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The haematopoietic effect of *Panax japonicus* on blood deficiency model mice

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ABSTRACT

Ethnopharmacological relevance: The rhizome of *Panax japonicus C. A. Mey. var. major (Burk.) C. Y. Wu et K. M. Feng* (PJ) is a commonly used traditional Chinese medicine to promote hematopoietic effects, promote blood circulation and supporting healthy energy. Aim of the study is to investigate the haematopoietic effects of PJ and determine the mechanism of its haematopoietic activity.
Materials and methods: The crude extract from PJ (PJE) was separated into two fractions: polysaccharides (PJPS) and low-molecular-weight compounds (PJSM). PJPS, and PJSM were incubated with mice spleen cells, and their haematopoietic activities were evaluated by determining the haematopoietic growth factor levels (HGFs) in vitro. The in vivo experiments used anaemia model mice that were given hypodermic injections of *N*-acetyl phenylhydrazine (APH) and intraperitoneal injections of cyclophosphamide (CTX).
Results: Both PJPS and PJSM were significantly involved in the haematopoietic effect of PJE. The administration of PJPS and PJSM could accelerate the recovery of the white blood cell (WBC), red blood cell (RBC), and haemoglobin (HGB) levels in the blood deficiency model mice. Haematopoietic activity may result from stimulating the secretion of interleukin-3 (IL-3), interleukin-6 (IL-6), erythropoietin (EPO), GM colony-stimulating factor (CSF), and M-CSF and by the resistance of spleen cells to apoptosis.
Conclusions: The study results support the potential use of PJPS and PJSM for the treatment of anaemia.

1. Introduction

Anaemia, the most common blood disease, is a condition that develops when the blood lacks sufficient healthy red blood cells or haemoglobin. Increased erythrocyte damage, decreased or faulty RBC production, and blood loss are the three major causes of anaemia (Bruno et al., 2008). Impaired haematopoietic function is a common adverse reaction to malignant tumour chemotherapy. After chemotherapy, many patients exhibit leucopoenia and thrombocytopenia (Abeloff et al., 2001), which cause them to discontinue chemotherapy, thereby seriously undermining the effects of chemotherapy and increasing the treatment time. Therefore, methods that treat or prevent haematopoiesis in anaemia patients and prevent the side effects of malignant tumour chemotherapy have attracted tremendous attention in recent years.

Haemopoiesis is the process by which haematopoietic stem cells develop into mature blood cells through the stimulation of various cytokines in the haematopoietic microenvironment, including EPO, CSF, IL-3 and IL-6, among other cytokines (Harmening, 1997).

The PJ is belonging to more than five families, is a commonly used traditional Chinese medicine (Jing-Yi and Qi-Bing, 1989) that was first developed in "Ben Cao Gang Mu Shi Yi" (Supplements to compendium of materia medica, published in 1765 A.D.). It is a commonly used traditional Chinese medicine to promote hematopoietic effects, promote blood circulation and supporting healthy energy (Duan and Chen, 1996; Hui-Lan and Cun-De, 1994). PJ contains a complicated mix of bioactive constituents, including polysaccharides, organic acids, and saponins, such as oleanolic acid-type triterpenoid saponins and darma alkane-type triterpenoid saponins (Chen et al., 2006; Jing-Yi and Qi-Bing, 1989). The PJ crude extract and polysaccharides extracted from rhizomes of PJ (PJPS) have been the subject of great interest because of their apparent ability to replenish blood (Duan and Chen, 1996), prevent haematopoietic decrease induced by cyclophosphamide (Chen et al., 2008; Miao et al., 2007), and anti-tumour (Tao et al., 2007) effects. Saponins reportedly have anti-inflammatory effects (Tao et al., 2007) and decrease the effects of myocardial ischemia

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reperfusion injuries (Tao et al., 2004). The various ingredients in PJ that are associated with the haematopoietic activity and the haematopoietic-recovery mechanism(s) of PJ need to be further investigated.

Hot water extraction is traditionally used to prepare PJ (Tao et al., 2007), we also extracted PJ using this method, and then divided the crude extraction (PJE) into two parts (PJPS and PJSM) by alcohol precipitation method.

In vitro experiments were used to determine the major chemical components of PJ that enhance haematopoietic function. Subsequent in vivo experiments were performed to investigate the effects of PJE, PJPS, and PJSM on blood deficiency model mice and the possible mechanism(s) behind the associated haematopoietic activity.

2. Materials and methods

2.1. Chemicals

The *Panax japonicus C. A. M ey.var. major (Burk.) C. Y. Wu et K. M. Feng* (Zhu-zi-shen, 珠子参) used in this study was obtained from Shaanxi province in China and identified by Professor Yang (Shaanxi Academy of Traditional Chinese Medicine, Xi'an, China). A voucher specimen was deposited in the herbarium stock room at Shaanxi Academy of Traditional Chinese Medicine, under the number 03478. APH, CTX, MTT and DMSO was obtained from Sigma–Aldrich (St. Louis, MO, USA). Ginsenoside Re (Batch No. 110754-200822) was obtained from National Institutes for Food and Drug Control (Beijing, China).

