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Betulin attenuates lung and liver injuries in sepsis

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ABSTRACT

Sepsis is a complex condition with unacceptable mortality. Betulin is a natural extract with multiple bioactivities. This study aims to evaluate the potential effects of betulin on lung and liver injury in sepsis. Cecal ligation and puncture was used to establish the rat model of sepsis. A single dose of 4 mg/kg or 8 mg/kg betulin was injected intraperitoneally immediately after the model establishment. The survival rate was recorded every 12 h for 96 h. The organ injury was examined using hematoxylin and eosin staining and serum biochemical test. The levels of proinflammatory cytokines and high mobility group box 1 in the serum were measured using ELISA. Western blotting was used to detect the expression of proteins in NF-κB and MAPK signaling pathways. Betulin treatment significantly improved the survival rate of septic rats, and attenuated lung and liver injury in sepsis, including the reduction of lung wet/dry weight ratio and activities of alanine aminotransferase and aspartate aminotransferase in the serum. In addition, levels of tumor necrosis factor-α, interleukin-1β, interleukin-6 and high mobility group box 1 in the serum were also lowered by betulin treatment. Moreover, sepsis-induced activation of the NF-κB and MAPK signaling pathway was inhibited by betulin as well. Our findings demonstrate the protective effect of betulin in lung and liver injury in sepsis. This protection may be mediated by its anti-inflammatory and NF-κB and MAPK inhibitory effects.

1. Introduction

Sepsis, a serious condition with a very high mortality in intensive care, is characterized by a systemic uncontrolled hyper-inflammatory response, disseminated intravascular coagulation and multiple organ dysfunctions [1–5]. The pathogenesis of sepsis is complicated. Till now, the physiopathological mechanisms of sepsis have been studied well. Many mediators such as proinflammatory cytokines, chemokines and free radicals were found to be involved in the processes of sepsis [6,7]. However, despite the investigation of various therapeutic strategies to improve sepsis care, an effective compound has seldom been found. Antibiotics are still the main strategy in clinical care and the mortality of sepsis is still as high as 30% to 40% [8,9].

Multiple organ failure is one of the important reasons that cause death in septic patients [10]. Multiple-organ dysfunction syndrome (MODS) frequently occurs in severe sepsis and sepsis shock. The severity of organ dysfunction often determines the prognosis of sepsis [11]. As a systemic inflammatory response syndrome, in the process of sepsis, an inflammatory cascade was activated and excess proinflammatory cytokines were secreted into periphery blood and organs. Uncontrolled inflammation causes capillary permeability, endothelial damage, interstitial edema and hyperdynamic circulation, which subsequently lead to tissue hypoxia, metabolic failure and finally MODS [10]. Therefore, compounds that have an effective anti-inflammatory profile and target multiple-organs would be beneficial in sepsis therapy.

Betulin (lup-20(29)-ene-3β, 28-diol) is a triterpene extracted from birch tree bark. It can be converted to betulinic acid by chemosynthesis or biotransformation. The three active positions in their structure empower these two compounds with various pharmacological activities, such as antitumor, anti-HIV, anti-inflammatory, antiviral and antibacterial activities [12]. Moreover, previous studies have shown that betulin could decrease LPS-induced inflammation by preventing nuclear factor kappa-B (NF-κB) activation [13]. Betulin also inhibited the activation of p38 and c-Jun N-terminal kinase (JNK) transduction pathways in ethanol-induced liver stellate cells [14]. These two signaling pathways have high correlations of inflammation and cell stress. In addition, they were found to be activated in sepsis, and pharmacological inhibition of these two pathways was involved in the anti-inflammatory effects [15,16]. In the present study, a sepsis model was established by cecal ligation and puncture (CLP) and the potential effect of betulin on lung and liver injury in sepsis was evaluated.

2. Materials and methods

2.1. Animals

A total of 92 male Sprague–Dawley rats (8 weeks old) were supplied by the Experimental Animal Centre of China Medical University (Shenyang, China). The animals were housed in cages located in a room with stable temperature (22–24 °C) and a 12/12 h light/dark cycle and

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received water and food *ad libitum*. All animal protocols were approved by the ethics committee of China Medical University (Shenyang, China).

2.2. Induction of sepsis

Sepsis was induced by CLP according to a method described previously [17]. Briefly, after fasting for 12 h, rats were anesthetized with 3.5 ml/kg chloral hydrate. A 1.5 cm midline incision was performed and the cecum was exposed and ligated at the root. The cecum was punctured three times using a 5 ml syringe needle. Light pressure was applied to expel a small amount of fecal material. The cecum was then returned and the wound was closed. All the animals received 3 ml/100 g saline subcutaneously immediately after the operation for fluid resuscitation. Rats in the sham group received only cecum exposure but not ligation or puncture on the cecum.

