EXPERIMENTAL RESEARCH

Effect of Jianpi Huoxue Decoction (健脾活血方)–containing Serum on Tumor Necrosis Factor-α Secretion and Gene Expression of Endotoxin Receptors in RAW264.7 Cells Induced by Lipopolysaccharide

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ABSTRACT Objective: To evaluate the effect of Jianpi Huoxue decoction (健脾活血方, JHD)-containing serum on tumor necrosis factor-α (TNF-α) secretion and endotoxin receptor gene expression in RAW264.7 cells induced by lipopolysaccharide (LPS). Methods: The cytotoxicity of blank-control serum and JHD-containing serum at different concentrations were evaluated through the lactate dehydrogenase (LDH) assay in RAW264.7 cells. RAW264.7 cells were divided into six groups: 5% blank-control serum group (C1, n=3), 5% blank-control serum plus LPS group (L1, n=4), 5% JHD-containing serum plus LPS group (J1, n=4), 10% blank-control serum group (C2, n=3), 10% blank-control serum plus LPS group (L2, n=4), and 10% JHD-containing serum plus LPS group (J2, n=4). After cultured with the corresponding serum for 1 h, cells in L1, L2, J1 and J2 were treated with LPS (0.1 μg/mL) for 12 h without rinse. The supernate, cells, protein and RNA were collected for assay. TNF-α in the culture supernate was assayed by the enzyme linked immunosorbent assay (ELISA). Protein expression of TNF-α in RAW cells was detected by Western-blot. TNF-α, Toll-like receptor 2 (TLR2), TLR4 and CD14 mRNA expression in RAW cells were detected by real-time RT-PCR. Results: The LDH assay supported that cultured for 24 h or less with the JHD-containing serum at the concentration of 10% or lower, RAW264.7 cells showed no cytotoxicity. After stimulation with LPS for 2 h, TNF-α in the culture supernate of the 5% blank-control serum plus LPS group (L1, P=0.03), 10% blank-control serum plus LPS group (L2, P=0.002) and in the cell layer (P=0.01) of these groups increased remarkably. After stimulation with LPS for 1 h, the mRNA expression of TNF-α (P=0.004), TLR4 (P=0.003), CD14 (P=0.004) was up-regulated obviously. In the 10% JHD-containing serum plus LPS group (J2), the protein expression of TNF-α in both supernate (P=0.04) and cell layer (P=0.04), gene expression of TNF-α (P=0.03), TLR4 (P=0.001), CD14 (P=0.001) were all inhibited. On the other hand, the TLR2 mRNA expression was not up-regulated after LPS stimulation in the 10% blank-control serum plus LPS group (L2). Conclusion: JHD-containing serum inhibited the LPS-induced cytokines expression in RAW264.7 which was probably associated with its inhibitory effect on the mRNA expression of LPS receptors TLR4 and CD14.

KEY WORDS Jianpi Huoxue decoction-containing serum, lipopolysaccharide, cytokine, endotoxin receptor

The theory of "two-hits" has been thoroughly studied with great advancement obtained in the pathogenesis research of alcoholic liver disease (ALD). At present, most attention in this field was focused on the mechanism of Kupffer cell activation by endotoxin or lipopolysaccharides (LPS). The endotoxin receptors, such as CD14, Toll-like receptor 4 (TLR4), Toll-like receptor 2 (TLR2), have been identified and proved to be involved in the endotoxin signal pathway which activates the nuclear factor-κ B (NF-κ B) promoting the gene expression of cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, in Kupffer cells and ultimately resulting in liver injury.

In previous researches, it was demonstrated that Jianpi Huoxue decoction (健脾活血方, JHD) inhibited intestine and liver injury, gut-leakage, Please cite this article in press as: PENG Jing-hua (彭景华), HU Yi-yang (胡义扬), FENG Qin (冯 琴), CHENG Yang (成 扬), XU Li-li (许丽莉), CHEN Shao-dong (陈少冬), TAO Qing (陶 庆), and LI Feng-hua (李凤华), Chin J Integr Med (2009) 15:198-203 DOI: 10.1007/s11655-009-9001-x
endotoxin concentration and protein or gene expression of TNF-α in the liver of rats induced by Lieber-DeCarli ethanol liquid or LPS. However, it was not clear whether JHD directly inhibited the expression of Kupffer cell-derived cytokines or endotoxin receptors, besides reducing the LPS-gut-leakage. In the following research\(^{15}\), the effect of JHD on LPS-induced cytokine secretion pathway was observed and its inhibition on protein or gene expression of cytokines or endotoxin receptors was confirmed. To further investigate the effect of JHD-containing serum on cytokine secretion and gene expression of endotoxin receptors in vitro induced by LPS, murine macrophage RAW264.7 was used in the present study.

