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Original Research Article

Triptolide mitigates rheumatoid arthritis through Notch1/Jagged1 pathway

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Abstract

Purpose: *To determine the effect of triptolide on rheumatoid arthritis, and the underlying mechanism of action.*
Methods: *Sixty Sprague Dawley (SD) rats were assigned to blank control, model, methotrexate, and triptolide groups (n = 15/group). A rat model of rheumatoid arthritis was established. Each group was given the corresponding treatment. Toe edema and arthritis index of rats were assessed in all the groups. Levels of IL-2, IFN-γ, TNF-α, IL-6, IL-8, and IL-10 were evaluated by enzyme-linked immunosorbent assay (ELISA).*
Results: *Serum levels of IL-2, IFN-γ, TNF-α, IL-6 and IL-8 were significantly higher in model rats than in blank control, while IL-10 level was significantly lower ($p < 0.05$). In triptolide group, serum IL-2, IFN-γ, TNF-α, IL-6 and IL-8 were significantly lower than the corresponding levels in model group, but IL-10 was significantly higher. The relative mRNA and protein concentrations of Notch1 and Jagged1 were significantly higher in model rats than in blank control rats, but significantly lower, relative to model rats.*
Conclusion: *Tripterygium wilfordii exerts a good therapeutic effect in the treatment of rheumatoid arthritis in rats, most likely by inhibiting the activation of Notch1/Jagged1 signal pathway. Thus, it regulates inflammation by regulating the expressions of various inflammatory cytokines, and may be potential agent for the management of rheumatoid arthritis.*

Keywords: *Tripterygium wilfordii, Triptolide, Notch1, Jagged1, Rheumatoid arthritis, Toe edema, Arthritis index, Methotrexate*

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INTRODUCTION

Rheumatoid arthritis is an autoimmune disease with clinical manifestations of aggressive and multiple arthritis [1]. Rheumatoid arthritis is associated with very high disability and deformity rates. It easily causes joint deformation and structural changes, resulting in joint dysfunction which severely reduces quality of daily life [2]. At present, the clinical treatment of patients with rheumatoid arthritis is aimed at alleviating clinical symptoms and arresting deterioration of the disease.

Western medicines such as anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, and glucocorticoids are used to mitigate the disease. However, the clinical application of Western

medicines alone is greatly limited due to the associated serious side effects and high costs [3]. Therefore, it would be of great clinical significance to develop newer and cheaper anti-rheumatoid arthritis drugs with low side effects and high degrees of effectiveness. The pathogenesis of rheumatoid arthritis has not yet been fully understood. However, latest research findings suggest that it is related to dysfunction in immune regulation, especially dysfunction in T helper cells (Th1/Th2) [4]. The Notch1/Jagged1 pathway mediates T cell activation, causes imbalance in Th1/Th2 cytokine ratio, and promotes cell proliferation, inflammatory cell infiltration, proteolysis and inflammatory reaction [5]. Rheumatoid arthritis belongs to the category of "arthralgia" in traditional Chinese medicine (TCM). Therefore, the treatment is focused on *cold dispersion* and pain relief, activation of blood circulation, and detumescence.

Tripterygium wilfordii is one of the TCM herbs, with triptolide as an important bioactive component. Triptolide exerts significant anti-inflammatory, antioxidant, and immune-regulating effects. It has been widely used in research, and it is often employed for treating immune diseases, but not much is known about the mechanism involved in its application for treating rheumatoid arthritis [6]. This study was carried out to investigate the effect of triptolide on rheumatoid arthritis and the involvement of the Notch1/Jagged1 pathway in the process.

EXPERIMENTAL

Animals and reagents

Sixty male Sprague Dawley (SD) rats aged 6 - 8 weeks, weighing 180 – 220 g, were purchased from the animal management center of Fujian Provincial Government Hospital. The reagents used, and the suppliers (in parenthesis) were: methotrexate and Freund's complete adjuvant (Amresco, USA); triptolide tablets (Lunan Houpu Pharmaceutical Co. Ltd.); qRT-PCR kit, and immunohistochemical goat anti-rabbit secondary antibody (Shanghai Biyuntian Company); H&E kit (Shanghai Biyuntian Company); ELISA kits for IL-2, IFN-γ, TNF-α, IL-6, IL-8, and IL-10 (Shanghai Biyuntian Company), and antibodies for Notch1, Jagged1 antibody (Abcam Biotechnology Co. Ltd, UK).

