In vivo and in vitro anti-sepsis effects of physcion 8-O-β-glucopyranoside extracted from Rumex japonicus

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[ABSTRACT] The present study was designed to investigate the anti-sepsis effects of physcion 8-O-β-glucopyranoside (POG) isolated from Rumex japonicus and explore its possible pharmacological mechanisms. POG was extracted from R. japonicus by bioactivity-guided isolation with the anti-sepsis agents. Survival analysis in septic mouse induced by LPS and heat-killed Escherichia coli were used to evaluate the protective effect of POG (40 mg·kg⁻¹, i.p.) on sepsis. Cytokines including TNF-α, IL-1β and IL-6 in RAW 264.7 cells induced by LPS (100 ng·mL⁻¹) were determined by ELISA. In addition, the proteins expressions of TLR2 and TLR4 were determined by Western blotting assay. Our results demonstrated that POG (40 mg·kg⁻¹, i.p.) possessed significant protective activity on the endotoxemic mice. The POG treatment (20, 40, and 80 μg·mL⁻¹) significantly decreased the TNF-α, IL-1β and IL-6 induced by LPS (P < 0.01) in a concentration-dependent manner. Furthermore, the TLR4 and TLR2 proteins were also down-regulated by POG at 20 (P < 0.01), 40 (P < 0.01), and 80 μg·mL⁻¹ (P < 0.01). The present study demonstrated that the POG extracted from R. japonicus possessed significant anti-sepsis effect on endotoxemic mice, and can be developed as a novel drug for treating sepsis in the future.

[KEY WORDS] Anti-sepsis; TLR-4; TLR-2; Pro-inflammatory cytokines; LPS

[Introduction] Currently, the increasing incidence of sepsis remains a major clinical threat to for modern medicine [1-3]. Sepsis, the generalized inflammatory response to infection induced by various microorganisms and virus, can easily lead to multiple organ dysfunction syndromes (MODS), septic shock, and even death in clinic [4-5]. It’s estimated that approximately 2%–11% of intensive care unit (ICU) patients are attributed to sepsis in the USA and Europe [6], and sepsis is a commonly severe clinical syndrome with mortality ranging from 30% to 50% [7-9]. Despite great improvements have been achieved in diagnosis and supportive care of sepsis, the mortality rates are still not significantly improved [2, 10-11]. Therefore, it is urgent to develop novel and alternative strategies for treating sepsis.

Rumex japonicus Houtt., belonging to the Polygonaceae, is a perennial herb plant and widely distributed in China. In traditional Chinese medicine, R. japonicus is commonly used in treating infections, malignant sores, tumors, and constipation, etc [12-13]. However, systemic research regarding its active constituents is lacking. Currently researches have revealed that this plant possesses plenty of anthraquinones including emodin, physcion, chrysophanol, emodin-8-O-β-D-glucoside, physcion-8-O-β-D-glucoside, and chrysophanol-8-O-β-D-glucoside [12, 14]. We have firstly studied the anti-sepsis effect of physcion-8-O-β-D-glucoside (POG) which is one of the major anthraquinones in R. japonicas. In the present investigation, septic mouse models induced by LPS and heat-killed Escherichia coli were established, and the protective effect of POG was investigated. Pro-inflammatory cytokines and TLRs are important targets for treating sepsis [6-7]. To explore the possible mechanism of action, we studied the pro-inflammatory cytokines releases and expressions of TRLs in RAW 264.7 cells induced by LPS. Our results indicated that POG was a promising adjuvant candidate compound in treating...
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Plant materials

Materials and Methods

**Plant materials**

The whole plant of *R. japonicas* was collected from the Yong-An country, Pu-Xing township, E’Mei, Sichuan Province, and identified by the department of traditional Chinese medicine of our Hospital. A herbarium specimen of *R. japonicas* (20130621-1#) was deposited at our laboratory.