**METHODS**

**JHD Preparation**

JHD is composed of *Altractylodes macrocephala* Koidz., *Salvia miltiorrhiza* Bge., *Citrus aurantium* L., *Paeonia lactiflora* pall., *Pueraria lobata* (willd.) Ohwi, *Alisma orientalis* (Sam.) Juzep., *Schisandra chinensis* (Turcz.) Baill. and *Curuma longa* L. *Altractylodes macrocephala* Koidz., *Citrus aurantium* L. and *Curuma longa* L. were distilled with ethanol to get the volatilizable components for three times, each lasting for 1-2 h. *Schisandra chinensis* (Turcz.) Baill. alone was also extracted with ethanol twice for 1-2 h each time. The other herbs were boiled with water for three times after being soaked in water for 1 h. The final density of the aqueous extract was 1.08-1.12 \( (80^\circ C) \) and the aqueous extract was purified with ethanol. Finally, the volatilized components, ethanol-extraction of *Schisandra chinensis* (Turcz.) Baill. and aqueous extract were mixed as the JHD. The concentration of 0.9 g crude drug/mL was used in the experiments.

**JHD-containing and Blank Control Serum Preparation**

Normal male SD rats weighing 300 ± 10 g were divided into JHD and control groups and administrated with JHD or normal saline via gastrogavage respectively, 5 mL/kg, twice a day, for 3 days. One hour after the last administration, the JHD-containing and blank-control blood samples were collected through celiac vein steriley. After settling down for 3-4 h at room temperature, the JHD-containing and blank-control sera were extracted through centrifuge at 3 000 r/min under 4°C for 20 min and inactivated at 56°C for 30 min, and then stored at -70 °C.

**RAW264.7 Cell Line Culture and Treatment**

RAW264.7 cells (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in RPMI-1640 (GIBCO Invitrogen Corporation, California, USA) containing 10% fetal bovine serum (GIBCO Invitrogen Corporation, California, USA) in 5% CO₂ incubator (Heraeus Holding GmbH, Germany) at 37 °C and rinsed twice with minimum essential medium (MEM) (GIBCO Invitrogen Corporation, California, USA) before testing.

Cultured for 24-48 h, RAW264.7 cells were divided into 6 groups: 5% blank-control serum group (C1), 5% blank-control serum plus LPS group (L1), 5% JHD-containing serum plus LPS group (J1), 10% blank-control serum group (C2), 10% blank-control serum plus LPS group (L2) and 10% JHD-containing serum plus LPS group (J2). After cultured with corresponding serums for 1 h, cells in L1, L2, J1 and J2 were treated with LPS (Escherichia coli 0111:B4, Sigma-Aldrich Co., USA) 0.1 μg/mL for 1-2 h without rinse. The supernate, cells, protein and RNA were collected for assay.

**JHD-containing Serum Toxicity Test**

Cells were cultured with 10%, 20% and 30% JHD-containing serum or blank-control serum respectively. At time points of 0 h, 12 h and 24 h, the activity of LDH in the supernate was detected.

**Measurement of LDH Activity with Biochemistry Assay**

The activity of LDH in the supernate was determined with an LDH biochemistry assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacture’s instruction. Results were proofread with the protein concentration of cells and expressed as U·μg pro.⁻¹.

**Measurement of TNF-α in the Supernate by ELISA**

Concentration of TNF-α in the supernate was determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA) according to the manufacturer’s instruction. TNF-α was determined from a standard curve for the combination of these cytokines. Results were proofread with protein concentration of cells and expressed as pg·μg pro.⁻¹.