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Protective effects of *Punica granatum* (pomegranate) peel extract on concanavalin A-induced autoimmune hepatitis in mice

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ABSTRACT

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease of an unknown etiology, glucocorticoid therapy is currently recognized as an effective treatment for AIH, but conventional application and patient compliance are both hindered by its side effects. The exploration of the AIH pathogenesis and the searching for the new candidate drugs that exert potential activity and low toxicity are urgently needed. Pomegranate peel extract (PoPx) is a natural extract of *Punica granatum* and has been reported to have anti-inflammatory and antioxidative properties. The present study aimed to clarify the effect of PoPx on the concanavalin A (ConA)-induced autoimmune hepatitis in a mouse model that is well established at 12h after tail vein injection with a dose of 20 mg/kg of ConA. C57BL/6 female mice were pretreated with PoPx (250 mg/kg, once daily for 3 days) followed by a ConA challenge. Pretreatment with PoPx significantly alleviated ConA-induced liver injury by down-regulating the levels of plasma alanine transaminase (ALT), aspartate transaminase (AST) and cytokine, including TNF-α, interferon (IFN) -γ and interleukin (IL)-6. Moreover, liver hematoxylin and eosin (H&E) staining displayed a lighter inflammatory infiltration around the portal area in the PoPx-pretreated mice. In addition, the flow cytometry (FCM) data showed that the immune response in the liver was died down in the PoPx-pretreated condition. Specially, pretreatment with PoPx reduced the infiltration of activated $CD4^+$ and $CD8^+$ T cells in the liver. Taken together, these findings contributed to a better understanding of the actions of PoPx against acute AIH and indicated that PoPx might be a potential compound in treating T cell-mediated autoimmune liver injury.

1. Introduction

Autoimmune hepatitis is an uncommon idiopathic syndrome of immune-mediated destruction of hepatocytes. It exhibits a female predominance, occurrs at all age and can progress to liver cirrhosis and failure if untreated. AIH is characterized by serum autoantibody positivity, elevated aminotransferase levels, high immunoglobulin G (IgG) and/or high gamma-globulinemia, and hepatic pathologies with the presence of interface hepatitis [1,2]. Immunosuppression (prednisone and azathioprine) and liver transplantation are effective therapeutic strategies for AIH [3].

However, AIH is a relatively rare and very heterogeneous disease with an incompletely understood etiology, what's more, the clinical studies of AIH are hampered by the limited numbers of patients who can be included in the trials, and thus, the disease remains a major diagnostic and therapeutic challenge [4]. Furthermore, topical corticosteroid agents have steroid-specific side effects. Changes associated with Cushing's syndrome are the most common side effects, including facial rounding, dorsal hump formation, striae, weight gain, acne, alopecia and facial hirsutism [5]. Other relatively rare but more serious adverse reactions include osteopenia with vertebral compression, brittle diabetes, psychosis, pancreatitis, opportunistic infection, labile hypertension, and malignancy [6,7]. Similarly, some azathioprine-related side effects occur, for example, bone marrow suppression, which is the main reason for drug withdrawal [5,8]. Although liver transplantation (LT) is a successful and effective treatment for AIH, the recurrence rate is as high as 30% [9]. Meanwhile, the long waiting cycle for LT, high risk of infections after surgery and other intractable post-

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operative complications limit its application [10]. Despite promising results, alternative therapies for AIH have progressed slowly, and none have been incorporated into the standard care of AIH [5]. Therefore, the exploration of the AIH pathogenesis and the searching for the new candidate drugs that exert potential activity and low toxicity are urgently needed.

Recently, the protective and curative effects of Traditional Chinese Medicines (TCMs) have been confirmed by medical research [11–14]. TCMs have increasingly received public attention. Among these medicines, pomegranate, which is well known as a safe food nutrient, is gradually attracting the attention of researchers. For nearly half a century, scientists have conducted extensive research on the antioxidant effects of pomegranates and found that pomegranate peel had the strongest antioxidant activity [15–17]. It has been confirmed that the main components of antioxidant properties in pomegranate peel extract (PoPx) are punicalagin (PC) and ellagic acid (EA) [18,19]. PoPx has also been reported to have certain bioactivities, including regulating blood glucose levels [20], antifungal [21], anti-tumor [22,23] and some other activities [24,25].

Nevertheless, no reports have investigated the effect of PoPx on AIH. To evaluate the potential action of PoPx on AIH, we examined the effect of PoPx in an AIH murine model *in vivo*. In this study, we prepared the AIH model by injecting concanavalin A (ConA) via the tail vein, which is a well-accepted experimental model of immune-mediated liver injury [26], and clarified the effect of PoPx on the ConA-induced liver injury.

