serum and blood for evaluating specific immune responses in long-term heavy-load exercising mice. The mice were divided into cages with five per cage, and were fed a diet that was in accordance with the national standard for routine feeding of rodents. The investigation was conducted in an animal husbandry environment of 23 °C ± 2 °C with humidity kept at 40 %–60 %. The rodents followed the natural circadian rhythm.

After the 100 mice underwent adaptive feeding for 3 days, they were randomly divided into a saline group (S), a saline + exercise group (S + T), a high-dose GLP + exercise group (H + T), a medium-dose GLP + exercise group (M + T), and a low-dose GLP + exercise group (L + T) (n = 20 for each group). At the end of intragastric administration and training, each group of mice was randomly divided into A and B subgroups. Subgroup A was used to determine complete blood count (CBC) and macrophage phagocytosis to evaluate non-specific immune response; subgroup B was used to determine serum agglutination valence (antibody relative content) and the PFC, which were used to evaluate specific immune response.

Experimental animal model design

Dose of GLP

GLP was administered by gavage. The standard human dosage of GLP is 3 g/day. Surface coefficient is a comprehensive parameter for comparing the conversion of the GLP between humans and animals according to body surface area. This coefficient considered a variety of factors, including metabolism. According to

body surface areas of humans and mice, the equivalent dose in the mice was calculated as follows:

Animal dose (per kg) = the known daily animal dose × body surface coefficient / unknown animal body weight. The body surface coefficient was assigned to be 0.0026 [12], thus the dose for mouse (per kg) = 3000 mg × 0.0026/0.02 kg = 390 mg/kg.

Gavage dose: the H + T group was dosed at 800 mg/kg/day; the M + T group was dosed at 400 mg/kg/day; and the L + T group was dosed at 200 mg/kg/day. GLP solutions were prepared with normal saline to concentrations of 800 mg/10 mL, 400 mg/10 mL, and 200 mg/10 mL, respectively. GLP aqueous solutions were intragastrically administered at 0.2 mL/day, and the S and S + T groups were given the same volumes of saline. Intragastric administration was performed daily between 8:00 am and 9:00 am for 4 weeks.

Exercise conditions and training designs

The mice were placed in a plastic swimming pool with dimensions of 100 × 60 × 50 cm^3. Water depth was set at 40 cm. The water depth was more than twice the mouse body length and water temperature was 27–30 °C. The water temperature was similar to that used in other studies [13].

Mice in the S group without exercise underwent normal physiological activities and did not swim. Mice in the S + T, H + T, M + T, and L + T groups swam with loads of 5 % of body weight (a fine copper wire equivalent of 5 % of mouse weight was tied to the root of the mouse tail). Flowing water forced the mice to swim. GLP 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 minutes with daily increases of 5 minutes until 45 minutes per day was reached. Exhausted mice that exercised less than 45 minutes were quickly picked up, dried off, allowed to rest for 5 minutes, and then put back into the water to continue swimming. Exhaustion is accompanied by increased serum-free radical levels and decreased hypoxia tolerance time. The cumulative 45 minutes/day training time was required. At the end of the training, mice were quickly dried off with a hair dryer.

A total of 14 mice died due to improper gavage, untimely judgment of mice exhaustion, and failure to pick the mice up from drowning. One mouse died in the saline group, four died in the S + T group, two died in the H + T group, four died in the M + T group, and three died in the L + T group.

Experiment measurements

Subgrouping

At the end of intragastric administration and training, each group of mice was randomly divided into A and B subgroups. Subgroup A was used to determine CBC and macrophage phagocytosis to evaluate non-specific immune responses; subgroup B was used to determine serum agglutination valence (relative antibody content) and the PFC, which are used to evaluate specific immune response.

Evaluation of WBC, NEUT

Two to 3 drops of EDTA were transferred into 1.5 mL centrifuge tubes which were dried for 2 days at 50 °C for later use. Four days before the end of the training, tail blood was collected from mice in subgroup A. The mouse was placed in the restraint holder with tail exposed. The tail was immersed in 45ºC water for a few minutes, so that the tail vein became full. The tail was sterilized and dried with a sterile gauze. Five mm was snipped off the tip of the tail. The tail was pushed from the root to the tip, and the blood sample was collected at the tip of the tail with a pipette. The blood was pipetted into the centrifuge tubes containing EDTA, and the bottom of the tube was continuously tapped to give the blood full access to the EDTA. About 0.1 mL of blood was collected from each mouse [12]. CBC analysis was performed using three automatic classification hemocytometers (M-2100; Blue Bridge Medical Technology Co., Ltd.).

