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Comparative immune regulatory effects of *Cordyceps militaris*, *Flammulina velutipes* extracts along with glutamine on rat's body after exhaustive exercise

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ABSTRACT

Exhaustive exercise can impair immune balance in the rat's body due to change of Th1- and Th2-related cytokines, and some natural products can regulate immune banlance. The aim of this study is to compare the immunoregulation action of *Cordyceps militaris* cordycepin, *Flammulina velutipes* polysaccharide, and glutamine on rats that were subjected to an intense running session by analyzing Th1- and Th2-related cytokines and transcription factors. All rats were subjected to the exercise protocol except for a subgroup rat that was used as the negative control group. Blood samples were collected for the evaluation of biochemical parameters. The protein expression of cytokines and transcription factors were examined by ELISA and Western blot. Meanwhile, the mRNA expression of the cytokines and transcription factors in the spleen of rats was assessed via real-time PCR Furthermore, the ratio of Th1/Th2 was determined via flow cytometry. The blood analysis results show that cordycepin was more effective than polysaccharide and glutamine in regulating all kind of indexs. Three immunomodulator could adjust the expression of the transcription factors and cytokines to normal levels, whereas *Flammulina velutipes* polysaccharide and glutamine could only induce several of these transcription factors or cytokines. Thus, this study implies that the action of cordycepin had the most significant effect on the immunoregulation of rats that were subjected to exhaustive exercise.

KEY WORDS: CORDYCEPIN; *FLAMMULINA VELUTIPES* POLYSACCHARIDE; GLUTAMINE; EXHAUSTIVE EXERCISE; IMMUNOREGULATION

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The dual effect of exercise on the immune system has been previously studied in great detail, and many epidemiological studies have demonstrated that moderate exercise improves the immune function and increases resistance to infections. On the other hand, exhaustive exercise had opposite effects(Dos Santos, *et al.*, 2009, Nieman, 2007), most of which have been associated with an increased incidence of respiratory tract infections(Nieman, 1997). The cytokine from the T cell has a critical function in the development of immune responses against invading pathogens. The cellular basis for this heterogeneity in cytokine is due to the presence of two distinct cytokine-producing CD4+ T helper and CD8+ T cytotoxic cell phenotypes. The CD4+ T helper has been designated as Th1 and Th2 cells based on their distinct cytokine profile(Mosmann, *et al.*, 1986). Th1 cells secrete IFN-γ, IL-2, and tumor necrosis factor-γ, and are responsible for defense against intracellular pathogens, whereas Th2 predominantly secrete IL-4, IL-5, and IL-13, and are responsible for defense against extracellular pathogens (Schoenborn, *et al.*, 2007, Seder, *et al.*, 1994, Kutukculer, *et al.*, 2016).

The imbalance of the Th1/Th2 ratio, which is attributed to exhaustive exercise, can cause an imbalance in the immune function of athletes. Thus, researchers should use special drugs to recover the Th1/Th2 ratio of athletes that are prone to exhaustion.

In terms of effector T-cell differentiation, Th2 cells development was the first process to be linked to the actions of a cytokine. IL-4 was recognized early on to promote the development of Th2 cells subset. Later, this activity was shown to operate through the actions of STAT6. Th cells stimulated with interleukin-4 (IL-4) and antigen, through the T-cell receptor (TCR), upregulate GATA3 transcription. The GATA3 protein induces heritable remodelling of the IL-4 locus, which is characteristic of fully differentiated Th2 cells. Th cells activated under Th1-inducing conditions are exposed to IFN-γ signalling during TCR engagement, leading to the activation of signal transducer and activator of transcription 1 (STAT1). T-bet was identified as a Th1 cells specific factor that can induce the production of IFN-γ by developing Th2 cells. T-bet seems to be expressed in developing and committed Th1 cells (Murphy, *et al.*, 2002, Tian, *et al.*, 2015 and Kutukculer, *et al.*, 2016).

Thus, the change of Th1 and Th2 number can through the levels of these transcription factors. *Cordyceps militaris* is a traditional Chinese medicinal mushroom that has been shown to have a variety of benefits on human health, such as antitumor, antimutagenic, and hypoglycemic effects (Thomadaki, *et al.*, 2008). Cordycepin from *Cordyceps militaris* is involved in many pharmacological activities, which include immunological stimulation, anticancer, antivirus, and anti-infection activities(Nakamura, *et al.*, 2005). Recently, the level of several cytokines from Th1 and Th2 has been reported to all increase after mouse splenocytes were exposed to purified cordycepin (Jeong, *et al.*, 2012, Tian, *et al.*, 2015 and Kutukculer, *et al.*, 2016).

