**Immunoprotective Activity of Polysaccharides from Eucommia Ulmoides on Intensive-exercise Mice**

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**Abstract.** The aim of this study is to investigate the immunoprotective effect of Eucommia Ulmoides Polysaccharide (EUP) on intensive exercise mice. EUP were isolated and its physicochemical properties were analyzed. Meanwhile, an intensive exercise model of mice was established and EUP were orally treated during exercise and some immunological parameters were measured at the end of the training session, such as thymus and spleen index, lymphocytes proliferation, natural killer (NK) cells activity. The results showed that EUP was a polysaccharide-protein conjugates and significantly increased the spleen index and activities of NK cells. In conclusion, EUP can ameliorate the exercise-induced immunosuppression.

**Introduction**

The moderate and suitable physical activity positively influences the immune system, whereas the excessive and exhausting physical load can lead to chronic immunosuppression in athletes, placing them at a greater risk from opportunistic infections, particularly infections affecting the upper respiratory tract [1]. Nutrition undoubtedly plays a critical role to influence exercise-induced immunosuppression [2]. Many researchers have attempted to modify, through nutritional manipulation, the negative changes observed in the immune function in athletes following heavy exertion [3]. The value of polysaccharides in food and medicine has been well documented and polysaccharides have been shown to be potent immune modulators and emerging as potentially new options for combating immunosuppression [4].

Eucommia Ulmoides is commonly used for the treatment of hypertension, rheumatoid arthritis, lumbago and ischialgia in traditional Chinese medicine [5]. Crud polysaccharides from Eucommia Ulmoides possessed anti-inflammatory, immunological and antioxidant activity [6, 7].

In the present study, Eucommia Ulmoides polysaccharides (EUP) were isolated and its physicochemical properties were characterized, and then were orally administrated to intensive exercise mice for 4 weeks. The findings indicate EUP can ameliorate the exercise-induced immunosuppression.
Materials and Methods

Materials and Reagents

Eucommia Ulmoides were obtained from Shangluo city, Shaanxi province, China, authenticated by Prof. Xingbin Yang (College of Food Engineering and Nutritional Science, Shaanxi Normal University). DEAE-52 cellulose and Sephadex G-150 were purchased from Beijing Dingguo Reagent Co., China. Mannose, ribose, rhamnose, d-glucuronic acid and d-galacturonic acid, glucose, xylose, galactose, arabinose, and Fucose were obtained from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) and 1-Phenyl-3-methyl-5-pyrazolone (PMP) were purchased from the Shanghai Ziyi Reagent Co., China. RPMI1640, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were from Gibco BRL (Gaithersburg, USA). CCK-8 and CCK-8 assay kit was from Daojindo Laboratories, Japan. YAC-1 cells were purchased from Sigma (St. Louis, USA).

Extraction and Purification of Polysaccharides

Dried sample was mashed into powder soaked in 95% alcohol (1: 5, w/v) for 2 h. Then the residues were dried by airing and then extracted in hot water (1:10, w/v) at 80 °C three times, 1 h each time. The extracted solution was concentrated and the free proteins were deproteinized 7 times using the Savage method [8], and then polysaccharides were precipitated with 4-fold volume of ethanol at 4 °C for 24 h, then dialysis against running water for 24 h using the membranes with molecular weight cut-off of 10 kDa. The retentate was collected and lyophilized to afford crude polysaccharide. The main component of crude polysaccharide, named EUP, was purified by DEAE-52 cellulose column (2.0 × 80 cm) and Sephadex G-150 column (2.0 × 40 cm), respectively.

