Effect of Miao medicine, Jinwujiangu decoction, on IL-17/IL-23 inflammatory axis of fibroblast-like synoviocytes in rheumatoid arthritis

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Abstract

**Purpose:** To explore the influence of the Miao medicine, Jinwujiangu decoction, on the interleukin (IL)-17/IL-23 inflammatory axis of fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA).

**Methods:** Synovial tissue samples were randomly divided into a blank control group, high-dose (0.06mg/mL), medium-dose (0.6mg/mL), and low-dose (6.0mg/mL) groups of Jinwujiangu decoction, a leflunomide group, and a tripterygium glycosides group. Proliferation of RA synovial cells was detected by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Enzyme-linked immunosorbent assay (ELISA) was used to determine the secretion of IL-6, transforming growth factor beta (TGF-β), and IL-17. Real-time polymerase chain reaction was used to evaluate the expression of IL-23R, IL-17R, RAR-related orphan receptor alpha (RORα), RORγt, and signal transducer and activator of transcription (STAT3) mRNA. The protein activities of IL-17R, STAT3 and pSTAT3 were assessed by Western blot assay.

**Results:** Jinwujiangu decoction inhibited the proliferation of RA synovial cells. Treatment with different drug concentrations resulted in downregulation of IL-6, TGF-β, and IL-17 secretion. The expression levels of IL-23R, IL-17R, RORα, RORγt, and STAT3 mRNA in RA-FLS were significantly reduced after intervention with different drugs. Protein expression levels of STAT3, pSTAT3, and IL-17 in the different drug treatment groups were significantly decreased.

**Conclusion:** Jinwujiangu decoction inhibits the secretion of IL-6 and TGF-β in RA-FLS, and intervenes to regulate gene expression of IL-23/IL-17 inflammation axis and suppress immune inflammation. The results of this study provide new evidence for the study of anti-inflammatory mechanism of TCM compound prescription.

**Keywords:** Jinwujiangu decoction, IL-17/IL-23, Fibroblast-like synoviocytes, Rheumatoid arthritis, Ethnomedicine

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease, in which inflammatory synovitis and synovial joint erosion is the main reason for bone destruction and joint deformity [1]. Fibroblast-like synoviocytes (FLS) are stimulated to proliferate by the inflammatory process [2,3]. FLS secrete...
large amounts of inflammatory factors and mediate joint destruction in RA. Therefore, inhibiting the abnormal proliferation of FLS and preventing the release of inflammatory factors are crucial in the treatment of RA.

Recently, some reports have shown that the interleukin (IL)-17/IL-23 axis plays a key part in RA development [4,5]. IL-17 is an important pro-inflammatory cytokine in the pathogenesis of RA. It mainly exists in the synovial fluid of RA patients. Its secretion is closely related to anticyclic citrullinated peptide antibodies, IgM, IgA and IgG. IL-17 is valuable as a marker to confirm active RA disease [6]. Additionally, IL-17 has a strong inflammatory effect and may play a critical role in aggravating joint destruction and synovial inflammation in RA. It stimulates stromal cells to secrete inflammatory factors such as IL-6, TNF-α and chemokines, s, and increase synovial inflammation in RA [5,7].

IL-23 is a recently discovered cytokine that is expressed in a variety of cells, which is necessary for Th17 cell development and induces interferon (IFN) production [8]. In recent years, some studies have shown that IL-23 is a key cytokine in the pathogenesis of RA [9]. IL-23 gene-targeted mice were resistant to the development of osteoarthritis, so it may be a promoter of joint autoimmune inflammation.

The Miao medicine, Jinwujiangu decoction, has been used in the treatment of clinical experience of RA for a long time. It consists of Rhizoma cibotii, Periploca forrestii schltr, Caulis sinomenii, Homalomena occulta, Curcuma longa, Zaocys dhunnade, pseudo-ginseng, and licorice, with strong effects on kidney function, strengthening sinewes, bone activation, expelling pathogenic wind, and tongluo. Previous studies showed that Jinwujiangu decoction could improve the clinical symptoms of joint pain and morning stiffness in RA patients [10]. It would reduce the protein activity of synovial cells and the secretion of IL-17 and IL-23 in serum of CIA rats.

This study further investigated the effects of the Miao medicine, Jinwujiangu decoction, on the IL-17/IL-23 axis in RA-FLS, to explore the anti-inflammatory effects of Jinwujiangu decoction on RA - FLS.

EXPERIMENTAL

Patients

The samples were obtained from Guiyang Second Affiliated Hospital of Traditional Chinese Medicine and Guiyang Orthopaedic Hospital. All RA patients and healthy controls according to the 1987 ACR classification criteria were enrolled and provided written informed consent [11].