Flammulina velutipes is as an edible and medical resource, and many of its bioactive molecules have been identified. Polysaccharides are the best known and the most potent *Flammulina velutipes*, from which substances with antitumor and immunomodulating properties can be derived(Wasser, 2002). In addition, studies have shown a decrease in plasma glutamine concentration after exhaustive exercise in humans and animals, (Castell, 2003). These small decreases in the plasma glutamine concentration are sufficient to promote immunosuppression (Hiscock, *et al.*, 2002 Dos Santos, *et al.*, 2009 and Harriss, *et al.*, 2011 and Tian, *et al.*, 2015).

The objective of the present study want to use some sensitive biochemical criterions that can response immune injury and recover after injury to compare that cordycepin, polysaccharide from *flammulina velutipes*, and glutamine, which are natural medicine with the possible of immunomodulatory effect on rat's body after a high-intensity running session.

MATERIAL AND METHODS

CONSTRUCTION OF EXHAUSTIVE ANIMAL MODEL

A total of 50 eight-week-old Sprague Dawley (SD) (160–200 g) rats were purchased from Shandong University. The rats were routinely screened for common rat pathogens. Immediately after arrival, the rats were weighed and randomly divided into five subgroups (n = 10) as follows: control group (C; the group was not subjected to the exercise protocol); exhaustive exercise (EE; after exercise, all rats were perfused with 2 mL of sterile saline); cordycepin (CM; after exercise, all rats were perfused with 2 mL of aqueous solution of cordycepin based on a dosage of 400 mg/kg weight); *Flammulina velutipes* (FV; after exercise, all rats were perfused with 2 mL of aqueous solution of *Flammulina velutipes* polysaccharide based on a dosage of 150 mg/kg weight) and glutamine (G; after exercise, all rats were perfused with 2 mL of aqueous solution of glutamine based on a dosage of 100 mg/kg weight). Except for the C subgroup, all rats were accustomed to running on a rodent treadmill based on the exercise protocol, as shown in Table 1. On the tenth week, the exercise was stopped. All animals were killed by decapitation after having recovered on the tenth week. All animal procedures were approved by the Sports Science Research Center of Shandong Prov-

ince Animal Investigational Committee, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of People's Republic of China and IJSM's ethical standards document(Harriss, *et al.*, 2011), This study has been approved by the ethics committee of Sports Science Research Center of Shandong Province.

HEMATOLOGICAL INVESTIGATIONS

Blood samples were collected from the killed rats for hematological investigations. The parameters investigated were leucocyte count, red blood cell count, hemoglobin content, hematocrit value (PCV), creatine kinase, and urea nitrogen(Silva, *et al.*, 2007, Spiess, *et al.*, 1998, Tilak, *et al.*, 2007).

QRT-PCR

Total RNA was isolated from less than 100 mg of spleen tissue by using a TRI-zol Reagent (TaKaRa) according to the manufacturer's instructions. cDNA was obtained according to Wang's method(Wang, *et al.*, 2011). The cDNA product was stored at -20 °C until use. The oligonucleotides for real-time PCR (Table 2) were designed using the Primer Premier 5.0 software (PREMIER Biosoft International). BLAST analysis was then performed against other organism genome sequence for specificity confidence (http://www.ncbi.nlm.nih.gov/BLAST/). The Mfold web server was used to avoid positioning on risky secondary structures. Primer specificity was analyzed before qRT-PCR.

The reaction mix (final volume of 20 µL) consisted of 10 µL of SYBR Ex Taq II, 0.4 µL of ROX Reference Dye, 0.8 µL of each primer (final concentration of 250 nM), 7 µL of ddH_2O, and 1 µL of cDNA (dilution factor of 1/10). The thermocycling program consisted of two phases, which include one cycle of 94 °C for 3 min, 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. After completion of these cycles, the melting-curve data were obtained to verify PCR specificity, contamination, and the absence of primer dimers. Each sample was tested in triplicates in an ABI 7300 real-time PCR apparatus (Applied Biosystem). The relative expression levels of MTs were normalized against the GAPDH gene as an internal standard. The relative quantity levels of MT mRNA expression were obtained using the formula $2^{-\Delta\Delta Ct}$.

