Therapeutic effect of tripterine on adjuvant arthritis in rats

Hong Li, Yun-Yi Zhang*, Hui-Wen Tan, Yong-Feng Jia, Duan Li

Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 200032, China

A R T I C L E   I N F O

Article history:
Received 27 August 2007
Received in revised form 15 April 2008
Accepted 17 May 2008
Available online 3 June 2008

Keywords:
Tripterine
Adjuvant arthritis
Cytokine
Immune response

A B S T R A C T

Aims of the study: Tripterygium wilfordii Hoog f., a perennial vine, is used in traditional Chinese medicine for treatment of rheumatoid arthritis. This study was to determine whether tripterine, isolated from Tripterygium wilfordii Hoog f., had therapeutic effects on adjuvant arthritis.

Materials and methods: Adjuvant arthritis (AA) was induced in rats on day 0. Tripterine 5, 10 and 20 mg kg\(^{-1}\) day\(^{-1}\), or prednisone 10 mg kg\(^{-1}\) day\(^{-1}\) was given to rats intragastrically from day 19 to day 24.

Results: Tripterine significantly inhibited paw swelling and bone destruction in AA rats. Serum level of IgG anti-Mycobacterium tuberculosis antibodies and delayed-type hypersensitivity (DTH) induced by Mycobacterium tuberculosis were also decreased by tripterine. The effects of tripterine were associated with decreased interleukin-1β (IL-1β) mRNA expression in ankle joint synovial membrane and tumor necrosis factor-α (TNF-α) mRNA expression in homogenized paws from adjuvant-induced arthritic rats.

Conclusions: These findings suggested that tripterine had a therapeutic effect on adjuvant arthritis.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Rat adjuvant arthritis (AA) is a chronic, polyarticular, erosive type of arthritis induced by an injection of killed mycobacteria (Pearson and Wood, 1959). AA in rat is an experimental model that shares some features with human rheumatoid arthritis (RA). One of the most important features of AA is the chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion. AA is widely used for studying the pathogenesis of RA and for searching new drugs for treatment of rheumatoid disease (Greenwald, 1991; Schmidt-Weber et al., 1999; Zheng and Wei, 2005).

Tripterine (Fig. 1) is one of the main bioactive components of Tripterygium wilfordii Hoog f. (Celastraceae) that has been traditionally used for rheumatoid arthritis, and now widely used in chronic nephritis, thrombocytolytic purpura and dermatologic disease in Chinese medicine. It inhibits not only humoral and cellular immune responses but also some inflammatory reactions (Zhang et al., 1986, 1990). In vitro, tripterine inhibited lipopolysaccharide induced interleukin-1 production from murine peritoneal macrophages and human monocyte, concanavalin A-activated interleukin-2 production from murine splenocytes, and prostaglandin E\(_2\) released from synovial cells (Xu et al., 1991; Huang et al., 1998). Tripterine has shown interesting disease-modifying activity in the streptococcus cell wall induced model of arthritis, collagen induced arthritis in rats and systemic lupus erythematosus-like syndrome in mice (Li et al., 1997, 2005; He et al., 1998). Tripterine was reported to inhibit NF-kB activation, nitric oxide production, interleukin-6, interleukin-8, and prostaglandin E\(_2\) formation and lipid peroxidation (Jin et al., 2002; Pinna et al., 2004).

Here, the therapeutic effect of tripterine was studied in adjuvant-induced arthritic rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (Shanghai Slac Laboratory Animal Co., Ltd.) weighing 160±20 g were used. They were housed under standard laboratory conditions. Food and water were provided ad libitum. All experimental protocols described in this study were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University.

2.2. Drugs and reagents

Tripterine (red cubic crystal, mp 199–201 °C) was isolated by Prof De-jie Pan (Department of Pharmaceutical Chemistry, School of Pharmacy, Fudan University) and was used in the experiments. The purity was greater than 97% as determined by high performance liquid chromatography (HPLC). Immunosorbent assay (ELISA) kits for TNF-α, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and nuclear factor-kB (NF-kB) were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Bovine serum albumin (BSA), Mycobacterium tuberculosis, Mycobacterium bovis BCG, Freund's complete adjuvant, Freund's incomplete adjuvant, Freund's adjuvant (FCA), and Freund's incomplete adjuvant (FIA) were purchased from Wuhan Institute of Biological Products Research (Wuhan, China). Concanavalin A (Con A) was purchased from Sigma-Chemical Co., USA. Phospho-buffered saline (PBS), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), horse serum, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco BRL Co., USA. Cytokine ELISA kits were purchased from BioSource Co., USA. 