Isolation and culture of synoviocytes

Synovial cells were obtained from patients with RA who underwent arthroplasty. The RA synovial tissue was placed in a culture dish to remove fat and blood clot, and the tissue was cultured with PBS for three times. Then, the tissue was cut into 1 mm pieces and inoculated into 25 mL culture flask. At 37 °C and 5 % CO₂, 5 mL complete culture medium was added and cultured for 4 hours. To remove non-adherent lymphoid cells, the cell culture medium was changed after 3 days. They were passaged till monolayer cells spread to 70 – 80 % of the bottom of the culture flask after enzymatic digestion with 0.25 % trypsin. Cells at passages 3 – 5 were used for the experiment [12-14].

Experimental medicine

Miao medicine, Jinwujiangu decoction, consists of Rhizoma cibotii 10 g, Periploca forrestii schltr 10 g, Caulis sinomenii 10 g, Homalomena occulta 10 g, Curcuma longa 10 g, Zaocys dhunnade 10 g, pseudo-ginseng 3 g, and Radix paeoniae alba 15 g. The herbal medicine was obtained from Guizhou Tongjitang pharmacy.

Experimental groups and drug delivery

The experiment was conducted with six groups, in which cells were treated respectively with low-, medium-, and high-dose Jinwujiangu decoction (0.06 mg/mL, 0.6 mg/mL, 6.0 mg/mL, respectively), leflunomide, tripterygium glycosides (0.03 mg/mL), and blank control. The cells were inoculated into sterile 96-well plates and cultured in a 37 °C incubator (Thermo Fisher, 3100) in an atmosphere of 5 % CO₂. After the cells adhered, different concentrations of experimental drugs were added into 96-well plates and added such as volume DMEM to blank control group. At 37 °C and 5 % CO₂, the 96-well plates were cultured for 24 h. Then, cell supernatants were collected for subsequent experiments.

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay

Synoviocytes in the logarithmic phase were collected, 15 % fetal bovine serum (GIBCO, 10099-133) was added to the cell suspension, and 6 x 10⁴ cells were added to each well in 96-well plates at 37 °C incubator (Thermo Fisher, 3100) for 24 h in an atmosphere of 5 % CO₂.
Then, 200 μL of different concentrations of Jinwujiangu decoction were added to each well of the 96-well plates. Each group had five repetitions. An equal volume of DMEM was added to the wells in the blank control group. At 37 °C and 5 % CO₂, the 96-well plates were cultured for 24 h. Subsequently, DMEM and MTT were added to each hole. After waiting for 4 h, discard the supernatant and add formazan. The absorbance at a wavelength of 490 nm was monitored by EnzymeSign while cell proliferation rate was measured based on the reference cells [12].

**Enzyme-linked immunosorbent assay (ELISA)**

The serum levels of IL-6, TGF-β, and IL-17 were determined by ELISA kits (Abcam, USA), according to the manufacturer’s instructions.

**Real-time PCR**

RNA was prepared by RNA extraction kit (QiAGEN). mRNA was synthesized into the cDNA through the cDNA synthesis kit (Applied Biosystems, Japan). ABI Prism 7500 was used to quantify RT-PCR. Primers for IL-23R, IL-17R, RORα, RORγt, and STAT3 were purchased from Applied Biosystems. Each sample was processed in triplicate, and the experiment was repeated three times. All primers are presented in Table 1.

**Western blot assay**

Cells were inoculated on a 60 mm plate, cultured at room temperature for 24 hours. Then, the cells were collected and washed three times with PBS, and the precooled lysate was added to the cells. The solution were thoroughly mixed, placed on ice about 20 minutes, and centrifuged for 20 minutes. The protein was blocked overnight at 4°C with 5% skim milk without antibodies after being transferred to a membrane. After washing with TBSH three times, the membranes were incubated with anti-STAT3 (EPR787Y), anti-STAT3 (phospho Y705), and anti-IL17RD (ABCAM, UK), at a dilution of 1:2000, at 37 °C for 1 h. Then, protein bands were developed using ECL reagents, and images were acquired using the ChemiDoc Imaging system.

**Statistical analysis**

The data were expressed as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). The threshold was set to $p < 0.05$. SPSS 17 software was used for statistical analysis. Significance threshold was set at $p < 0.05$. SPSS 17.0 software was used for statistical analyses.

**RESULTS**

**Jinwujiangu decoction inhibited proliferation of RA synovial cells**

MTT testing results show that with increasing drug concentrations, the OD value gradually dropped, and the inhibition rate rose gradually (Figure 1). With drug concentrations in the 0.3 – 0.6 mg/mL range, half-maximal inhibition of RA-FLS proliferation was reached. At a concentration of 6.0 mg/mL, the inhibition rate reached its peak value.