ELISA ASSAYS

The tested rats were killed by breaking their necks on the tenth week after a week of rest. Their serum were collected from whole blood and immediately frozen at -80 °C for ELISA assays. IL-4 and IFN-γ were measured using ELISA kits according to the manufacturers' instructions (Dakewe Biotech Co. Ltd., China). Microtitration plates were coated overnight with 100 µL of a 10 µg/mL solution of anti-IL-4 or IFN-γ fragments in a carbonate buffer (pH 9.6). After blocking and washing, 100 µL of the tissue supernatant was incubated for 1 h at room temperature. The plates were washed and incubated with peroxidase-linked anti-IL-4 or IFN-γ fragments for 1 h at room temperature. After washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added to the plates for color development. The reaction was stopped after 15 min by adding 20 µL of a 1:20 sulfuric acid solution. The absorbance at 450 nm was measured using an ELISA plate reader. Duplicate readings were taken for all samples, and the means were calculated. The cut-off was determined using the values from the control group.

WESTERN BLOT

Western blot analysis tissue were lysed with sample buffer [62.5 mmol Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mmol dithiothreitol, and 0.1% bromophenol blue] and heated at 100°C for 5 – 10 min before being loaded and separated on 10% SDS-polyacrylamide gels. The proteins were electrotransferred to a nitrocellulose membrane in transfer buffer containing 48 mmol Tris-HCl, 39 mmol glycine, 0.037% SDS, and 20% methanol at 4°C for 1 h. Non-specific binding to the membrane was blocked for 1 h at room temperature with blocking buffer [5% non-fat milk in Tris-buffered saline (TBS), and 0.1% Tween 20]. The membranes were incubated for 16 h at 4°C with various primary antibodies (STAT6, STAT1, GATA3, T-bet and Actin) in blocking buffer at the dilutions specified by the manufacturers, followed by incubation with horseradish peroxidase–conjugated secondary antibody for 1 h. Membranes were then washed, and visualized using the enhanced chemiluminescence system.

FLUORESCENT LABELING AND FLOW CYTOMETRY

Whole blood (200 µL) was directly pipetted into a 12×75 mm fluorescence-activated cell sorting tube containing 20 µL of monoclonal antibodies for the T-helper surface antigen CCR5 and CCR8 (CD4-PerCP, Becton Dickinson, San Diego, CA), and was then incubated at room temperature in the dark for 10 min. About 0.5 mL of 1% paraformaldehyde was then added for 8 min to stabilize the monoclonal antibody-surface antigen complex. RBCs were lysed using 3 mL of a 1×fluorescence-activated cell sorting lysing solution (Becton Dickinson) for 8 min. After centrifugation at 1,930 rpm for 5 min, the supernatant was aspirated. A 1×permeabilizing solution (500

μL, Becton Dickinson) was added into the pellet, and the solution was incubated for 10 min at room temperature in the dark. After washing with 3 mL of the buffer (1% bovine serum albumin, 0.1% NaN_3, 1×PBS), cytokine-specific antibodies (20 μL, CCR5-FITC, CCR8-PE, Becton Dickinson) were added to the cells and incubated for 30 min at room temperature in the dark. After one final wash, the cells were resuspended in 1% paraformaldehyde (500 μL) and stored at 4 °C until flow cytometry analysis. The cells were obtained using a Beckman-Coulter EPICS XL flow cytometer (Miami, FL), and the data were analyzed using the CellQuest software. The percentages of Th1 and Th2 cytokine-producing cells were identified as the number of CCR5- and CCR8-positive cells present, respectively, in the total population of CD4+ T-helper cells(Chanova, *et al.*, 2016) A minimum of 5,000 CD4+ cells was counted from each sample.

STATISTICAL ANALYSIS

All results are presented as mean ± SD. The data were evaluated using the SPSS software (SPSS company, USA). Statistical differences were determined using Student's t-test with a significance level set at $P < 0.05$ or $P < 0.01$.