Physicochemical Properties Analysis of Polysaccharides

Total carbohydrate content was determined according to the phenol-sulfuric acid method using glucose as a standard [9]. EUP was ground with KBr powder and then pressed into pellets for Infrared (IR) measurement using a Tensor 27 Bruker instrument in the frequency range of 500-4000cm⁻¹. The ultraviolet spectrogram of EUP was measured from 200 to 800 nm of wavelength with an ultraviolet and visible spectrophotometer (Hitachi U-3900, Hitachi Ltd., Japan). The monosaccharide compositions were analyzed according to the previous method [10]. Briefly, EUP was hydrolyzed into 2 ml of 3 M TFA at 100 °C for 6 h to release monosaccharides, which were derivatized with 40 μL of 0.5 M PMP and 40 μL of 0.3 M NaOH at 70 °C for 2 h. The analysis of the PMP derivatives was performed by an HPLC (LC-2010A, Shimadzu, Japan) system and a RP-C₁₈ column (4.6 mm × 250 mm, 5 μm, Venusil, USA) was used for the separation of monosaccharide. Amino acids were analyzed according to our previous method [10]. Briefly, 20 mg of EUP was hydrolyzed under vacuum at 110 °C in 6 M hydrochloric acid for 24 h. The hydrolysis products were detected with an amino acid analysis analyzer (Hitachi L-8900, Japan) with a group of standard amino acids as the markers. The content of each kind of amino acids was calculated using the standard curves.

Exercise Model

50 male Kunming mice (20-23g), 60-day-old, were purchased from Experimental Animal Centre of Xi'an Jiaotong University (Xi'an, China) and were housed on a 12/12 h light-dark cycle at room temperature and allowed free access to standard rodent food and water during the
experiments. Animal handling procedures were conducted under National Institutes of Health animal care and use guidelines. All animals, at the beginning of the experiments, were randomly divided into 5 groups as following: Moderate-exercise control (MC) group, Intensive-exercise control (IC) group and three EUP treatment (EUP-L, EUP-M and EUP-H) groups. Each group contains 10 mice. Animals were administered orally (at 8:00 am) and swum (at 8:00 pm) daily for 4 weeks. EUP-L, EUP-M and EUP-H groups orally obtained 100, 200 and 400 mg/kg body weight of EUS in appropriate volumes of water, respectively. MC and IC groups received the same volume distilled water.

Protocol of exercise was carried out as described in our previous study[11] and made slight adjustment. Briefly, swimming exercise was carried out in pool (length 65 cm, width 50 cm, depth 50 cm, water depth 30 cm and temperature 28 °C) for 90 min/d in the 1st and 2nd week, 120 min/d in the 3rd and 4th week. Mice in IC, EUP-L, EUP-M and EUP-H groups were tied a wire of 5% body weight on their tails when swimming whereas those in MC without loads.

Mice were measured body weight and killed by cervical dislocation after anesthetization on the last day of the 4th week. Spleens and thymuses were immediately removed and weighted for calculation of their indexes (the weight of spleen/thymus relative to the body weight).

**Assay of Spleen Lymphocyte Proliferation**

Spleen of mouse was placed in 0.1 M cold phosphate-buffered saline (PBS) and gently homogenized, then passed through a 200-mesh sieve to generate single cell suspensions. Erythrocytes were rapidly washed by hypo-osmotic haemolysis, then the cells were suspended at a final density of 2×10^5 cells/ml in RPMI-1640 medium supplemented with 10% FBS, and then were seeded into a 96-well plate (100 µl/well) in the presence of Con A (7 µg/ml) and cultured at 37 °C in 5% CO2 incubator. After 72 h of incubation, 10 µl of CCK-8 was added to each well and the plate was incubated for an additional 3 h. Finally, the absorbance values (A) at 450 nm were measured using a Zenyth 3100 microplate reader (Anthos, Austria).

**Assay of Natural Killer (NK) Cells Activity**

NK cells activity was determined using CCK-8 assay kit. Splenocytes were prepared as above 2×10^5 cells/ml and YAC-1 cells (2×10^4 cells/ml) were seeded into 96-well plates with a ratio of 50:1 of effectors to target, then 10 µl of CCK-8 was added. Following another 4 h of co-culture, the optical density (OD) of each well was measured using the microplate reader. Meanwhile, absorbance measurements were recorded for the blank control, target cell control, and effectors cell control. The percentage of NK cell activity was determined by the following equation: NK cell activity (%) = 1 [(optical density value of test samples - optical density value of effectors cell control) / optical density value of target cell control] × 100.