Abbreviations: AA, adjuvant arthritis; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FCA, Freund’s complete adjuvant; GAPDH, glyceraldehyde phosphate dehydrogenase; IL-1β, interleukin-1β; NF-kB, nuclear factor-kB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction analysis; Pre, prednisone; RA, rheumatoid arthritis; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; Trip, tripterine.

* Corresponding author. Tel.: +86 21 5423 7343; fax: +86 21 6469 3495.
E-mail address: zhcheng@shmu.edu.cn (Y.-Y. Zhang).
animals were selected and distributed into groups (metatarsal footpad of rats (day 0). Sixteen days after inoculation, the Shanghai), desiccated in 100

2.3. Induction of adjuvant arthritis

Adjuvant arthritis was induced by an injection of 1 mg heat-killed Mycobacterium tuberculosis (Institute of Biological Products, Shanghai), desiccated in 100 μl paraffin oil, into the right hind metatarsal footpad of rats (day 0). Sixteen days after inoculation, the animals were selected and distributed into groups (n = 10) according to the severity of arthritis, so that each group had similar disease severity at the beginning of the treatment. One was given 0.5% CMC solution as vehicle-treated group, the others were given tripterine (5, 10 and 20 mg kg\(^{-1}\)) intragastrically from day 19 to day 24, respectively.

2.4. Quantification of paw edema

The severity of AA was quantified by measuring the volume of hind paws using a water plethysmometer. Paw volume (ml) was measured on days 0, 16, 18, 20, 22, 24, 26 and 28 after arthritis induction. Data were expressed as the volume of increase with respect to day 0 volume.

2.5. Blood sampling and anti-Mycobacterium IgG antibody quantification

Adjuvant arthritis was elicited in rats and treatment was given as described above. Rats were sacrificed on day 26 after arthritis induction and blood samples were obtained. Serum was stored at \(-70\)°C until use.

IgG anti-Mycobacterium tuberculosis antibodies were determined by enzyme-linked immunosorbent assay (ELISA) (Turull and Queralt, 2000). Briefly, 96-well plates (Costar, Corning, NY) were coated with 0.1 ml soluble fraction of Mycobacterium tuberculosis of 3 μg of protein/ml prepared in 0.15 M phosphate-buffered saline (PBS) and incubated for 2 h at room temperature in a moist chamber. Free sites of the plastic surface were blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Serum samples were diluted at 1:200 in PBS containing 0.05% Tween 20 and 1% BSA, and added to plates. Then, plates were washed with PBS containing 0.5% Tween 20 and 100 μl goat anti-rat IgG (whole molecule) -peroxidase (Zhongshan Golden Bridge Biotechnology Co., Ltd.), prepared in PBS containing 0.05% Tween 20 and 1% BSA diluted at 1:2500, was added. The plates were washed with PBS containing 0.5% Tween 20. After the assay was developed with 0-phenylenediamine, the plate was incubated for 30 min at room temperature. Sulfuric acid (50 μl, 2 M) was added to every well and the optical density was measured at 492 nm using a well scanner ELISA reader (Labsystems Dragon).

A pooled standard batch of sera from arthritic animals was used as positive control on every plate and used to calculate relative unit, which was expressed as U/ml, for the anti-Mycobacterium antibody response.

2.6. Delayed-type hypersensitivity (DTH)

DTH was induced on day 23 after AA induction by an intradermal injection into the left ear of 20 μl of a 100 μg/ml soluble fraction of Mycobacterium tuberculosis (Turull and Queralt, 2000). The right ear was injected with normal saline as a control. After 24 h, the thickness of the pinna was measured with a dial thickness gauge (Guanglu). The non-specific increase in ear thickness was also determined by injecting 20 μl soluble fraction of Mycobacterium tuberculosis in the left ear of normal rats. Results are expressed as a percentage of increase in the thickness of the left ear.