Establishment of animal model of rheumatoid arthritis

The 60 SD rats were assigned to four groups: blank control, model, methotrexate, and triptolide groups (n = 15/group). Approval for this study was given by the Animal Ethics Authority (approval no. FPGH202002) of Fujian Provincial Government Hospital and was conducted in line with NIH guidelines [7].

To establish the rat model of rheumatoid arthritis, inflammation was induced in the right hind foot of each rat in the model, methotrexate and triptolide groups through injection of 0.1 mL Freund's complete adjuvant. Control rats were given injection of physiological saline in place of Freund's medium. From the 12th day of induction of inflammation, rats in triptolide group were given triptolide daily at a dose of 3.0 mg/kg body weight via gavage. In methotrexate group, rats received methotrexate 1.0 mg/kg body weight via gavage every week, while model rats received physiological saline in place of methotrexate, for 4 weeks.

Joint function in rats

Arthritis index was evaluated in line with the five-grade scoring method. Foot edema, including ankle, was scored 4 points, while edema below the ankle was scored 3 points. A score of 2 points was given for the swelling of toe joint and plantar joint. Redness and little toe joint edema was scored 1 point, while 0 point was scored for absence of redness and swelling. The degree of plantar swelling in each group was calculated as shown in Eq 1.

$$D = (TVAME-TVBME)/TVBME \quad \ldots\ldots\ldots (1)$$

where *D* is degree of plantar swelling, *TVAME* is toe volume after model establishment, and *TVBME* is toe volume before model establishment.

Sampling and sample preparation

After collection of blood samples, the rats were anesthetized with 10 % chloral hydrate. The interphalangeal joints of the distal right hind feet of rats in each group were fixed overnight in 4 % paraformaldehyde solution, followed by routine processing and H&E staining.

Examination of joint morphology

The pathological changes in synovium were examined under a light microscope after H&E staining and sealing.

Determination of biochemical indices

Following 24-hour fast, 1 mL of abdominal aortic blood was centrifuged in a serum separator at 3000 rpm for 20 min. Serum levels of IL-2, IFN-γ,

TNF-α, IL-6, IL-8, and IL-10 were determined using ELISA kits, in strict compliance with the kit instructions.

Western blot assay

Total synovial protein was extracted with protein extraction kit, and the protein concentration was measured with BCA procedure. The proteins were resolved with SDS-PAGE and electro-transferred to PVDF membranes which were subsequently blocked with non-fat milk, followed by overnight incubation 4 ^{o}C with primary immunoglobulins for Notch1 and Jagged1 for 12 h, and incubation with secondary antibodies, and analysis with Bio-Rad image laboratory software.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total synovial RNA was extracted using RNA extraction kit, followed by reverse transcription into cDNA using one-step prime script miRNA cDNA synthesis kit. Then, miRNA fluorescence quantitative PCR detection kit was used for quantitative real-time PCR, and the cycle was completed according to the kit instructions. The relative expression levels of Notch1 and Jagged1 were calculated in terms of differences in CT between the gene of interest and the control gene, *GAPDH*, which was used as internal reference for data normalization.

Statistical analysis

This was done with SPSS 20.0 software package. ReDataare presented as mean ± standard deviation (SD). Analysis of variance was used for multiple group comparison while paired comparison was done with LSD test or Tamhane test. Values of $p < 0.05$ indicated significant differences.

RESULTS

Pathological changes in synovium

Histology results indicated that synovial cells in blank control group had fewer layers, clear boundaries, no inflammatory cell infiltration, no hypertrophy and processes, and clear synovial tissue structure. In the rheumatoid arthritis model group, there was increased number of synovial cells, and there was massive presence of inflammatory cells and increases in synovial blood vessels. In the methotrexate group, pannus formation was seen in some articular cartilage of rats; the articular surface was fuzzy, and the synovial cells of the joints were arranged orderly, with reduced degree infiltration by inflammatory cells. In the triptolide group, overall structure of the articular surface was relatively regular, with smaller population of inflammation-associated cells in synovial membrane and vascular wall of the joint. Moreover, the synovial cells were orderly arranged.