**Figure 1:** Inhibition of RA-FLS proliferation at different concentrations of Jinwujiangu decoction

**Effect of Jinwujiangu decoction on RA-FLS morphology**

When RA-FLS were treated with different drugs for 24 h, cell morphological changes were investigated by reverse microscopy.

**Table 1: Primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer forward (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>TCC TCC TGA GCG CAA GTA CTC T</td>
<td>GGT CAG TAA CAG TCC GCC TAG AA</td>
<td>153</td>
</tr>
<tr>
<td>IL-23R</td>
<td>TGG CTT GCA ATC TGA ACT TG</td>
<td>GAG CTC CCG GGA ATT CTT AC</td>
<td>244</td>
</tr>
<tr>
<td>IL-17R</td>
<td>CAGAAATGCCAGACACTCCA</td>
<td>CAGACGATGACAGGATGAC</td>
<td>115</td>
</tr>
<tr>
<td>STAT3</td>
<td>TGG AGG AGA GAA TCG TGG AG</td>
<td>TTT GAC CAG CAA CCT GAC TTT</td>
<td>149</td>
</tr>
<tr>
<td>RORα</td>
<td>TCC CTA CTG TTC GTT CAG CA</td>
<td>CAG GTT TCC AGA TGC GAT TT</td>
<td>102</td>
</tr>
<tr>
<td>RORγt</td>
<td>GGC TGT GGG ACA AGT TCA GT</td>
<td>GTC GGA GAA GGT CAT GGT GT</td>
<td>197</td>
</tr>
</tbody>
</table>
Cells of the blank control group were arranged in close parallel swirls and were bright and shuttle-shaped (Figure 2 A). Compared with the blank control group, RA-FLS growth slowed with increased concentrations of Jinwujiangu decoction, and the swirling pattern disappeared. Cells became rounded and smaller and were spaced further apart. Cell adherence was decreased and the cells lost cytoplasm (Figure 2 B to D). Compared with the tripterygium glycoside group (Figure 2 E), the medium-dose group did not show obvious differences. In the high-dose group, the cell morphology was obviously altered, the cells had very little cytoplasm, cellular size was reduced, and some cells underwent apoptosis. These changes were similar to those seen in the leflunomide group (Figure 2 F). Cellular cytoplasm was lost, growth polarity disappeared, the cells became rounded and underwent apoptosis, and some cells were detached and floating in suspension.

The results of ELISA (Figure 3) showed that the concentrations of IL-6, TGF-β, and IL-17 in cell culture supernatant were lower than those in blank control group \( (p < 0.01) \). Different concentrations of Jinwujiangu decoction resulted in decreased secretion of IL-6, TGF-β, and IL-17. The effect of high-dose treatment was more obvious than others \( (p < 0.01) \). Compared with the tripterygium glycosides group, the secretion levels of IL-6, TGF-β and IL-17 in the high-dose group of Jinwujiangu Decoction decreased \( (p < 0.05) \), while the secretion levels of IL-17 in the medium-dose group had no significant difference \( (p < 0.05) \). Compared with leflunomide group, the secretion levels of IL-6, TGF-β and IL-17 in high-dose Jinwujiangu decoction group were decreased \( (p < 0.01) \), but there was no significant difference in low-dose group and middle-dose group \( (p > 0.05) \).

The Figure 4 shown that the expression levels of IL-23R, IL-17R, RORα, RORγt, and STAT3 mRNA in RA-FLS were reduced following intervention with different drugs \( (p < 0.01) \). Compared with the tripterygium glycosides group, the expression levels of IL-23R, IL-17R, RORα, RORγt, and STAT3 mRNA in RA-FLS were lowest in the high-dose group \( (p < 0.01) \). Compared with the leflunomide group, the expression levels of IL-17R, RORγt, and STAT3 mRNA in RA-FLS in the low-dose group exhibited no obvious difference \( (p > 0.05) \). However, the decrease in expression of IL-23R, IL-17R, RORα, RORγt, and STAT3 mRNA in the high-dose group was less than in the leflunomide group \( (p < 0.01) \).

The Western blot test results showed that the protein expression levels of STAT3, pSTAT3 and IL-17R were significantly lower in high-dose treatment groups compared with the blank control group \( (p < 0.01) \).
level of IL-17R protein in the middle dose group had no significant difference (p > 0.05). The expression levels of STAT3, pSTAT3 and IL-17R in high dose group were significantly decreased (p < 0.05). However, compared with leflunomide group, the expression levels of STAT3, pSTAT3 and IL-17R in high dose group were lower (p < 0.01).