2.7. Hind limb mRNA extraction and cytokine mRNA polymerase chain reaction analysis (PCR)

Adjuvant arthritis was elicited in rats and treatment was given as described above. On day 26 of arthritis, four animals from each group were killed and the hind left limbs were amputated at about the ankle. Frozen synovial membranes of ankles and hind paws were crushed and homogenized in liquid nitrogen and total RNA was extracted using Beyozol reagent (Beyotime Biotechnology) according to the manufacturer’s recommendation. The RNA was reverse transcribed into cDNA with random hexamers (Promega Corporation). Then PCR was performed in a total volume of 50 μl containing 3 units of rTag and 50 pmol of primers specific for murine interleukin-1β (5′-CAC CTA CCT ATG TCT TGC CC-3′, 5′-GTC GTT GCT CTC TCT TT-3′), for GAPDH (5′-GTT GGG GGG CCC AGG CAC CA-3′, 5′-GTC CCT AAT GTC GAC CAG CAC GAT TTC-3′) and for murine tumor necrosis factor-α (5′-ACC CCC AAT CTA TGA AGA AA-3′, 5′-TCC AGC CAA AAC GGA ATG AA-3′). Cycling conditions were as follows: 30 s of denaturation at 94°C, 1 min of annealing at 61°C and 1.5 min of elongation at 72°C for 40 cycles. Equal volume products of reverse transcription-polymerase chain reaction (RT-PCR) were separated on an agarose gel (1.5%) and visualized with ethidium bromide staining by a gel documentation system. The extent of interleukin-1β or tumor necrosis factor-α expression was quantitated using a densitometer with multi gauge software (Gel Doc GIS-2008, Tianneng Technology Co., Ltd., Shanghai). Glyceraldehyde phosphate dehydrogenase (GAPDH) levels were also analyzed as a control. Sample PCR product values of interleukin-1β or tumor necrosis factor-α were expressed as a percentage of the density of GAPDH in the same sample.

2.8. Histological examination

Rats were sacrificed on day 29 after immunization. The legs and hind paws were removed, fixed with 10% formaldehyde in normal saline, and then decalcified for 10 days with ethylenediamine tetraacetic acid and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin. Ankle joints were examined. The histopathological alteration of joints was blindly graded by a pathologist and assigned a score of 1–4 based on the following criteria: 1, minimal synovitis, primarily infiltration of mononuclear inflammatory cell into synovial membrane; 2, mild synovitis, pannus formed, cartilage degeneration; 3, proliferation and infiltration of a large amount of mononuclear cells, subchondral bone erosion; superficial cartilage damage; 4, severe destruction of cartilage and subchondral, complete disorganization of the joint space, fiber thickening and severe fibrosis, bony ankylosis.
2.9. Statistical analysis

Quantitative variables were expressed as mean ± S.D. One-way analysis of variance (ANOVA) was used. If any significant change was found, post hoc comparisons were performed using Fisher's PLSD. Non-normal and ranked distribution data were analyzed by method of Mann–Whitney U-test. P-Values < 0.05 was considered significant.

3. Results

3.1. Effects of tripterine on hind paw swelling in adjuvant arthritis rats

Arthritis was induced reproducibly in all animals injected the adjuvant, with onset of injected hind paw (right paw) erythema and swelling (arthritis onset) occurring on day 9, swelling of non-injected hind paw (left paw) began on day 11 and persisted to the end of the experiment.

Treatment with tripterine (5, 10, 20 mg kg\(^{-1}\) day\(^{-1}\), days 19–24) and prednisone (10 mg kg\(^{-1}\) day\(^{-1}\)) diminished the right hind paw swelling from day 20 to 28 (P<0.05–0.001) and left hind paw swelling from day 24 to 28 after immunization (P<0.05–0.001) (Fig. 2A and B). Maximum inhibitory rates in the drug-treated groups were 35.8%, 43.2%, 78.6% (Tripterine 5, 10 and 20 mg kg\(^{-1}\) day\(^{-1}\)) and 62.5% (prednisone 10 mg kg\(^{-1}\) day\(^{-1}\)) for the right paw (injected), and 59.6%, 67.3%, 84.6% (tripterine 5, 10 and 20 mg kg\(^{-1}\) day\(^{-1}\)) and 72.1% (prednisone 10 mg kg\(^{-1}\) day\(^{-1}\)) for the left paw.

The reduction of edema was sustained throughout the experiment even the treatment ended on day 24. These in vivo results demonstrated that tripterine was effective in suppressing the development of AA in rats.