Joint function

The degree of paw swelling and arthritis index were significantly higher in model rats than in blank control rats. However, the degree of paw swelling and arthritis index in rats were significantly lower in triptolide-exposed rats than in model rats. These results are shown in Table 1.

Table 1: Comparison of joint function of rats amongst the groups (mean ± SD, n = 15)

Group	Plantar swelling (%)	Arthritis index (score)
Blank control	39.24±9.52	0
Model	75.00±12.34a	5.75±1.26a
Methotrexate	41.38±10.04ab	2.92±0.75ab
Triptolide	42.68±10.54ab	3.02±0.90ab

[a]$P < 0.05$, vs. blank control; [b]$p < 0.05$, vs. model

Levels of Th1 cytokine

Serum levels of IL-2, IFN-γ, TNF-α, IL-6, IL-8 and IL-10 were significantly higher in model rats than in the blank control rats, but they were lower in triptolide-exposed rats than in the model group ($p < 0.05$; Table 2).

Levels of Th2 cytokines

The serum levels of IL-6 and IL-8 in the model group were significantly higher than those in the blank control group, but the level of IL-10 was lower in model rats than in the blank control rats. However, serum levels of these cytokines were significantly lower in triptolide group than in

Table 2: Comparison of Th1 cytokines in rats of each group (mean ± SD, n = 15)

Group	IL-2 (ng/L)	IFN-γ (ng/L)	TNF- α (ng/L)
Blank	42.56±8.23	63.42±10.37	97.41±17.58
Model	73.21±10.25[a]	90.37±17.42[a]	147.82±31.10[a]
Methotrexate	54.30±9.12[ab]	70.52±12.82[ab]	110.38±23.14[ab]
Triptolide	58.53±9.24[ab]	74.13±13.73[ab]	115.42±24.85[ab]

[a]$P < 0.05$, vs. blank control rats; [b]$p < 0.05$, vs. model

Table 3: Comparison of Th2 cytokines in rats amongst the groups (mean ± SD, n = 15)

Group	IL-6 (ng/L)	IL-8 (ng/L)	IL-10 (ng/L)
Blank control	41.42 ± 8.67	70.44 ± 12.56	102.68 ± 18.62
Model	85.65 ± 15.46[a]	124.34 ± 19.92[a]	49.25 ± 14.84[a]
Methotrexate	45.62 ± 10.02[ab]	76.30 ± 15.12[ab]	86.52 ± 16.82[ab]
Triptolide	47.62 ± 10.44[ab]	80.53 ± 15.24[ab]	85.13 ± 17.73[ab]

[a]$P < 0.05$, vs. blank control; [b]$p < 0.05$, vs. model

model rats, but the level of IL-10 in the triptolide group was significantly higher than that in the model group ($p < 0.05$). These results are shown in Table 3.

Relative mRNA expression levels of Notch1 and Jagged1

The relative mRNA expressions of Notch1 and Jagged1 were higher in model rats than in the blank control rats. However, relative mRNA expressions of Notch1 and Jagged1 were lower in triptolide-treated rats than in model rats ($p < 0.05$). These data are presented in Table 4.

Table 4: Relative mRNA expressions of Notch1 and Jagged1 in rats of each group (mean ± SD, n = 15)

Group	Notch1 mRNA	Jagged1 mRNA
Blank control	1.24±0.32	1.76±0.43
Model	1.65±0.40[a]	2.59±0.67[a]
Methotrexate	1.32±0.34[ab]	1.85±0.46[ab]
Triptolide	1.33±0.34[ab]	1.83±0.45[ab]

[a]$P < 0.05$, vs. blank control; [b]$p < 0.05$, vs. model

Protein expression levels of Notch1 and Jagged1

As shown in Table 5, relative protein concentrations of Notch1 and Jagged1 were significantly higher in model rats than in blank control rats, but they were significantly lower in triptolide group, relative to model rats.