**Statistical Analysis**

Data are presented as the mean ± standard deviation (SD). Statistical differences between groups were evaluated using a t-test. P values < 0.05 were considered significant.
Results

Physicochemical Property of Polysaccharide

The results suggested that contents of total carbohydrate in EUP were 82.65%. EUP was easily soluble in water but not soluble in organic solvents such as ethanol, ether, acetone, and chloroform. As shown in Fig. 1(A), the absorption peak at 280 nm in the ultraviolet spectrogram was characteristic absorption peak of protein, which indicated that EUP might be a conjugate with portions of polysaccharide and protein. IR spectrum of EUP exhibited some different absorption peaks (Fig. 1(B)). The absorption bands within the range of 3600–3200 cm\(^{-1}\), 3000–2800 cm\(^{-1}\), 1400–1200 cm\(^{-1}\) and 1200–1000 cm\(^{-1}\) were the characteristic absorption peaks of polysaccharides. The peak at 3436.20 cm\(^{-1}\) was attributed to the stretching vibration of O–H. The peaks at 2918.00 cm\(^{-1}\), 1408.46 cm\(^{-1}\) and 1246.16 cm\(^{-1}\) were due to the C–H stretching vibration absorption. The band at 1640.42 cm\(^{-1}\) was the characteristic IR absorption of protein. The absorptions around 1744.33 cm\(^{-1}\) and that at 1408.46 cm\(^{-1}\) were indicative of the presence of carboxyl groups and carbonyl groups that indicated the characteristic IR absorption of uronic acid. These observations further confirmed that EUP was composed of polysaccharide, protein and uronic acids [12, 13, 14].

As shown in Fig. 1(C) b, the result of monosaccharide composition suggested that EUP was composed of mannose, rhamnose, glucose, galactose and Galacturonic acid in the molar percentages of 16.64%, 10.31%, 27.84%, 21.66%, 9.19% and 10.59%, respectively. This result was in agreement with monosaccharide standard (Figure 1(C)a). The content of total amino acid was 2.91% in EUP (Figure 1(D)), in which 5 amino acids were identified. His was the major amino acid (1.46%), followed by Glu (0.54%), Leu (0.06%), Tyr (0.84) and Lys (0.01).

Figure 1. Wavelength, wave numbers, monosaccharide composition and the content of total amino acid.
A: the absorption peak at 280 nm in the ultraviolet spectrogram was characteristic absorption peak of protein, which indicated that EUP might be a conjugate with portions of polysaccharide and protein. B: spectrum of EUP exhibited some different absorption peaks; C: monosaccharide composition; D: content of total amino acid.

Effects of EUP on Body Weight and the Relevant Immune Parameters

As shown in Fig. 1, intensive exercise led to a decrease in body weight, the indices of spleen and thymus, and NK cells activity in comparison with MC group. On the contrary, the spleen index and NK cells activity were significantly (P<0.05) increased in EUP (200 and/or 400 mg/kg) treatments mice in comparison with those in IC group. However, there was no difference observed in thymus index and Lymphocyte proliferation between mice in IC and EUP treatments groups (P>0.05).

Discussion

The number of factors influencing the immune system during exercise, a multitude of findings, from immunodepression to immunostimulation has been reported [15]. The function and number of NK and B lymphocytes was reduced after a prolonged and intense bout of exercise [16]. The present study established an intensive exercise model of mice. The results demonstrated that the indices of spleen and thymus, spleen lymphocyte proliferation and NK cells activity were significantly decreased in intensive-exercise group mice when compared with those in moderate-exercise group which without load. It is clear that exercise modifies the cellular and humoral branches of the immune system, and that regular physical activity of light to moderate levels can increase host resistance to disease, whereas heavy exertion enhances the risk of illness [17]. Our results indicated that the intense exercise suppressed the immune function. In addition, the body weight was significantly decreased in IC group mice when compared with those in MC which without load. The result suggested the intense exercise might hinder the normal physical growth of mice.

Many studies have reported that plant polysaccharides could activate immune cells through some signaling pathways [18, 19] and further enhance spleen lymphocyte proliferation, macrophage and NK cells activity [20]. The mechanism was demonstrated that polysaccharides can not only activate immune cells as signal molecular but also protect immune system by attenuating exercise-induced oxidative stress [21]. Based on the above exercise model and views, we tried to investigate the effect of EUP supplementation upon immune functioning in intensive exercise mice. The results showed that EUP treatment dramatically increased NK cells activity as well as improved spleen index.

In summary, EUP supplementation can significantly increase immune function of intensive exercise mice. Further studies are necessary on determination of structure and active mechanism of EUP, which has already been underway in our lab.

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References