3.2. Effects of tripterine on delayed-type hypersensitivity and anti-Mycobacterium IgG antibody

Ear thickness was significantly increased in the vehicle-treated adjuvant arthritis group compared with the normal group (P<0.001). Tripterine significantly decreased the delayed-type hypersensitivity induced by Mycobacterium at all three doses assayed (P<0.05–0.001) (Fig. 3A).

In the vehicle-treated adjuvant arthritis group, serum levels of IgG anti-Mycobacterium antibodies were significantly increased...
3.3. Effects of tripterine on mRNA expression

While interleukin-1β mRNA was detectable in synovial membrane, tumor necrosis factor-α mRNA could not be detected at all. The antisense primer is all right because it successfully gave tumor necrosis factor-α mRNA in hind paw.

In the vehicle-treated adjuvant-induced group, interleukin-1β mRNA in synovial membrane and tumor necrosis factor-α mRNA in hind paw was significantly increased compared with the normal group (P < 0.001) (Fig. 3B). Tripterine 20 mg kg⁻¹ day⁻¹ significantly decreased anti-Mycobacterium antibodies as compared with the vehicle-treated adjuvant arthritis group (P < 0.05) (see Fig. 3B).

3.4. Effect of tripterine on joint destruction in adjuvant arthritis rats

In normal rats, synoviocytes were monolayer and articular cartilages were not infiltrated with inflammatory cells (Fig. 5A). In vehicle-treated adjuvant-induced rats, articular cartilages were destructed and infiltrated with inflammatory cells; the joint spaces were replaced with mononuclear cells and thickened fiber. Bony ankylosis was also formed in some animals (Fig. 5B).

In adjuvant-induced rats given tripterine (10, 20 mg kg⁻¹ day⁻¹) and prednisone, the proliferation and infiltration of mononuclear cells and pannus were partly inhibited and the destruction of articular cartilages was alleviated (Fig. 5C and D). Tripterine 20 mg kg⁻¹ day⁻¹ markedly ameliorated the joint injury when compared with the vehicle-treated adjuvant-induced group although the treatment was ended on day 24 (P < 0.05) (Table 1).

4. Discussion

AA in rat is an experimental model that shares some features with human RA, such as swelling, cartilage degradation, and loss of joint function. One of the most important features of AA is chronic synovitis, including inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion (Zheng and Wei, 2005).

In this model, at least 14–15 days are needed before polyarthritis is reached. The appearance of polyarthritis initiates a secondary stage of the inflammatory response and the lesions in the paws can be seen by radiography on day 21 and day 25 (Noguchi et al., 2005; Simoes et al., 2005). In order to evaluate the therapeutic effect of tripterine on arthritis, tripterine was given to AA rats from day 19 to day 24. In the present study, we found that although the treatment stopped on day 24, the second inflammatory reactions and bone destructions were still inhibited by tripterine until the end of the experiment, especially in tripterine 20 mg kg⁻¹ day⁻¹ treated group. All these illustrated the therapeutic effect of tripterine on AA rats.

Autoantigens that cross-react with Mycobacteria have been implicated in the pathogenesis of adjuvant arthritis in the rat. Antibody to Mycobacterial antigen is reported in AA and also in rheumatoid arthritis. Adjuvant arthritis appears as a consequence of an immune response to cell wall of Mycobacterium. Compared with normal rats, AA rats had higher levels of IgG anti-Mycobacterium antibodies and the delayed skin reactions induced by the soluble fraction of Mycobacterium (Turull and Queralt, 2000). Tripterine at dose 20 mg/kg significantly decreased humoral
immune responses. At the same time, treatment with tripterine at all three doses assayed inhibited the delayed-type hypersensitivity seen in arthritic animals. This work suggested that tripterine might exert its effect through its influence on the cellular and on the humoral immune response to *Mycobacterium* in adjuvant-induced arthritic rats.