**Animals**

ICR mice (weighing approximately 18–22 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed at constant room temperature (25 °C) and relative humidity (70%). Food and tap water were available *ad libitum*. Each experimental group was consisted 10 animals. The animal protocol in the present study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of our hospital. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

**Chemicals**

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and Lipopolysaccharide (LPS) were purchased from Sigma Chemicals (Shanghai, China). Mouse TNF-α, IL-1β and IL-6 ELISA kits were purchased from Beyotime Biotech. (Jiangsu, China); GAPDH antibody, goat-anti-rabbit/rat horseradish-peroxidase-conjugated (HRP) secondary antibody, and BCA protein assay reagents were purchased from Beyotime Biotech. (Jiangsu, China). The Dulbecco modified Eagle medium (DMEM) and FBS were purchased from Invitrogen (Carlsbad, CA, USA). Silica-gel (100–200, 200–300 mesh) was purchased from Qingdao Haiyang Chemical Co. (Qingdao, China). Sephadex LH-20 was purchased from GE Healthcare Co. (Shanghai, China). All other chemicals used in the present study were of analytical reagent grade.

**Cell line and cell culture**

Murine macrophage RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 U of penicillin/ml, and 100 μg/mL of streptomycin at 37 °C with 5% CO₂.

**Cell culture and cell viability assay**

The RAW 264.7 cells were plated at a density of 5 × 10⁴ and pre-incubated for 24 h with 5% CO₂ at 37 °C. Then the cells were cultured with POG or total extracts of *R. japonicas* (TE) in the presence of 100 ng·mL⁻¹ LPS for 24 h at 37 °C. After that, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with 100 μL of 0.5 mg·mL⁻¹ MTT for 2 h to measure the cell viability. The medium was then discarded, and 100 μL of DMSO was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader (BIO-RAD, CA, USA) [15].

**Determination of TNF-α, IL-1β, and IL-6**

The RAW264.7 (1 × 10⁴) cells were grown in 48-well plates and incubated for 4 h. Then, POG or TE was added immediately after addition of 100 ng·mL⁻¹ LPS. After incubation for another 4 h, the supernatants were collected, and the levels of TNF-α, IL-1β and IL-6 were determined according to the instructions of commercial ELISA kits.

**Preparation of POG**

Dried whole plant of *R. japonicas* was crushed and extracted with 70% aqueous ethanol by reflux, and the filtrates were evaporated under vacuum to afford crude total extract (TE). Then the extract was suspended in water and subjected to macroporous adsorptive resins AB-8 eluted with aqueous ethanol of 20%, 40%, 60%, 80%, and 100%, respectively. The eluate was collected and evaporated under vacuum to yield five fractions, F20, F40, F60, F80 and F100, respectively. By determining the inhibitory effect of TNF-α, the F60 was selected to be investigated in the subsequent experiments. For the isolation of F60, the fraction was determined using HPLC. The HPLC separation was performed on a Agilent 1200 HPLC system with a C18 chromatographic column (250 mm × 4.6 mm, 5 μm) eluted with MeOH–formic acid–water (70 : 0.3 : 29.7) at 254 nm. The flow rate was set at 0.8 mL·min⁻¹, the sample injection volume was 10 μL, and the column temperature was set at 30 °C. Finally, the major constituent of POG was isolated by using HPLC with reversed phase column eluting with MeOH–formic acid–water (70 : 0.3 : 29.7) at a flow rate of 1.0 mL·min⁻¹ (Fig. 1). The chemical structure of POG was identified by NMR as physcion 8-glucopyranoside [13].

**Survival analysis in septic mice induced by LPS and heat-killed E. coli**

The survival analysis was carried out according to the previous method [9]. ICR mice were randomly divided into three groups (20/group): normal, model, and POG-treated groups. The POG was given at 40 mg·kg⁻¹ by intraperitoneal injection (i.p.) and 0.5% DMSO was used to enhance the POG solubility. The mice were injected intravenously (i.v.) with 15 mg·kg⁻¹ LPS and heat-killed E. coli [EC, 1.0 × 10¹⁰ CFU·kg⁻¹, *E. coli* ATCC 35218 (Manassas, VA, USA)]. The general conditions and mice mortalities were observed up to 7 days.

**Western blot analysis**

The RAW 264.7 cells (1 × 10⁶/mL) were seeded in 6-well polystyrene plates. LPS (100 ng·mL⁻¹) was added into the cells and various concentrations of POG (20, 40, and 80 μg·mL⁻¹) were added. The cells were incubated for additional 4 h. Subsequently, the RAW 264.7 cells were harvested and treated with lysis buffer. Total protein was extracted and the concentration was determined by BCA assay. The total protein was separated by SDS PAGE, and transferred to PVDF membranes. The membranes were then blocked with 1% BSA, incubated with TLR2...
and TLR4 monoclonal antibodies. Protein bands were detected by incubating with HRP-conjugated secondary antibodies and visualized by chemiluminescence detection. The proteins expression levels were expressed as a relative value to that of GAPDH expression.