Table 5: Relative protein levels of Notch1 and Jagged1

Group	Notch1 mRNA	Jagged1 mRNA
Blank control	0.18±0.04	0.23±0.05[a]
Model	0.44±0.11[a]	0.49±0.17[a]
Methotrexate	0.25±0.07[ab]	0.27±0.06[ab]
Triptolide	0.20±0.05[ab]	0.25±0.07[ab]

[a]$P < 0.05$, compared with the blank control group; [b]$p < 0.05$, compared with model

DISCUSSION

Rheumatoid arthritis is characterized by symmetrical polyarticular synovitis which causes joint pain, swelling, movement disorders, joint deformity, and loss of joint function. A clinical epidemiological survey has shown that the incidence of rheumatoid arthritis increases yearly, with 0.24 % prevalence in the world, and 0.28 - 0.45 % prevalence in China. The incidence of rheumatoid arthritis is about three times higher in women than in men [8]. The basic pathological changes in rheumatoid arthritis comprise chronic synovial inflammation, pathological angiogenesis, exudation, cell proliferation, destruction of articular cartilage, pannus formation, and joint deformity. The clinical management of patients with rheumatoid arthritis is mainly aimed at alleviating clinical symptoms and delaying the development of the disease. Western medicine treatment drugs such as anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, and glucocorticoids are used to mitigate the disease. Although new anti-rheumatic drugs significantly delay the progression of rheumatoid arthritis, western medicine use is associated with adverse side effects such as tumors, severe infection, and hepatorenal toxicity [9]. Therefore, there is a need to develop new anti-rheumatoid arthritis drugs with low side effects, low cost, and high efficiency.

Triptolide is a lipophilic extract of the rhizome of *T. wilfordii.* It contains alkaloids, diterpenoid lactones, and other bioactive ingredients. The extract which *clears heat* and removes toxins, is also applied for detoxification, detumescence and pain, *wind removal,* and dehumidification [9]. Triptolide produces good clinical efficacy in treatment of rheumatoid arthritis patients. Triptolide enhances the quality of life of patients by effectively relieving the clinical symptoms of rheumatoid arthritis [10]. Although the use of triptolide in treatment of rheumatoid arthritis is effective, the underlying mechanism is not yet fully understood. Data from H&E staining revealed that synovial cells in blank control group had a reduced number of layers, clear boundaries, no inflammatory cell infiltration, no hypertrophy and processes, and a clear synovial tissue structure. In the rheumatoid arthritis model rats, the population of synovial cells was increased, with massive infiltration of inflammatory cells, as well as increased number of synovial blood vessels. In the methotrexate group, pannus formation could be seen in some

articular cartilages of rats; the articular surface was fuzzy, and the synovial cells of the joints were arranged orderly, with a small amount of inflammatory cell infiltration. In contrast, in the triptolide group, the overall structure of the articular surface was relatively regular, and there was lower level of infiltration of inflammatory cells in the synovial membrane and vascular wall of the joint. Moreover, the synovial cells were orderly arranged. These results suggest that the rat model of rheumatoid arthritis was successfully established, and triptolide produced a good therapeutic effect in the treatment of rheumatoid arthritis. The degree of paw swelling and arthritis index were significantly higher in model rats than in the blank control rats, but degree of paw swelling and arthritis index in triptolide-exposed rats were lower than model rat values. Thus, triptolide reduced stenosis of joint space in rheumatoid arthritis inhibited the proliferation of synovial tissue and joint fibrous tissue and alleviated the destruction of articular cartilage.