Based on the cytokine profile of RA, paracrine and autocrine networks may participate in disease perpetuation. Pro-inflammatory cytokines like interleukin-1β and tumor necrosis factor are important molecular mediators of immune and inflammatory responses. Excessive production of interleukin-1β and tumor necrosis factor-α is believed to contribute to the onset and progression of a number of inflammatory and autoimmune pathologies, such as rheumatoid arthritis and septic shock (He et al., 1998; Pinna et al., 2004; Table 1). Interleukin-1 and tumor necrosis factor-α promote induction of adhesion molecule, proteinase gene expression and play major role in the progression of joint destruction and proliferation of synoviocytes. They can also increase production of interleukin-6, granulocyte-macrophage colony-stimulating factor, interleukin-8, and other chemokines (Sweeney and Firestein, 2004). These cytokines, in turn, can activate macrophages in the environment and lead to continued cytokine production. This creates a positive feedback mechanism between the fibroblast- and macrophage-like synoviocytes that perpetuates synovial inflammation RA (He et al., 1998; Sweeney and Firestein, 2004; Zheng and Wei, 2005). Interleukin-1β receptor antagonists and monoclonal antibodies to tumor necrosis factor-α were shown to be efficacious in animal models of inflammatory diseases and demonstrated to be effective drugs in RA treatment. Therefore, the inhibition of the production of both cytokines represents a good strategy for therapeutic intervention of immune and inflammatory diseases (Smith, 2005; Zheng and Wei, 2005).

Considering the contribution of interleukin-1β and tumor necrosis factor-α to AA severity, the mRNA expression of these two cytokines was analyzed in the synovial membrane. Unexpectedly, the synovial membrane lacked tumor necrosis factor-α gene transcription. As tumor necrosis factor-α protein was detectable in homogenized paw from adjuvant-induced arthritic rats (Lucas et al., 2003), we decided to detect the mRNA in hind paw and found that mRNA expression was significantly increased in arthritic rats compared with the normal rats. Schmidt-Weber et al. (1999) also obtained negative results for tumor necrosis factor-α mRNA in the synovial membrane of arthritic rats and assumed that the presence of tumor necrosis factor-α protein in joint homogenates may reflect uptake from circulation, popliteal lymph node and spleen. But from our results, local tumor necrosis factor-α gene transcription existed

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Joint lesion score</th>
<th>Right paw</th>
<th>Left paw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>–</td>
<td>4 (3–4)</td>
<td>3 (2–4)</td>
<td></td>
</tr>
<tr>
<td>Tripterine</td>
<td>5</td>
<td>3.5 (3–4)</td>
<td>3 (2–4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.5 (2–4)</td>
<td>2.5 (1–4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3 (2–4)</td>
<td>2 (1–3)</td>
<td></td>
</tr>
<tr>
<td>Prednisone</td>
<td>10</td>
<td>3 (3–4)</td>
<td>2 (2–3)</td>
<td></td>
</tr>
</tbody>
</table>

Rats were immunized with Freund’s complete adjuvant on right paw on day 0. Immunized rats were grouped randomly and treated with tripterine 5, 10 and 20 mg kg⁻¹ day⁻¹, prednisone 10 mg kg⁻¹ day⁻¹ or placebo from day 19 to day 24; rats were sacrificed on day 29 and samples were collected for detection. Data expressed as median (minimum–maximum) by Mann–Whitney U-test; n = 10 rats for each group.

*P* < 0.05 vs. placebo-treated group, tested by Mann–Whitney U-test.

Fig. 5. The extent of foot pathological lesions was graded on a semiquantitative scale. Light microscopy 100×. Joint tissue was fixed in 3% formaldehyde and 5-μm paraffin sections were stained with hematoxylin and eosin (A–D). (A) Grade 0: Joint space with normal synovial lining, from normal rat. (B) Grade 4: Destruction of cartilage and subchondral bone, disorganization of the joint space and replacement with mononuclear cells and fiber thickening from vehicle-treated group. (C) Grade 3: Proliferation and infiltration of a large amount of mononuclear cells; subchondral bone erosion; superficial cartilage damage, from tripterine 10 mg kg⁻¹ day⁻¹ treated group. (D) Grade 2: Pannus forming with superficial cartilage damage, from tripterine 20 mg kg⁻¹ day⁻¹ treated group.
and may contribute to pathophysiology of inflammatory processes in hind limb in the chronic phase.

In our study, AA rats expressed high levels of interleukin-1β mRNA in synovial membrane and tumor necrosis factor-α mRNA in hind paw when compared with normal animals. The inhibitory effect of tripterine on interleukin-1 and tumor necrosis factor-α mRNA correlated with its effect on joint lesion reduction.

From the present study, we found that tripterine has therapeutic effect on AA, and the effect might relate to its suppressive effect on the production of pro-inflammatory cytokines and on humoral and cellular immune responses.

References