2.2. Sample preparation

The PJ (400 g) were pulverised and soaked in distilled water at 80 °C for 2 h. After the aqueous extracts were pooled and concentrated under reduced pressure, the crude extract (PJE) was obtained. The concentrated extracts were further precipitated by adding 4 volumes of 95% ethanol. The supernatant was concentrated and lyophilised, and the small-molecule fraction (termed PJSM) was obtained, the Total saponins content was 62.4% that was determined using the UV spectrophotometry (Gao et al., 2006) with Ginsenoside Re as standard. The resulting precipitate was collected and re-dissolved in distilled water. The aqueous solution obtained was deproteinated using the sevage method (Zhang and YE, 2010), concentrated under reduced pressure, and finally lyophilised to obtain the crude polysaccharide (termed PJPS), the total carbohydrate content was 75.2% that was determined using the phenol–sulfuric acid method with glucose as the standard (Lin et al., 1996).

2.3. in vitro experiments

2.3.1. The spleen cells toxicity of the PJE, PJPS and PJSM

In order to determine toxicity of the PJE, PJPS and PJSM, 96-well plate was used. Eight thousands normal mice spleen cells (the calculated amount) and 80 μL culture medium were added to 48 wells. Seven doses of PJE, PJPS and PJSM (2, 4, 8, 16, 24, 36, and 60 μg mL^{-1} for each fraction) were injected into 45 wells (three repeats for each group) and no drug was added to the other 3 wells (control group). The total volume reached 200 μL by adding the additional culture medium. The plate was put in 37 °C incubator for 72 h. After 72 h, 20 μL of MTT solution was added to each well. The plate was covered with an aluminum foil (reaction in darkness) and was put in 37 °C incubator for 3 h. Then, the supernatant was discarded and 100 μL of DMSO solution was added to each well. Finally, the absorbance of each well was measured in ELASA reader machine at 570 nm.

2.3.2. The spleen cells activity of the PJPS and PJSM

Normal mice spleen cells were cultured in RPMI-1640 medium (with 20% foetal bovine serum, 2 mmol mL^{-1} L-glutamine and 100 IU mL^{-1} penicillin) and treated with phosphate buffer solution, PJPS (12, 18, 24, 36 and 60 μg mL^{-1}), or PJSM at different concentrations (8, 12, 18, 24 and 36 μg mL^{-1}) at 37 °C in the CO_2 incubator. After incubating for 3 days, the supernatants were individually collected using 0.22-μm filters, and the concentrations of IL-3, IL-6 and GM-CSF in filtrates were determined separately using an enzyme-linked immunoassay. The operational approach was performed according to manufacture specification (Wuhan Colorful Gene Biological Technology Co., LTD, China). In brief, 96-well ELASA kit were used, 40 μL of standard diluting agent and different amounts of Standard (120, 80, 40, 20, and 10 μL, separately) were added to the wells of standard, and 40 μL of sample diluting agent and 10 μL of testing sample were added to the wells of testing. After 30 min incubation at 37 °C, the supernatant was discarded, and the plate was dried prior to washing with washing buffer (repeated five times). The HRP-Conjugate reagent 50 μL was added to each well, except blank well. After 30 min incubation at 37 °C, the liquid was discarded, the plate was dried by swing, and then was washed by washing buffer, repeat five times. Chromogen Solution A 50 μL and Chromogen Solution B was added to each well, the plate was incubated for 15 min at 37 °C under dark conditions. Then Stop Solution 50 μL was added to each well, OD450 of each well was measured using Microplate spectrophotometer **(**Labsystems Multiskan MS, Finland**).**

2.4. in vivo experiments

2.4.1. Animals

A total of 81 Male Kunming mice (weight, 20 ± 2 g) were purchased from the Laboratory Animal Breeding and Research Centre of Xi'an Jiaotong University and maintained in a specific pathogen-free environment (SPF).

2.4.2. Dose selection of PJE, PJPS and PJSM

According to Ch. P. 2010, the daily dose of PJ is 0.1 g kg^{-1} body weight per day on human. In general, the experimental doses on animals need to be kept between 30 and 50 times of those on human. In consideration of the yield of PJ crude extraction (PJE) of 39.82%, we selected 40 times and 20 times of 0.1 g kg^{-1} body weight as the high dose and the low dose of PJ on mice, respectively, namely 1.6 g kg^{-1} body weight per day and 0.8 g kg^{-1} body weight per day. In addition, in the preliminary experiments, we found 150 mg kg^{-1} body weight per day and 75 mg kg^{-1} body weight per day of PJPS or PJSM could increase WBC, RBC and HGB levels on blood deficiency mice. Therefore, the above two doses were used as the experimental doses in this study.

2.4.3. Grouping and administration of PJE, PJPS, and PJSM

The mice were fed food and water ad libitum for 5 days. They were randomly placed into one of the following nine groups: the normal group, model group, rhG-CSF group, PJE high-dose group, PJE low-dose group, PJSM high-dose group, PJSM low-dose group, PJPS high-dose group, and PJPS low-dose group. In the normal group, the mice were treated with distilled water. The mice in the other 8 groups received hypodermic injections of APH (20 mg kg^{-1}) the 1st day, twice that dosage on the 4th day, and daily intraperitoneal injections of CTX (25 mg kg^{-1}) for 4 days.