The pathogenesis of rheumatoid arthritis is complex and has not yet been fully elucidated. However, it is generally believed that rheumatoid arthritis is caused by interaction of multiple factors. At present, it is generally accepted that immune system dysfunction is vital in the development of this disease, and an imbalance in Th1/Th2 cytokine ratio is crucial in this process [11]. The occurrence of inflammatory reactions in the immune system has an impact on osteoblasts and osteoclasts, leading to aggressive and destructive lesions in the joints, and ultimately resulting in rheumatoid arthritis [12]. The differentiation of Th1 cells and Th2 cells is one of the important links in the regulation of the immune system. The Th1 cells are CD4 + positive cells that produce TNF-α, IFN-γ, IL-2, and other cytokines that participate in immunity regulation. They mediate the differentiation of cytotoxic T cells and cellular immune response and also promote the release of inflammatory cytokines, enhance the expression of adhesion molecules, and induce activation of synovial cells, leading to synovial hyperplasia, cartilage damage, and ultimately, rheumatoid arthritis [13]. The Th2 cells produce IL-8, IL-6, IL-10 and IL-13. These cytokines secreted by Th2 cells promote the proliferation, differentiation, and antibody production by B cells, and they participate in the activation of B cells. Interestingly, IL-6 accelerates the destruction of cartilage and bone, stimulates the proliferation of synovial tissue, and induces the differentiation of osteoclasts which is inseparable from the pathogenesis of rheumatoid arthritis [14]. On the other hand, IL-10, an anti-inflammatory cytokine, inhibits massive infiltration of lymphocytes and lymphocyte mediators around the synovium of bone and joint, terminates inflammatory reaction, directly inhibits the generation of osteoclasts and the differentiation of osteoclast precursors, and repairs injured tissues around the joint [15]. Pharmacological studies have revealed that triptolide exerts anti-inflammatory effect by suppressing the activation of inflammatory cells, reducing the production and secretion of inflammatory reaction mediators, and decreasing inflammatory response [16]. There were significantly higher serum levels of IL-6 and IL-8 in model rats than in blank control rats, but IL-10 level in model rats was lower than that in blank control rats. However, serum levels of IL-6 and IL-8 in triptolide-treated rats were significantly reduced, relative to model rats, but IL-10 level in triptolide-treated rats was significantly higher than that in model rats. These results suggest that triptolide dose-dependently corrected the imbalance in Th1/Th2 cytokine ratio in rats with rheumatoid arthritis, thereby reducing inflammatory response.

Research has shown that the Notch pathway, a conservative signal transduction pathway between cells, mediates T cell activation, causes an imbalance in Th1/Th2 cytokine ratio, and induces cell proliferation, cell recruitment, proteolysis, and inflammatory reaction [17]. The main receptor in Notch pathway is Notch1, which, along with its ligand, constitutes one of the key signaling pathways that regulate cell differentiation. Studies have revealed that the Notch1/Jagged1 pathway is associated with the occurrence, prognosis, and development of rheumatoid arthritis [18]. The activation of the Notch1/Jagged1 signaling pathway stimulated arthritic mice and enhanced their immune inflammatory response [19]. In that study, inhibition of Notch1 signaling, as well as IL-6, TNF-αand other inflammatory cytokines in arthritic mice led to significant mitigation of the severity of arthritis [19]. The results of the present study showed that the relative mRNA and protein expressions of Notch1 and Jagged1 were significantly up-regulated in model rats, relative to blank control rats, but they were significantly down-downregulated in the triptolide-exposed rats, relative to model rats. These findings suggest that the Notch1/Jagged1 signaling pathway is abnormally activated during induction of rheumatoid arthritis, and it is closely related to the occurrence and development of rheumatoid arthritis. Triptolide inhibited the activation of Notch1/Jagged1 signaling pathway, thereby regulating the expressions of various cytokines linked to inflammation. The bioactive alkaloid component of triptolide is a non-steroidal immunosuppressant that affects the immune

response from multiple links, inhibits cellular and humoral immunity, and regulates Th1/Th2 cytokine ratio, thereby regulating the expressions of a variety of inflammatory cytokines [20].

CONCLUSION

Triptolide exerts good therapeutic effect in the treatment of rheumatoid arthritis through a mechanism linked to suppression of the activation of Notch1/Jagged1 signaling pathway. It plays an anti-inflammatory role by regulating the expressions of a variety of inflammatory cytokines, and hence has a potential for the clinical management of rheumatoid arthritis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yidan Gao conceived and designed the study, Yi Zhang and Lili Chen collected and analyzed the data, Yidan Gao wrote the manuscript. All authors read and approved the manuscript for publication.

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