Figure 4: Expression of IL-23R, IL-17R, RORα, RORγt, and STAT3 mRNA in different groups; **p < 0.01

Figure 5: Protein expression levels of IL-17R, STAT3, and pSTAT3 in different groups. *P < 0.05; **p < 0.01

DISCUSSION

RA is a systemic inflammatory disease. Most patients with RA will eventually have different degrees of joint dysfunction, which causes serious harm to human health. Some reports have shown that IL-17/IL-23 inflammatory axis has great significance for the improvement of joint synoviocytes in RA [15]. JAK2/STAT3 signaling pathway plays a bridge role in the transmission of IL-23/IL-17 inflammation axis. STAT3 plays a significant role in the differentiation process of Th17. Following the binding of IL-23 with its receptor, the JAK2/STAT3 signaling pathway was activated, causing the phosphorylation of STAT3 and promoting the expression of inflammatory mediators.

RORγt is a transcription factor for Th17 cells to secrete IL-17 and IL-23. Th17 differentiation is usually mediated by IL-17 and IL-23[16,17]. JAK2 is an essential factor in the phosphorylation process of STAT3, and the phosphorylation level of STAT3 is greatly reduced through the inhibiting activity of JAK2 [18]. Therefore, IL-23 activates JAK2/STAT3 pathway to upregulate the expression of RORγt. This study found that Jinwujiangu decoction could up-regulate the expression of IL-23R, STAT3, PSTAT3RO, and RORγt in RA-FLS. We speculate that Jinwujiangu decoction could weaken the bridge role of the JAK2/STAT3 genes in IL-23/IL-17 inflammation axis, and thereby inhibit the inflammatory effect of the IL-23/IL-17 axis. In addition, regulation of STAT3 is closely related to cell proliferation and apoptosis [19]. Studies have confirmed that STAT3 directly inhibits the proliferation of RA-FLS [20]. This study shows that compared with the blank control group, different concentrations of Jinwujiangu decoction have an inhibitory effect on RA-FLS proliferation, which could upregulate the gene and protein expression levels of STAT3.

RORα and RORγt are members of a family of steroid hormone receptors. RORγt is an important transcriptional regulator that mediates the function of IL-23/IL-17 inflammation axis and promotes the secretion of IL-17 [21]. Among them, RORγt is more effective in promoting the secretion of IL-17, and both RORα and RORγt in combination are more effective than either in isolation. In the absence of both RORα and RORγt, IL-23/IL-17 axis would be unable to produce an inflammatory effect. This study found that Jinwujiangu decoction could up-regulate the expression of RORα and RORγt in RA patients, enhance the inhibitory effect of IL-17 secretion, and prevent the persistent inflammation of RA. In normal circumstances, proinflammatory and anti-inflammatory cytokines are balanced to maintain homeostasis in the body. They form a complex cell network and mediate a variety of important physiological functions of the body. Increase in immune dysfunction and proinflammatory cytokines is one of the important mechanisms of
RA progression. IL-6 is a proinflammatory factor that has a variety of biological activities. It regulates the activity of other cytokines and of multiple signaling pathways [22]. Transforming growth factor-β (TGF-β) is a multifunctional cell regulatory factor, and has a dual role in RA. The binding of TGF-β with TGF-β receptors on the surface of osteoblast precursor cells promotes the differentiation and expression of osteoblasts [23,24].

This study suggests that Jinwujiangu decoction can inhibit the secretion of IL-6 and TGF-β in RA-FLS, interfere with the gene expression of IL-23/IL-17 inflammation axis and inhibit immune inflammation. However, due to the different biological effects of IL-6 and TGF-β, the effects of Jinwujiangu decoction on RA pannus hyperplasia and bone destruction need to be further study. We will further understand the detailed mechanism of Jinwujiangu decoction in the treatment of rheumatoid arthritis.

**CONCLUSION**

In this study, the in vitro culture models of different concentrations of Jinwu Jiangu Decoction for RA-FLS were successfully established, and the mechanism of Jinwu Jiangu Decoction on the inflammation axis of RA IL-23/IL-17 was discussed. They suggested that Jinwu Jiangu Decoction plays an important role in immunopathology of rheumatoid arthritis. The results of this study open up new avenues for the development of ethnomedical therapeutics for RA.

**DECLARATIONS**

**Acknowledgement**

This work was supported by Natural Science Foundation of China which entitled, ‘Discuss the Mechanism of Miao Medicine Jinwu Jiangu Capsule to Rheumatoid Arthritis Synovial Cell Autophagy and Apoptosis Based on mTOR Signal’ (grant no. 81760907); Guizhou province high-level innovative talent training program by Guizhou Provincial Department of Science and Technology(grant no. [2016]5650).

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xueming Yao and Wukai Ma designed all the experiments and revised the paper. Rong Li, Qiaoyi Ning, Ying Huang, Fang Tang, and Hui Xu performed the experiments. Daoming Lu, Jiang Liang, and Yang An wrote the paper.

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