Statistical analysis
All data were expressed as means ± SD. The Chi-square test was performed to analyze the significance of mice mortality differences among different groups. All other differences in means between two groups were analyzed with two-tailed Student’s t-test. Results were considered statistically significant at a level of $P < 0.05$.

Results

**TE possess inhibitory effects on TNF-α release**
As shown in Fig. 2A, in the presence of 100 ng·mL$^{-1}$ LPS, TE at concentrations of 50–800 μg·mL$^{-1}$ did not affect the proliferation of RAW 264.7 cells, indicating that TE with LPS (100 ng·mL$^{-1}$) didn’t possess obvious cytotoxic effect on RAW 264.7 cell line. In addition, our present results demonstrated that TE (ranging from 50 to 800 μg·mL$^{-1}$) significantly inhibited the release of TNF-α induced by LPS ($P < 0.01$). Besides, our study also revealed that for the inhibiting TNF-α release, no obvious difference was observed at 200, 600, and 800 μg·mL$^{-1}$ ($P > 0.05$). Therefore, in the subsequent investigations, all the sub-fractions of TE were studied at 200 μg·mL$^{-1}$.

**F60 possess the strongest inhibitory effects on TNF-α release**
In the present study (Fig. 3), all the sub-fractions prepared by macroporous adsorptive resins including F20, F40, F60, F80, and F100 at the concentration of 200 μg·mL$^{-1}$ didn’t show obvious cytotoxic effect on the RAW 264.7 cells. In addition, our results demonstrated that all the sub-fractions of TE could inhibit TNF-α release ($P < 0.01$), and in particular, the F60 showed a notable suppression of TNF-α release induced by LPS (100 ng·mL$^{-1}$) in RAW 264.7 cells.

**POG significantly protects mice challenged by lethal dose of LPS and heat-killed E. coli**
As shown in Fig. 4, 80% of the normal mice challenged with lethal dose of LPS (15 mg·kg$^{-1}$) were dead within 2 days, and all the mice died at 2.5 days after LPS injection. However, the survival rate of POG treated (40 mg·kg$^{-1}$) mice was obviously improved with a survival rate of 70% at the end of observation period ($P < 0.01$), indicating that POG possessed
Fig. 3  Effect of sub-fractions of TE on release of TNF-α in RAW 264.7 cells induced by LPS. RAW264.7 cells were incubated with sub-fractions (200 μg·mL⁻¹) and LPS (100 ng·mL⁻¹), and the release of TNF-α were detected by using ELISA method. Data were represented as mean ± SD (n = 4). *P < 0.05, and **P < 0.01 vs control

Fig. 4  Effects of POG on the survival rate of mice challenged with lethal dose of LPS (A) and heat-killed E. coli (B). Mice were randomly divided into three groups (n = 20), and observed for 7 days. The mice were injected intravenously (i.v.) with 15 mg·kg⁻¹ LPS and heat-killed E. coli (EC, 1.0 × 10¹¹ CFU·kg⁻¹). POG (40 mg·kg⁻¹) was administered by intraperitoneal injection (i.p.)

notable protective effect on the endotoxemic mice. In addition, the endotoxemic mouse model induced by lethal dose of heat killed E. coli was also established, and our results demonstrated that POG (40 mg·kg⁻¹) had a notable protective effect against sepsis in this model (P < 0.01).

POG suppresses TNF-α, IL-1β and IL-6 induced by LPS in RAW 264.7 cells

As shown in Fig. 5A, in the presence of LPS (100 ng·mL⁻¹), POG (0–320 μg·mL⁻¹) showed no obvious cytotoxic effects on the RAW 264.7 cells. Based on the cytotoxic assay of POG, our present investigation demonstrated that POG at the 20, 40, and 80 μg·mL⁻¹ significantly inhibited the release of TNF-α, IL-1β and IL-6 induced by LPS (P < 0.01), in a concentration-dependent manner. The results suggested that POG possessed powerful ability of suppressing the release of pro-inflammatory cytokines.