Determination of macrophage phagocytic function

Six g of soluble starch was dissolved into 100 mL of broth culture fluid. It was mixed and boiled to sterilize for later usage. Four days before the end of training and after tail blood collection and CBC analysis, each mouse from subgroup A was intraperitoneally injected with 6 % soluble starch, 1 mL per mouse, and then continued with gavage and training. Three days later, the mouse was intraperitoneally injected with 2 mL of 5 % chicken red blood cell suspension, with their abdomens rubbed softly after injection. Thirty minutes after the injection, 1 mL of normal saline was injected intraperitoneally. To collect ascites, the mice were placed in their natural standing position. At the leading edge of the pubis, both sides along the abdominal midline were sheared. After sterilization

and anesthesia, the skin was tightened around the puncture and then pierced vertically. The ascites were withdrawn slowly with a syringe. A droplet was placed onto a clean glass slide and pushed into thin slices. Once the drop air-dried, it was fixed with methanol for 4–5 minutes. Swiss Giemsa dye was added and allowed to stain for 3 minutes. The slide was washed with running water, air-dried, and observed using a microscope.

One hundred macrophages were counted randomly. The percentage of macrophages that engulfed the chicken red blood cells was used to determine the phagocytosis rate. The number of macrophages that engulfed chicken erythrocytes divided by 100, yielding the phagocytic index [13].

Determination of serum agglutination valence (relative antibody content)

1st step: Prepare SRBC. Sheep blood was collected from the jugular vein, and it was stored in the EDTA solution at a ratio of 1:5. The mixture was washed three times with saline, centrifuged at 2000 revolutions/minute for 5 minutes and the supernatant was removed. The SRBC solutions with hematocrit ratios of 1 %, 1.25 %, and 5 % were prepared with saline. The solution of the suspended SRBC with a density of 2×10^9 cells/mL was prepared using Hanks solution, pH 7.2.

2nd step: Immunize mice. At the end of gavage and training, each mouse in subgroup B was injected intraperitoneally with 0.2 mL of 5 % SRBC for the mice to generate PFC and produce antibodies.

3rd step: Determination of serum agglutination valence. Four days after immunization, mice from subgroup B were hung upside down and had blood samples taken from the neck (mice were immunized with 5 % of SRBC suspension 4 days prior). Blood samples were incubated in a 37 °C water bath for 2 hours and then transferred to the refrigerator for 2 hours to separate the serum and perform antibody agglutination. Fifty μL of saline was added to 1 to 10 wells of each row, and then 50 μL of a 1:20 dilution of serum sample was added to the first well of each row. A series of 1:2 dilutions was made from well 2 to well 9. Fifty μL was removed from the ninth well after the dilution. The tenth well contained only saline. In each row, the serum dilutions in wells 1 through 9 were 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, and 1:10240, respectively. Fifty μL of 1.25 % SRBC solution was added to each well. Each well was mixed with a micro-oscillator for 3 minutes and then placed into a moist box and covered. The box was insulated in 45 ºC water for 1 hour and transferred to room temperature for 15 minutes for observation. There was no agglutination in saline control wells. Agglutination from the first well of each row was observed with the liquid turbidity and the size of sediment in the bottom of the well. The serum agglutination valence was defined as the final serum dilution at which an obvious agglutination was visible with a certain amount of antigen.

PFC assay (spleen hemolytic plaque formation)

To prepare the guinea pig serum (complement), the guinea pig's ear was disinfected, cut with scissors, and coated with a 20 % sodium citrate solution. The blood was then collected, and the serum separated.

