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Cytoprotective effects of glycyrrhetinic acid liposome against cyclophosphamide-induced cystitis through inhibiting inflammatory stress

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ABSTRACT

This study was designed to investigate the pharmacological efficacy of glycyrrhetinic acid liposome (GAL) against female mice with nonbacterial cystitis induced by cyclophosphamide (CPS). Mice in different groups were subjected to tests for lactate dehydrogenase (LD), cytokine contents (IL-6, TNF-α) in serum, and histological changes in bladder tissue and to immunoassays. As a result, cyclophosphamide-induced cystitis in mice showed an increased LD level in serum, and the contents of cytokines (IL-6, TNF-α) were elevated. Interestingly, GAL-treated mice showed decreased LD and inflammatory cytokines of IL-6 and TNF-α in blood. Inflammatory infiltration and cell death in bladder tissue were reduced by GAL treatments. In addition, intravesical mRNAs of NF-κB and TNF-α were lowered dose-dependently in GAL-treated mice. As shown in cytohistological staining, the number of intravesical caspase-3, PARP-positive cells decreased in GAL-treated mice. Furthermore, a GAL-treated bladder showed down-regulated NF-κB and TNF-α expressions in a dose-dependent manner. In conclusion, our current findings may be the first to provide scientific evidence demonstrating that glycyrrhetinic acid liposomes provide benefits against cyclophosphamide-induced cystitis, which possibly occurs through underlying mechanisms that inhibit cell death and inflammatory stress.

1. Introduction

Bladder infection (cystitis) is a type of chronic pain that affects bladder function [1]. Clinically, some medications may induce non-bacterial/pathological cystitis, such as cyclophosphamide [2]. Cyclophosphamide is used in chemotherapy and is one of the most common medicines in most health systems. However, most patients experience unwanted adverse effects, including low immune cell counts, hair loss, vomiting, and bladder bleeding [3]. In bladder impairment, cyclophosphamide produces acrolein that is toxic to the bladder epithelium and causes hemorrhagic cystitis. Bladder function impairment can be linked to lower urinary tract symptoms, including dysuria, hematuria, and hemorrhage [4–5]. Risks of hemorrhagic cystitis can further induce immune disturbances and inflammatory disorders over time [6]. In clinical practice, sodium 2-mercaptoethane sulfonate is used to detoxify acrolein, but some dose-dependent side effects still occur [7]. Therefore, inhibition of cystitis may enhance drug effects of cyclophosphamide and reduce pain in patients. Meanwhile, the potential alternative for interstitial cystitis treatment needs to be further developed and investigated. Glycyrrhetinic acid (GA), which is obtained from the herb liquorice, is a beta-amyrin type pentacyclic triterpenoid derivative that is obtained from the hydrolysis of glycyrrhizic acid. GA has some additional pharmacological properties, including antiviral, antibacterial, and anti-inflammatory activities [8]. GA inhibits specific enzymes that metabolize the prostaglandins PGE-2/2α to the respective 15-keto-13,14-dihydro metabolites that are inactive. This inhibition causes an increased level of prostaglandins in the digestive system [9]. A liposome is a spherical vesicle that has at least one lipid bilayer. The liposome can be used as a vehicle for administration of nutrients and pharmaceutical drugs [10]. Based on the possible benefits above, we hypothesized that the glycyrrhetinic acid liposome (GAL) might have potent action against cyclophosphamide-induced cystitis. Therefore, our current study aimed to assess the beneficial actions of GAL against cyclophosphamide-induced cystitis in mice and to discuss the underlying mechanisms that are involved.

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2. Materials and methods

2.1. Key materials

GA (purity > 98%) was obtained from Succhi Shiqi Pharmaceutical Co. Ltd. (Guangdong, China). Subsequently, preparation and identification of glycyrrhetinic acid liposome was completed by the Department of Pharmaceutical Preparation at Guilin Medical University. Clinical lyophilized powder of cyclophosphamide was purchased from Jiangsu Shengdi Pharmaceutical Co., Ltd. (Lianyungang, China) and dissolved in normal saline before use.

2.2. Animals and drug administration

Mature female Kunming mice (6–7 weeks, 20 ± 1 g) were purchased from the Experimental Animal Centre at Guilin Medical University (Guilin, China). All mice were housed in an animal room with a designated temperature of 25 ± 2 °C, a relative humidity of 60 ± 10%, and a room with a 12 h light/dark cycle. All mice had access to water and standard chow ad libitum. This study was approved by the Institutional Animal Care and Use Committee at Guilin Medical University.