Fig. 5  Effect of POG on releases of TNF-α, IL-1β and IL-6 in RAW 264.7 cells induced by LPS. RAW 264.7 cells were incubated with POG and LPS (100 ng·mL⁻¹), and the release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) were detected by using ELISA method. Data were represented as mean ± SD (n = 4). *P < 0.05, and **P < 0.01 vs control
POG down-regulates the expressions of TLR2 and TLR4

For the investigation the possible mechanism of POG on sepsis, Western blotting assay was performed to study the effects of POG on the expressions of TLR2 and TLR4. As shown in Fig. 6, LPS can notably up-regulated the expressions of TLR-2 and TLR-4, which were reversed by POG at the concentrations of 20, 40, and 80 μg·mL⁻¹, (P < 0.01) in a concentration-dependent manner.

**Fig. 6 Effect of POG on expressions of TLR2 and TLR4 in RAW 264.7 cells induced by LPS. RAW 264.7 cells were incubated with POG and LPS (100 ng·mL⁻¹), and the expressions of TLR-2 and TLR-4 were detected by using western-blot assay. Data were represented as mean ± SD (n = 4). † P < 0.05, and ‡ P < 0.01 vs control**

Discussion

In recent years, increasingly researches have revealed that natural agents isolated from plants or herbs are important resource for finding novel drugs with low toxicity [10, 15-18]. It’s over 2000 years for Chinese people to use herb medicines to treat inflammation and infection, and previous evidences have indicated that the use of natural drugs with anti-inflammatory effect is a feasible approach to treating sepsis [16, 19]. To the best of our knowledge, this was the first report demonstrating that POG isolated from R. japonicus possessed potential protective effect on sepsis model mice challenged with lethal dosage of LPS and heat-killed E. coli. Furthermore, our investigation also indicated that the possible mechanism was closely related to down-regulation of pro-inflammatory cytokines and TLR2 and TLR4.

LPS is the major component of outer membrane of the gram-negative bacteria, and it’s well known that LPS is a common and crucial trigger of sepsis. In addition, endotoxemic mice induced by lethal dose of LPS and heat-killed E. coli are two commonly used sepsis models for evaluating the protective effects of anti-sepsis candidate drugs [7, 20]. In our present study, the POG showed notable protective activity on endotoxemic mice challenged with lethal dose of LPS and heat-killed E. coli, suggesting that POG possesses potential value for developing as anti-sepsis drug.

Inflammation plays a crucial role in occurrence and development of sepsis. Increasingly researches have demonstrated that pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, are markedly increased in sepsis patients [21]. In addition, it’s commonly considered that over-released pro-inflammatory cytokines induced by LPS are the important reasons of sepsis and septic shock [22]. TNF-α is recognized as an important early pro-inflammatory cytokine in the process of inflammatory reaction, whereas IL-6 and IL-1 are considered as the later pro-inflammatory cytokines [23]. These cytokines above are important for the pro-inflammatory cascade reaction, and therefore inhibiting pro-inflammatory cytokines releases is also considered as a promising strategy for treating sepsis [19]. Importantly, our results demonstrated that POG significantly decreased the releases of TNF-α, IL-1β, and IL-6 in RAW264.7 cells induced by LPS in a concentration-dependent manner.

TLR2 and TLR4 are pattern recognition receptors for LPS, and it’s well known that TLR2 and TLR4 are important for the inflammatory reactions and the release of pro-inflammatory cytokines [24-25]. Currently, investigations have revealed that TLR2 and TLR4 are two targets for finding novel strategies to treat sepsis [19, 25]. In our present study, we demonstrated that POG significantly down-regulated the protein expressions of TLR2 and TLR4, which was another evidence for validation the anti-sepsis effects of POG.

In conclusion, our study revealed that the POG extracted from R. japonicus possessed anti-sepsis effects on the endotoxemic mice, and the anti-sepsis effects of POG were exerted via decreasing the releases of pro-inflammatory cytokines and down-regulating the TLR4 and TLR2 expressions. However, all the results merely based on animal and cell experiments, and thus more work should be devoted to clinical investigations of the therapeutically effect of POG. What’s more, the further molecular mechanism of POG should be studied in the future.

References