Spleen cells from mice (mice were immunized with 5 % of SRBC suspension solution 4 days prior to collection) in subgroup B were re-suspended with saline to a density of 1×10^7 cells/mL. The 0.1-mL spleen cell suspension and 5 % of SRBC suspension were rapidly mixed with 3.5 mL of 0.7 % agarose in a tube that was insulated in the 47ºC water bath. Immediately after shaking, the mixture was poured into a plate layered with agarose at the bottom. The plate was jiggled and the cell/agarose mixture was spread until the agar solidified. Plates were incubated at 37 ºC for 1 hour. Two mL of 1:10 dilution of guinea pig serum was added as a complement so that it would evenly cover the surface of the plate. The plates were incubated at 37 ºC for 30 minutes, followed by room temperature incubation for 1 hour and overnight incubation at 4 °C in the refrigerator. The next day, the complement was poured out and the plates were observed under low-magnification. The centers of the plaques were lymphocytes that were surrounded by a transparent area. The number of plaques in each plate defined the plaque number per million cells.

Data processing

The data was presented as mean ± standard deviation (X ± SD). SPSS11.5 statistical software was used for data processing. S, S + T, H + T, M + T, and L + 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 defined to be 0.05, while that of a very significant level was defined to be 0.01.

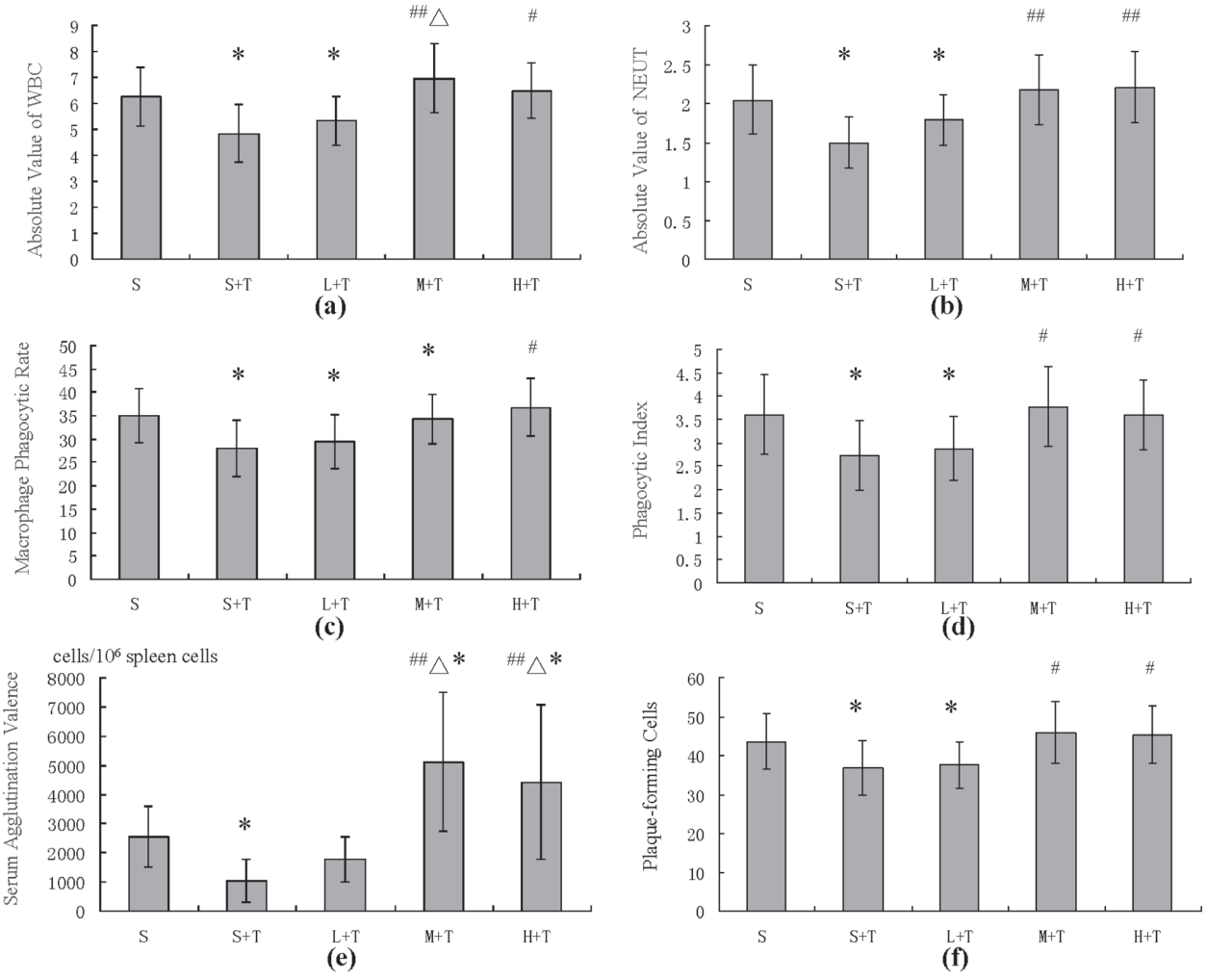

Figure 1: Comparisons between groups S, S + T, L + T, M + T, and H + T for (a) WBC, (b) NEUT, (c) macrophage phagocytic rate, (d) phagocytic Index, (e) serum agglutination valence, and (f) plaque-forming cells. Each value is represented with mean ± standard deviation. * denotes $p < 0.05$ in comparison with S group, # and ## denote $p < 0.05$ and $p < 0.01$ in comparison with S + T group, respectively. Δ denotes $p < 0.05$ in comparison with L + T group.

Experimental Results

Effect of heavy-load exercise training and GLP gavage on non-specific immune response in mice

After 4 weeks of heavy-load exercise training, peripheral WBC counts and absolute neutrophil counts for mice in the S + T group were significantly lower than those in the S group (Figures 1a, 1b).

The peripheral WBC counts and absolute neutrophil counts for mice in the M + T and H + T groups were significantly higher than that of the S + T group, and showed little difference from peripheral WBC counts and absolute neutrophil counts in the S group. The mean values of WBC for S, S + T, L + T, M + T, and H + T were 6.27, 4.85, 5.34, 6.97, and 6.50×10^9 cells/L, respectively (Figure 1a). The mean values of NEUT for S, S + T, L + T, M + T, and H + T were 2.05, 1.50, 1.79, 2.18, and 2.21×10^9 cells/L, respectively (Figure 1b). It is also noted that the normal ranges for WBC and NEUT are $4.0 \sim 12.0 \times 10^9$ cells/L and $0.7 \sim 4.0 \times 10^9$ cells/L, respectively.

After 4 weeks of heavy-load exercise training, the peritoneal macrophage phagocytic rate and the phagocytic index decreased significantly among the S + T group of mice (Figures 1c, 1d). Similar to Figure 1a, Figure 1b, the mouse peritoneal macrophage phagocytic rate in the H + T group (36.8 %), was also observed to be higher than that of the S + T group (28 %), and at the same level as measured for the

S group (35 %) (Figure 1c). The mouse peritoneal macrophage phagocytic index for different groups in Figure 1d showed the same changes as the peritoneal macrophage phagocytic rate in Figure 1c. The mean values of the phagocytic index for S, S + L, L + T, M + T, and H + T were 3.61, 2.74, 2.88, 3.78, and 3.59, respectively.

Effect of heavy-load training and GLP gavage on specific immune response in mice

After 4 weeks of heavy-load exercise training, the agglutination valence was significantly lower in the S + T group. In comparison, the serum agglutination valences for mice in the M + T and H + T groups were 2–3 times higher than in the S + T group and L + T groups (Figure 1e). The mean values of serum agglutinate valences for S, S + T, L + T, M + T, and H + T groups were 2560, 1040, 1778, 5120, and 4410, respectively.

In comparison to 43.6×10^6 cells for the S group, the PFC in the S + T group was lower at 36.74×10^6 cells. The PFC number was also observed to be higher among mice in the M + T and H + T groups than in the S + T group (Figure 1 f). The PFC number was 45.3×10^6 cells with a SD of 7.34×10^6. This value was similar to the number of plaque-forming cells for the S group.

Discussion

Long-term heavy-load exercise on immune response

Prolonged endurance exercise or long-term intensive training can suppress immune function, including reducing the number of lymphocytes in the circulating blood, changing the relative proportion of the subtypes of lymphocytes, inhibiting the cytotoxic activity of natural killer (NK) and lymphokine-activated (LAK) cells, and reducing the secretion of mucosal secretory IgA [14]. Fatiguing exercise has also been associated with a decrease in certain functions of neutrophils and immunosuppression [15]. Excessive and exhausting physical loads in elite athletes significantly decrease natural killer (NK) cell activity (NKCA) [16].