RESULTS

CHANGES OF THE HEMATOLOGIC PARAMETERS

The blood parameters mentioned in the above section between the C and EE subgroups show a significant difference ($P<0.05$ or $P<0.01$). The results of the EE subgroup show an imbalance in the immune function of the rats. CV subgroup could significantly elevate RBC quantity, Hb concentration, and PCV to normal levels (C subgroup) when compared with FV or G subgroups after the tenth week ($P<0.05$ or $P<0.01$). However, these three blood parameters between FV and G subgroups also had an obvious increasing, as shown in Table 3. The leucocyte counts were 4.97±0.27×109 and 4.77±1.84×109/mL in the CM and G subgroups, respectively. These results suggest that the regulating ability of cordycepin and glutamine on leucocyte restoration was lower than that of *Flammulina velutipes* polysaccharides, which elevated the leucocyte quantity to normal levels (6.20±0.85×109/mL) on the tenth week. The blood parameters' analysis demonstrated that cordycepin was more effective than *Flammulina velutipes* polysaccharides and glutamine in alleviating the condition of the rats from exhaustive exercise because estimating the fatigue of athletes is usually determined by detecting serum urea nitrogen (BUN) and creatine kinase(CK) (indicators of muscle damage)(Haralambie, 1973, Shi, 2005).

As data of Table 3 show, although the concentration of urea nitrogen and creatine kinase for CV, FV and G subgroups that all decreased when compared to the EE subgroup, whereas *Flammulina velutipes* polysaccharide (7.78±0.27mmol/L) or glutamine (7.99±0.50mmol/L) is effective than cordycepin (8.99±0.15 mmol/L) in decreasing BUN ($P<0.05$ or $P<0.01$), but cordycepin (166.00±10.84U/L) is more effective than *Flammulina velutipes* polysaccharide (231.00±17.87U/L) or glutamine (206.33±18.96U/L) in decreasing CK ($P<0.05$ or $P<0.01$). These results show that three immunomodulator could repair the damaged muscle tissue, as well as reduce the release of creatine kinase from cells. Their treatment effects for tissue damage were very ideal, but the more effective treatment was cordycepin.

CHANGE OF TH1- AND TH2-RELATED CYTOKINES AND TRANSCRIPTION FACTORS

The production of cytokines associated with Th1 and Th2 cells in the serum was evaluated *via* ELISA. As shown in Table 3, we observed that level IFN-γ did icrease after running if the rats were treated with cordycepin, *Flammulina velutipes* polysaccharide and glutamine. Additionally, CM subgroups significantly increased the level of IL-4 compared to the EE subgroup.

The mRNA expression of Th1- and Th2-related transcription factors and cytokines in the spleen of rats was determined by real-time PCR assay (Fig. 1). After a week of rest, the expressions of IL-4, GATA3, T-Bet, IFN-γ, STAT1, and STAT6 in the EE subgroup remained higher than those in the C subgroup, especially STAT1 and STAT6. However, the expression levels of STAT1 and STAT6 in the CM, FV, and G subgroups were already close to those of the C subgroup. The mRNA levels of T-bet and GATA-3 in the CM, FV, and G subgroups also significantly decreased. But the mRNA concentrations of IFN-γ and IL-4 in the FV and G subgroups remained close to or higher than those of the EE subgroup, respectively. These results suggest that cordycepin was more effective in regulating Th1- and Th2-related transcription factors than *Flammulina velutipes* polysaccharide and glutamine.

The level of the transcription factors expression associated with Th1 and Th2 cells in the spleen was evaluated via western blot, show in Fig. 2. After a week of rest, the protein expressions of STAT6 and T-bet significantly increased, whereas the protein expressions of STAT1 and GATA3 were decreased remarkablely in the EE subgroup. CM subgroup can reduce the expressions of STAT6 and T-bet, in addition to elevate the expressions of STAT1 and GATA3. Four types of transcription

factors were mostly close to C subgroup. However, only the protein levels of STAT6 significantly decreased in FV and G subgroups, another level of the transcription factors did not change toward nomal levels(C subgroup).

CHANGE OF TH1 AND TH2 CELLS BALANCES

The amounts of CCR5 and CCR8 could be used to estimate the number of Th1 and Th2 cells because CCR5 and CCR8 are characteristic of Th1 and Th2 cells, respectively(Kutukculer, *et al.*, 2016). In the present study, Th1- and Th2-related cytokines, namely, CCR5 and CCR8, in the whole blood of rats were analyzed *via* flow cytometry. The CCR5 and CCR8 results for the five subgroups are summarized in Table 5, and were calculated to assess the regulating effect of cordycepin, *Flammulina velutipes* polysaccharides and glutamine. Under the effect of cordycepin, the ratio of CCR5/CCR8 (0.155±0.043) was mostly close to the normal level (0.163±0.032) than those of *Flammulina velutipes* polysaccharides and glutamine that also down-regulated the ratio of CCR5/CCR8.