To establish cyclophosphamide-induced cystitis, the mice were given 50 mg/kg cyclophosphamide intragastrically every 3 days out 10 days, as described in a previous study [11]. Subsequently, the bladder-impaired mice were randomly assigned to three groups (n = 8 per group). In the GAL-dosed groups (5 mg/kg, 10 mg/kg), the mice were given GAL daily for 10 days. In the cyclophosphamide (CPS) group, the mice were intragastrically injected with the same volume of cyclophosphamide (50 mg/kg) every 3 days out of 10 days. In addition, untreated mice in the vehicle group were intragastrically injected with an equivalent volume of normal saline for 10 days.

2.3. Sample collection

At the end of day 10, the mice were killed by cervical dislocation for blood collection. Bladder tissue was isolated immediately, and some of the bladders were fixed with 4% paraformaldehyde for histocytological observations and others were stored at −80 °C for further immunoassays.

2.4. Enzymatic measurement in serum

Blood levels of lactate dehydrogenase (LD) were measured using commercial test kits (Nanjing Jiancheng Bioengineering Institute, China). The final data were expressed as U/L. In addition, the serum contents of cytokines (IL-6, TNF-α) were determined using the commercial ELISA kits (Shanghai Elisa Biotech Co., Ltd., China). The final data were expressed as pg/ml [12].

2.5. Histomorphological examination

Five micrometer bladder sections were subjected to routine staining with hematoxylin and eosin (H&E). Morphological changes were detected and imaged (BX53F, Olympus, Tokyo, Japan). The inflammatory assessment was conducted by assaying the mean amounts of staining in different optical fields.

Bladder samples were pre-fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 2.5% glutaraldehyde at 4 °C for at least 8 h. After washing with PBS and deionized water for 3 times, samples were post-fixed with 1% osmium tetroxide for at least 4 h. Following by dehydration in a series of alcohol concentrations, resin-embedded samples were sectioned and stained with 2% uranyl acetate/lead citrate. In conclusion, all the sections were imaged, captured, assayed using transmission electron microscope (TEM) (Hitachi, H-7650, Japan) system working at 80 kV.

2.6. Immunohistochemical analysis

Deparaffinized bladder sections were processed through stepwise concentrations of 80%, 90%, 100% dimethylbenzene and ethanol for 5 min, and then rinsed 3 times with PBS. The sections were treated with 3% H_2O_2 to inactivate endogenous enzymes for 6 min at 37 °C prior to a hot-fixed antigen procedure. Next, 5% BSA blocking solution was added, followed by a reaction with a diluted primary antibody and a specific second antibody (cleaved-caspase-3, cleaved-PARP, 1:200, Cell Signaling Technology, Boston, USA) coincubated with fresh SABC solution (Wuhan Boster, China). The subsequent steps were followed by DAB dyeing and hematoxylin counterstaining before dehydrating, transparentizing, mounting, and scanning images using an Olympus microsystem [13].

2.7. Real time-PCR tests

Target RNA was isolated from bladder tissue using Trizol (Thermo Fisher Scientific, USA). The purity of the RNA was confirmed with a spectrophotometer at 260 nm. RNA-to-cDNA transcription was conducted using a PCR profiling kit (Life Technologies Corporation, USA) according to the manufacturer's procedures. The primer sequences of NF-κB and TNF-α were obtained using a protocol from a prior study [14]. In brief, the PCR reaction was conducted at 94 °C for 10 min, 30 cycles of 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 60 s, then a final extension at 72 °C for 10 min. Beta-actin was used as a housekeeping gene and normalized with β-actin to produce the relative expression data.

2.8. Western blot analysis

The bladder samples were homogenized with lysis buffer (pH 7.2) using 1 mM protease inhibitor, and then the protein concentration was measured using a protein assay reagent (Beyotime Biotechnology, China). The protein was separated by 10% SDS-PAGE. The protein was transferred to a PVDF membrane, and the membrane was blocked with PBST buffer (20% Tween-20, PBS) and incubated with the anti-NF-κB antibody and anti-TNF-α antibody (1:1000 Santa Cruz, USA; Wuhan Boster, China) overnight at 4 °C. The blots were washed three times with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotechnology, China) for 1 h at room temperature. The band densities were analyzed using Scion image software (Bio-Rad, MD, USA) [15].

2.9. Statistical analysis

All data were generated through statistical product and service solutions (SPSS) 19.0 software (USA). Differences between these groups were determined using the analysis of variance (ANOVA) followed by a Tukey's test for post hoc multiple comparisons. The results were expressed as the mean ± SD. The level of significance was set at $P < 0.05$.

3. Results

3.1. Effects of GAL on vital signs in CPS-damaged mice

In body weight records, CPS-lesioned mice showed visible body mass loss compared to untreated mice ($P < 0.05$). Followed by CPS exposure and GAL cotreatment for 10 days, increased body weight was observed in a dose-dependent manner ($P < 0.05$). In addition, CPS-exposed mice had elevated serological levels of LD ($P < 0.05$). Compared to the CPS controls, the abnormal changes in LD content were reversed following co-administration of GAL in CPS-exposed mice ($P < 0.05$) (Fig. 1).