Intense and exhaustive exercise is considered an important immunosuppressor while moderate regular exercise has been associated with significant disease protection [14, 17]. The mechanisms of exercise-induced immunosuppression are currently thought to relate to dysfunction of the neuroendocrine system in immune regulation, activation of immunosuppressive cells, and production of immunosuppressive cytokines [11]. Exhaustive exercise can inhibit the expression of major histocompatibility complex (MHC II) in peritoneal macrophages, thereby affecting the antigen-presenting role of macrophages and inhibiting macrophage function [8, 10]. Studies have found that repeated 90-minute, 75 % VO_2 max exercise for 3 days can cause a cumulative inhibitory effect on salivary IgA levels [18]. Another study also found that 12 hours after 60 minutes of 80 % VO_2 max exercise, the same exercise can cause an inhibitory increase of natural killer cytotoxic activity (NKCA) [19]. Total white blood cell and NK cell numbers declined during long-duration high-intensity exercise training. The reduction of white blood cell count may be due to the removal of cells from the circulation, increase of cell turnover rate, or a combination of both [20].

The results of this study showed that peripheral blood WBC count and absolute neutrophil count for mice in the S + T group were significantly lower than in the S group. Peritoneal macrophage phagocytic rate and phagocytic index were also remarkably reduced. The serum agglutination valence and PFC were also observed to be lower in the S + T group than in the S group. Our study was consistent with previous studies and indicated that long-term heavy-load exercising mice were in exercise-induced immunosuppression.

Effect of GLP on immune response on long-term heavy-load exercising mice

There are many other studies on the effect of GLP on immune function. GLP can significantly restore the effects of aging; examples include reductions in mouse PFC response, ConA (concanavalin A)-induced lymphocyte proliferative response, MLR (mixed lymphocyte reaction), and spleen lymphocyte DNA polymerase α activity [21]. GLP can also promote the ability of ConA-induced lymphocyte proliferation, improve the function of the reticuloendothelial system in mice, and enhance the body's nonspecific and specific cellular immune function [22]. An established immunosuppression mice model using cyclophosphamide showed that black GLP can increase spleen and thymus indexes, promote T and B lymphocyte proliferation, and increase IL-2 and TNF-α content in serum [23]. GLP extract capsules can help increase ConA-induced mouse spleen lymphocyte proliferative

capacity and the thickness of mouse left hind hock. GLP can help improve serum hemolysin and hemolytic plaque [24].

GLP chemical structure plays a vital role in its biological activity, including molecular structure, molecular weight, branched-chain, branched-chain groups, and main chain structure. The key mechanism for immune regulation may involve identifying the β-(1 → 3) GR (gene rearrangement) on the surface of the membranes of monocytes, macrophages, and granulocytes [25,26]. Of GLP's complex biological activity and functions, immunomodulatory activity is the most important. These polysaccharides and polysaccharide-protein complexes are thought to enhance cell-mediated immune responses *in vivo* and *in vitro* and act as biological response modifiers. They are considered to be multi-cytokine-inducing agents to regulate gene expression of cytokine and various cytokine receptor-induced immune activities [27].

This study has important implications for humans. According to body surface area comparisons of humans and mice, the equivalent dose in mice, calculated based on a known human dosage of GLP, is 3 g/day. Conversely the equivalent high dose in human is 6 g/day. This is at non-toxic level, less than the 15 g/day of the national standard, and well below the LD50 value of GLP [28].

Summary

Our study shows that long-term heavy-load exercising mice were in exercise-induced immunosuppression, and that GLP can improve non-specific immune responses in long-term heavy-load exercising mice. High and medium doses of GLP can both significantly increase peripheral WBC and absolute value of peripheral blood neutrophils in long-term heavy-load exercising mice to the same level as a control group. The peritoneal macrophage phagocytosis rate and peritoneal macrophage phagocytic index in long-term heavy-load exercising mice treated with high doses of GLP were also higher, and at the same level as measured for S group.

GLP can also improve specific immune responses in long-term heavy-load exercising mice. Both high and medium doses of GLP can drastically increase serum agglutination valence among long-term heavy-load exercising mice, and the effect was better than that of low doses of GLP. High and medium doses of GLP can significantly increase the number of PFC in long-term heavy-load exercising mice.

In conclusion, four weeks of heavy-load exercise can lead to exercise-induced immunosuppression in mice. A supplement of GLP enables improvement of non-specific and specific immune responses among long-term heavy-load exercising mice. Effects of high and medium doses of GLP supplementary were significant.

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