2. Materials and methods

2.1. Preparation of PoPx

Tunisia soft-seed pomegranate peel was provided by the Yuzhuang Ecological Green Industry Co. (Qianxi County, Guizhou Province, China). The preparation of the PoPx was performed as previously described with minor modifications [27]. The fresh peel (400 g) was collected and cut into 0.5 cm pieces and extracted with a combination of ethanol (1550 mL) and H_2O (480 mL) followed by soaking for 2 h at 60 °C. Then, the crude extracts were filtered and concentrated under a vacuum. Finally, 45 g brown powder solid was finally collected. The PoPx was stored at -20 °C and protected from light before use.

2.2. Quanlitative and quantitative determination of ellagic acid (EA) and punicalagin (PC) in PoPx by mass spectrometry (MS) and high-performance liquid chromatography (HPLC)

A Waters 2695 high performance liquid chromatography (HPLC) system (Waters Corp., Milford, Massachusetts, U.S.A.) was used in this experiment. The method and chromatographic conditions were performed as previously described [28,29]. The system control and data analysis were processed by Waters Empower 3 software. Chromatographic separation was conducted on a Waters Symmetry C18 column (5 μm, 4.6 × 250 mm). The mobile phase consisted of deionized glacial acetic acid (A; 99:1, v/v; pH 3.0) and methanol (B) with a flow rate of 1 mL/min. The gradient program was set as follows: 0–70 min, 10-45% B and 70-80 min, 45% B. The chromatogram was detected at a wavelength of 256 nm throughout the assay. An Absciex Qtrap 5500 was used for MS. We first established the standard working curves of EA and PC by MS. In the next, we detected the samples by HPLC.

2.3. Regents and antibodies

The vehicle solution consisted of 2.5% dimethyl sulfoxide (DMSO) + 17.5% polyethylene glycol 400 (PEG400) + 80% normal saline (NS). DMSO was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).The PoPx was dissolved in the solutions described above as needed. Antibodies to stain the single cell suspension for flow cytometry (FCM) detection used in our study included CD4, CD8 and CD69, which were purchased from Dakewe Biotech Co. (Beijing, China).

2.4. Animals

Female C57BL/6 mice (aged 8–10 weeks; 19–22 g) were obtained from the Sichuan University Laboratory Animal Center (Chengdu, China). The mice were housed in a specific-pathogen-free (SPF) facility with a consistent room temperature and humidity and provided standard laboratory chow and water freely one week before the experiment.All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number:20161009).

2.5. Experimental design

The AIH mouse model was established at 12h after an injection of ConA with a dose of 20mg/kg through the tail vein,which was based on our previous study and other reported articles [30–32]. Both the PoPx and vehicle mice group were administered by gavage.

Initially, we performed a preliminary experiment using a dose gradient of PoPx (125 mg/kg, 250 mg/kg) to test its bioactivity in two independent AIH mice groups compared to a vehicle control group. Then, a formal experiment was performed (3groups: PoPx, ConA control (CC), normal control (NC), 5 mice per group) to confirm the efficacy of PoPx in the AIH mouse model. The distribution of all the groups strictly followed the principle of randomization. The PoPx and Vehicle groups received intragastric administration of PoPx (250 mg/kg) or vehicle once daily for 3 days. Thereafter, the two groups of mice were intravenously injected with ConA (20 mg/kg) and sacrificed 12 h after.

2.6. Liver aminotransferase assay

Blood samples were collected by removing the eyeball. Plasma was separated after centrifugation at 300 g for 10 min. Alanine transaminase (ALT) and aspartate transaminase (AST) measurements were performed by an automatic dry biochemical analyzer (Hitachi Auto Analyzer7170, Japan).

2.7. Cytokine quantification (ELISA)

The levels of cytokines, including TNF-α, IFN-γ and IL-6, in murine plasma were detected by ELISA using commercially available kits (eBioscience, San Diego, CA) according to the manufacturer's instruction.

2.8. Histopathological analysis

The liver tissues were collected 12 h after ConA injection and the samples were fixed in 4% buffered paraformaldehyde for 48 h and then embedded in paraffin. The sections (4 μm) were mounted on slides, deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and subjected to hematoxylin and eosin (H&E) staining. The slides were examined by light microscopy for infiltrating leukocytes, tissue injury, necrosis and fibrosis. The pathological alterations of the stained specimens were evaluated and confirmed by at least two professional researchers in a double-blinded assessment.

2.9. Flow cytometry analysis

Single-cell suspensions of the blood, liver, and spleen tissues were obtained 12 h after ConA administration by mechanical and enzymatic dispersion as described previously [33]. Then, 1×10^6 of freshly prepared cells were suspended in 100 μL of PBS and stained with different combinations of fluorochrome-coupled antibodies (T lymphocytes: