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Therapeutic effects of total steroid saponin extracts from the rhizome of *Dioscorea zingiberensis* C.H.Wright in Freund's complete adjuvant induced arthritis in rats

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ABSTRACT

The aim of our present study is to explore the anti-arthritic potential effect of total steroid saponins (TSSNs) extracted from the rhizome of *Dioscorea zingiberensis* C.H.Wright (DZW) and to investigate the underlying mechanisms. This work was performed using adjuvant-induced arthritis (AIA) rats in vivo and lipopolysaccharide (LPS) simulated 264.7 macrophage cells in vitro. In AIA-induced arthritic rats, TSSN significantly alleviated the arthritic progression through evaluating arthritic score, immune organ indexes, paw swelling, and body weight. This phenomenon was well correlated with significant suppression of the overproduction of inflammation cytokines (IL-1, IL-1β, IL-6, and TNF-α), oxidant stress makers (MDA and NO), eicosanoids (LTB_4 and PGE_2), and inflammatory enzymes (5-LOX and COX-2) versus the AIA rats without treatment. On the contrary, the release of SOD and IL-10 was profoundly increased. What's more, TSSN could obviously ameliorate the translocation of NF-κB to the nucleus through phosphorylation of the p65 and IκBα in vivo and in vitro. The current findings demonstrated that TSSN could protect the injured ankle joint from further deterioration and exert its satisfactory anti-arthritis properties through anti-inflammatory and anti-oxidant effects via inactivating the NF-κB signal pathway. This research implies that DZW may be a useful therapeutic agent for the treatment of human arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic and relapsing systemic autoimmune disease characterized by synovial hyperplasia, vasculogenesis, cartilage destruction, bone deformity and functional disability of the joint [1,2]. This systemic disorder is caused by progressive inflammation of the joint lining tissue, which can cause pain, stiffness, swelling, as well as many other symptoms [3]. RA is prevalent throughout the world and affects some of the human population causing long-term disability and premature mortality. Therefore, it is important to continue pathophysiological and pharmacological studies on this disease to discover the new therapeutical drugs.

Currently, RA is clinically treated mainly by synthetic medicines belonging to non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen, aceclofenac, and naproxen combined with steroid hormones like cortisone and prednisone [4]. However, these drugs only transiently suppress inflammation and ameliorate symptoms, but they do not significantly improve the long-term disease outcome [5]. Moreover, during these therapeutic treatment, many patients eventually lose response to the drugs or they are forced to interrupt drug administration due to severe adverse side effects such as gastrointestinal ulcergenicity [6], cardiovascular complication, hematologic toxicity and renal morbidity [7,8], hence utility of these medicines are limited for the treatment of RA. Owing to these shortcomings, the exploration of new anti-RA drugs with high efficacy and less toxicity is eagerly needed.

Traditional Chinese medicine (TCM), a unique medical system characterized by the use of multi-component drugs, can hit multiple targets with its components, can improve therapeutic efficacy, can reduce drug-related side effects, and may also be an effective way of decreasing drug resistance [9,10]. Recently the study of TCM has aroused much interest due to its superiority in the treatment of complex multi-factor diseases [11]. Thus, herbal medicines may constitute a potentially important avenue leading to novel therapeutic agents for RA that may not only prevent structural damage of arthritic joints caused by tissue and bone breakdown, but also be safe, relatively inexpensive, highly tolerated and convenient for many patients. Therefore, naturally originated drugs with minimum side effects are highly desired to substitute chemical therapeutics.

In recent years, steroid saponins isolated from herbs have attracted scientific attention because of their structural diversity and significant

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biological activities. DZW, one of the most commonly used raw materials from a unique plant growing in China, contains a high level of steroid saponins which have been applied as a folk treatment for cough, anthrax, rheumarthritis, tumefaction, sprain as well as cardiac disease in the TCM for a long time [12]. Bioactivities of these steroid saponins, including antitumor, antifungal, antivirus, and anticoronary heart disease, have been reported [13]. However, no research has been reported on its anti-arthritic effect to our knowledge. Therefore, our current study was designed to confirm its anti-arthritic effect and explore its potential mechanism of the total steroid saponin extracted from the rhizome of DZW on AIA-treated rats in vivo and macrophage cells in vitro.

2. Materials and methods

2.1. Reagents

FCA was purchased from Difco Laboratories (Detroit, MI, USA). Methotrexate was obtained from Shanghai Sine Pharmaceutical Co., Ltd. (Shanghai, China). ELISA test kits were purchased from R&D Systems (Minneapolis, USA). All other chemicals and reagents used for study were of analytical grade procured from approved organizations.

2.2. Plant material and preparation of total steroid saponin extracts

The rhizomes of DZW were provided by Yangtze River Pharmaceutical Industry Co., Ltd. (Jiangsu, China), and authenticated by Prof. Y.Z. Wang (Northwest University, Xi'an of Shaanxi, China). A voucher specimen (HJ20100925-10) has been deposited in the School of Pharmacy, Fourth Military Medical University, Shaanxi, China.

Dried raw material of DZW was powdered and extracted with 70% ethanol three times. The ethanol extracts were combined and evaporated to dryness under reduced pressure with a rotary evaporator. The residue was redissolved in water and subjected to centrifugation. The supernatant was separated on a D-101 macroporous resin column by eluting with 60% ethanol. The eluate was concentrated under reduced pressure. The syrup thus obtained was dissolved in water again, and extracted with an equal volume of n-butanol six times successively. The pooled n-butanol extract was concentrated to obtain residues for the subsequent experimental use.

2.3. Phytochemical investigation of TSSN by HPLC-ELSD and HPLC-ESI-MS

The compounds in the TSSN have been analyzed by HPLC-ELSD and HPLC-ESI-MS in our laboratory [14]. The HPLC analysis was performed on a Waters Alliance 2695 equipment (Waters, Milford, MA, USA) including Alltech 2000ES (Alltech, USA), and the mass spectrometer was equipped with a Q-TOF Premier, a quadrupole and orthogonal acceleration time-of-flight tandem mass spectrometer with an electrospray ionization interface.

2.4. Cell culture and NF-κB expression

The RAW 264.7 macrophage cell line acquired from American Type Culture Collection (Rockville, MD, USA) was used in the current study. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotic namely penicillin (100 units/mL) and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2 [15]. They were incubated with TSSN (25, 50, and 100 μg/mL) and corresponding positive control Methotrexate (35 μg/mL), followed by adding with LPS for another 12 h (LPS, 1 μg/mL). Non-stimulated normal control cells were also simultaneously cultured as the control. After stimulating with LPS for 24 h, the culture supernatants were collected and the total protein was extracted according to the previous description [16]. When finishing this procedure, the total p65 of NF-κB and IκBα were determined by Western blotting.

2.5. Animal preparation

Healthy adult male Sprague–Dawley rats aged 8–10 weeks (weighing 250–280 g) were purchased from the Experimental Animal Center of The Fourth Military Medical University (Shaanxi, China). One week before the experiment, the animals were acclimatized in an environment at 24 °C ± 1 °C, with relative humidity of 45–55% and 12:12 h dark/light cycle under specific pathogen-free (SPF) conditions. Enough rat food rich in various necessary nutritional ingredients was supplied, and water was changed every day. What's more, their cages were cleaned every two days to make their home comfortable. All experimental procedures were in strict accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. Animal experiments were approved by the local institutional review board at the authors' affiliated institutions.

2.6. Induction of adjuvant arthritis and drug administration

Before the onset of arthritis, sixty Sprague–Dawley rats were randomly divided into six groups, namely the normal group, the FIA (Freund's adjuvant induced arthritis) control group, the positive control group of Methotrexate, the TSSN low-dose group, the TSSN middle-dose group and the TSSN high-dose group, with 10 rats in each group. The FIA control group is used to estimate the pharmaceutical effects of treated groups (including the TSSN-group and the Methotrexate-group). The positive control group of Methotrexate is to compare the efficacy between TSSN and Methotrexate. Except for the normal group, the arthritis was induced by a single injection of 0.1 mL of FCA, which contained 10 mg/mL of heat-killed *Mycobacterium tuberculosis* in liquid paraffin, into the palmar surface of the right hind paw [17]. This operation was conducted under gentle anesthesia with diethyl ether. After this primary immunization, the TSSN-treated groups were orally administered with TSSN extracts at three levels, which are high (200 mg/kg), middle (100 mg/kg), and low dose (50 mg/kg). Methotrexate (MTX, 3 mg/kg) was used as a reference drug of the positive control group and given by intragastric (ig) administration twice a week, while the normal control and FIA control groups were given an equal volume of normal saline at the same time. All groups were orally administered those items daily after arthritis induction until the end of the experiment (day 28).

After establishing the arthritis model, some related measures were taken to ameliorate this suffering during the subsequent experiment. Soft sawdust was placed in cage to avoid hard touching with the swelling leg, and this packing was changed to keep dry and soft every three days. The touch times with arthritic hinds were reduced as much as possible, when the drugs were administrated to rats. What's more, the arthritic rats were raised in a quiet environment to prevent them from activating pain by noise. During the period from the onset of arthritis to the end, some clinical signs such as body weight, fur color, diet, and changes in feces were monitored and recorded according to day-by-day observations. If any abnormal physical signs besides arthritis appear, the cause was analyzed to improve the condition.

2.7. Measurement of arthritis progression

2.7.1. Assessment of arthritis scores

The rats were assessed every three days for signs of arthritis between days 1 and 28 post-FCA using a well-established, widely used scoring system developed to evaluate the severity of AIA. Arthritis was examined and graded for severity and loci of erythema and swelling using a 4-point scale in which 0 = normal, 1 = mild swelling and erythema of digits, 2 = swelling and erythema of the digits, 3 = severe swelling and erythema, and 4 = gross deformity and inability to use the limb. The total score of each animal was calculated as the arthritic index, with a maximum possible score of 8 (4 points × 2 hind paws) [18]. Assessment of the arthritis score was carried out by a double-blind test.

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