DISUSSION

Over the past two decades, many accumulated evidence have demonstrated the diverse responses of T1 and T2 cells to exhaustive exercise or long-term training at moderate and high intensities(Zhao, *et al.*, 2012). Studies on the immune-regulating effect of natural preparations such as *Herbkines*, *WooKiEum*, *Bouum-Myunyuk-Dan*, *Cool-Cool*, *Pelargonium sidoides*, and so on have been previously reported(Luna, *et al.*, 2011, Shin, *et al.*, 2004). Cordycepin, *Flammulina velutipes*, and glutamine were also used in regulating the immune system(Ike, *et al.*, 2012, Jeong, *et al.*, 2012, Pohlenz, *et al.*, 2012). In the present study, the regulating ability of cordycepin, *Flammulina velutipes* polysaccharide, and glutamine on the immune system was compared and analyzed after the rats showed signs of immunity imbalance caused by exhaustive exercise.

The quick recovery of the immune imbalance of athletes caused by exhaustive exercise is a common strategy to prevent upper respiratory tract infections (URTIs) after the athletes have terminated their short-term intense training. In this study, the concentration level of serum urea nitrogen and creatine kinase in the EE subgroup remained higher than those of the C subgroup on the tenth week. The nitrogen and creatine kinase results show that the fatigue level of rats in the EE subgroup remained high because of exhaustive exercise (Lee, *et al.*, 2010). Cordycepin, *Flammulina velutipes* polysaccharides, and glutamine could regulate the concentration of urea nitrogen and creatine kinase, as shown from the results. Thus, cordycepin was more effective than *Flammulina velutipes* polysaccharides and glutamine in alleviating fatigue. Similarly, cordycepin also showed a remarkable ability in regulating RBC, Hb, and PCV. The induction of *Flammulina velutipes* polysaccharides on leukocytes was more remarkable than that of cordycepin and glutamine.

The JAK-STAT pathway is the major signaling pathway that regulates Th1 and Th2 differentiation and functions (Tian, *et al.*, 2015). IFN-γ reduction was suppressed by down-regulating the STAT1/STAT4/T-bet pathway, which is critical for Th1 differentiation, as well as the GATA3/STAT6 pathway, which is essential for Th2 differentiation(Jia, *et al.*, 2011, Murphy, *et al.*, 2002). Our results confirm that cordycepin was more effective in regulating Th1- and Th2-related transcription factors than *Flammulina velutipes* polysaccharides and glutamine. Thus, cordycepin could quickly regulate Th1- and Th2-related transcription factors to normal levels.

Studies have suggested that cordycepin purified from *Cordyceps militaris* could up-regulate the level of Th1 cytokine, IL-12, and Th2 cytokines, IL-4 and IL-10(Jeong, *et al.*, 2012). The production of cytokines in rats with appropriate complementary glutamine was enhanced, and symptoms of upper respiratory tract infection decreased(Dos Santos, *et al.*, 2009). However, the CM, FV, and G subgroups could all adjust the protein levels to normal level. But our results suggest that cordycepin can more effective restore immune balance that the levels of IL-4, GATA3, T-Bet, IFN-γ, STAT1, and STAT6 were mostly close to C subgroup.

In addition, we also assessed the change in the number of Th1 and Th2 cells in the whole blood of rat via flow cytometry. Flow cytometric detection is a functional assay that measures the ability of specific immune cells to express type 1 and type 2 cytokines after polyclonal stimulation with mitogens(Jung, *et al.*, 1993, Pala, *et al.*, 2000, Prussin, 1997). Two functionally distinct T-helper lymphocyte subsets are distinguished by their signature cytokines as follows: CCR5 for Th1 lymphocytes and CCR8 for Th2 lymphocytes(Kutukculer, *et al.*, 2016). We confirmed that cordycepin quickly adjusted the ratio of CCR5/CCR8 into a normal level (C subgroup level) a week after the rats ended their exercise. A similar effect was also observed in *Flammulina velutipes* polysaccharides and glutamine.

Based on the above analysis, we propose that cordycepin functions as a better modulator of immune imbalance than *Flammulina velutipes* polysaccharides and glutamine in rats that were subjected to exhaustive exercise by regulating Th1- and Th2-related cytokines and transcription factors. However, the regulation pathway of T helper cell differentiation induced by

cordycepin and the efficiency of the two or more food mixing for immunoregulation requires further research.

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