2.3. Betulin treatment and mortality experimental protocols

Rats were randomly divided into 5 groups: (1) sham group (n = 22); (2) CLP group (n = 24); (3) CLP + dexamethasone 2 mg/kg group (DEX) (n = 24); (4) CLP + betulin 4 mg/kg group (Betulin L) (n = 24); and (5) CLP + betulin 8 mg/kg group (Betulin H) (n = 22). Rats were administrated intraperitoneally with DEX (Tianjinxinzheng, Tianjin, China) or betulin (Meilun, Dalian, China, purity >98%) dissolved in saline immediately after the model establishment. Rats in the sham and CLP groups received only saline. The choice of administration time and dosage was based on our primary experiments.

Ten rats in each group were randomly selected to record the survival rate. Rats were monitored every 12 h for 96 h and the numbers of surviving rats were recorded. In the remaining 52 rats, one in the CLP group and one in the Betulin L group died at 24 h after the operation. One rat in the CLP group and one in the Betulin L group were randomly selected to perform primary experiments. Six rats in each group were randomly selected to measure the wet and dry weight of lungs at 24 h after treatment. The last six rats in each group were used to perform plasmic, biochemical and molecular biological assays.

2.4. Measurement of the lung wet/dry weight ratio

Lungs were weighed immediately after being isolated and dried in a microwave oven at 80 °C to constant weight. The wet/dry weight ratio was calculated.

2.5. Histological analysis

Twenty-four hours after betulin treatment, tissues of lung and liver were harvested and fixed with 4% paraformaldehyde overnight. The samples were then dehydrated in ascending grades of alcohols, embedded in paraffin, and cut into 5-μm sections. To perform hematoxylin and eosin (H&E) staining, sections were immersed in xylene for 15 min and hydrated through graded ethanol. Then sections were stained with hematoxylin (Solarbio, Beijing, China) for 5 min and with eosin (Solarbio) for 3 min. After being dehydrated with increasing concentrations of ethanol and cleared in xylene, sections were mounted in Permount and observed under an optic microscope (DP73, Olympus, Tokyo, Japan).

2.6. Serum analysis

Blood sample was obtained 24 h after betulin treatment. Whole blood was centrifuged at 7700 *g* for 10 min at 4 °C. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Serum tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and high mobility group box 1 (HMGB-1) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits specific for rat following the manufacturer's instructions (USCN, Wuhan, China).

2.7. Protein preparation

Lung and liver tissues were homogenized in icy NP-40 lysis buffer supplement with 1% Triton X-100 and 1 mM phenylmethanesulfonylfluoride. Tissue homogenate was centrifuged at 12,000 *g* for 10 min and the supernatants were collected. Nuclear and cytosolic proteins were separated using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's protocol. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime).

2.8. Western blot analysis

An equal amount of protein (40 μg) for each sample was separated in the SDS-PAGE and transferred onto the polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using the wet transfer method. Membranes were blocked in 5% non-fat milk for 1 h at room temperature and incubated in primary antibodies at 4 °C overnight. After a wash stage using Tris buffered saline, with Tween-20 (TBST), membranes were incubated in horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse IgG (1:5000, Beyotime) for 45 min at room temperature. Finally, membranes were immersed in enhanced electrochemiluminescence reagent (7 Sea Pharmtech, Shanghai, China) and exposed using X-ray film. Primary antibodies against extracellular regulated protein kinases (ERK) (bs-2637R), p-ERK (bs-1522R), JNK (bs-10562R), p-JNK (bs-1640R), p38 (bs-0637R), p-p38 (bs-5477R), inhibitors of NF-κBα (IκBα) (bs-1287R), and p-p65 (bs-0982R) from Bioss, Beijing, China, and p65 (BA0610) from Boster, Wuhan, China were used and the protein levels were standardized to β-actin (sc-47,778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for whole cell lysate and Histone H3 (bs-17422R, Bioss) for the nuclear fraction.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Survival data were analyzed by the Kaplan–Meier curve and log-rank test. Other data were analyzed by one-way analysis of variance, and the Fisher's least significant difference test was used for *post hoc* comparisons. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of betulin on survival rate in CLP rats

All the rats in the sham group survived at the end of monitoring. In the vehicle-treated CLP group, the survival rate for 48 h was 40% and reached 0% at 96 h. No rat survived at the end of the experiment in this group. Betulin 4 mg/kg reduced the mortality to some extent but was not significant. The survival rate was 70% at 48 h and 30% at 96 h. DEX 2 mg/kg and betulin 8 mg/kg significantly improved the survival of septic rats, compared with the CLP group ($P < 0.01$). In the DEX group, the survival rate was 90% at 48 h and 80% at 96 h. In the betulin H group, the survival rate was 80% at 48 h and 60% at 96 h (Fig. 1). Although the survival rate in the DEX group was higher than that in the betulin H group, there is no significant difference ($P > 0.05$).

3.2. Effect of betulin on CLP-induced lung and liver injuries

H&E staining illustrated the normal cell structures of the lung and liver in the sham operated rats (Fig. 1A). After CLP operation for 24 h, lung tissue showed marked inflammatory cell infiltration, edema,