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Research Article

Immunomodulatory activity of a Chinese herbal drug Yi Shen Juan Bi in adjuvant arthritis

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ABSTRACT

Objective: To investigate the immunomodulating mechanisms of a Chinese herbal medicine Yi Shen Juan Bi (YJB) in treatment of adjuvant arthritis (AA) in rats.

Materials and Methods: Levels of serum tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) were measured by the Enzyme-Linked Immunosorbent Assay (ELISA). Expression of TNF-α mRNA and IL-1β mRNA in synovial cells was measured with the semi-quantitative technique of reverse transcription-polymerase chain reaction (RT-PCR), while caspase-3 was examined by western blot analysis.

Results: The administration of YJB significantly decreased the production of serum TNF-α and IL-1β. It also decreased significantly the TNF-α mRNA, IL-1β mRNA, and caspase-3 expression in synoviocytes.

Conclusions: YJB produces the immunomodulatory effects by downregulating the over-activated cytokines, while it activates caspase-3, which is the key executioner of apoptosis in the immune system. This may be the one of the underlying mechanisms that explains how YJB treats the rheumatoid arthritis.

KEY WORDS: Synoviocytes, TNF-α, IL-1β, caspase-3

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by fibroblastic proliferation, infiltration of the synovial lining by inflammatory cells which leads to expression of proinflammatory cytokines, and a paucity of apoptosis resulting in bone and joint destruction.[1,2] For the treatment of RA, traditional Chinese medicines (TCM) were found to be effective clinically, although the mechanisms underlying their efficacy have not yet been fully studied.[3]

Yi Shen Juan Bi (YJB, dark brown pills, patent number: ZL200510040550) is a compound anti-rheumatic herbal drug. YJB formula has been recognized as a valuable traditional medicine used in the treatment of RA. Clinically, YJB has been shown to ameliorate symptoms and signs of RA and decreases the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and the rheumatic factor (RF).[4] However, the mechanisms underlying the efficacy of YJB have not yet been fully studied. Therefore, we have studied the pharmacological actions and mechanisms of YJB using an animal model of the disease. In this study, we used adjuvant arthritis (AA) in rat as an experimental model. Important features of this model are chronic synovitis, including inflammatory cell infiltration, panes formation, destruction of cartilage, and bone erosion.[5] Such characteristics of AA are useful in evaluation of the efficacy of therapeutic agents and the underlying mechanisms.

One of the aims of this study was to investigate the effects of YJB on immunomodulatory cytokine production and the mechanism by which YJB affects the process of AA. For that purpose we have examined the production of serum cytokines by the enzyme-linked immunosorbent assay (ELISA). In order to explore further, we have studied the synovial expression of tumor necrosis factor alpha (TNF-α) mRNA, and IL-1β mRNA by using RT-PCR. Apoptosis plays a key role in the imbalance between proliferation and growth arrest of synoviocytes in RA. In this experiment, we have also observed the effect of YJB on proliferation of synovium by inducing apoptosis of synovial cells.

Materials and Methods

Ingredients and Preparation of Yi Shen Juan Bi

The ingredients [Table 1] were concentrated to 1 kg by the method described below.

Scorpio and Scolopendra were pulverized to fine powder and sifted. Radix Angelica and Rhizoma Corydalis were heated under reflux with 60% ethanol for one hour, each time twice and then filtered. Ethanol was recovered and the filtrate concentrated to a thin extract with a relative density of 1.2 (50°C). The decoction of other ingredients was made in water thrice, 2h

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Table 1:

Ingredients and preparation of Yi Shen Juan Bi

Radix rehmanniae praeparata	262.5 g
Radix angelica	210 g
Herba epimedii	210 g
Scorpio	31.3 g
Scolopendra	31.5 g
Nidus vespae (stir-baking)	210 g
Rhizoma drynariae	31.5 g
Herba pyrolae	31.5 g
Zaocys (stir-fried with wine)	210 g
Radix rehmanniae	210 g
Eupolyphaga seu steleophaga	210 g
Bombyx batryticatus	210 g
Herba erodii	262.5 g
Rhizoma corydalis	210 g
Radix et rhizoma	210 g
Cynanchi paniculati	262.5 g
Polygoni cuspidati	262.5 g
Caulis spatholobi	262.5 g
Japanese hop	262.5 g

for the first time and 1.5 h for the second and third times. The decoction was mixed and filtered. The filtrate was concentrated to the relative density of 1.2 (50°C). Ethyl alcohol was added to prepare a solution containing 50% of ethanol and kept aside. The supernatant was separated and ethanol was recovered and then concentrated to a thin extract with a relative density of 1.2 (50°C). Then fine powder, thin extract, and a quantity of excipients were mixed well and made to a concentrated pill, dried, and polished. YJB pills were suspended in 0.5% sodium carboxymethylcellulose (CMC-Na). Cyclophosphamide (CPA), (Jiangsu Hengrui Medicine Co., Ltd, batch number - 07012821) was dissolved in distilled water.

Animals

Male Spargue-Dawley (SD) rats, weighing 180-220 g, were purchased from experimental animal laboratory of Nantong University, China. All of them were acclimatized under standard laboratory conditions. During the experimental period, the animals were kept in a temperature-controlled environment (22±2°C, 55±5% relative humidity with a 12 h: 12 h light-dark cycle) and fed with standard laboratory chow and water.

All experimental protocols described in this study were approved by the University Ethics Committee on Animal Research and conformed to the National Institute of Health Guideline for Care and Use of Laboratory Animals.

Drug Treatment and Induction of AA

Rats were divided randomly into six groups: the control group, the model group, the positive control group (CPA, 7 mg/kg), YJB high dose (YJB-H, 2.4 g/kg), middle dose (YJB-M, 1.2 g/kg), and low dose (YJB-L, 0.6 g/kg). Each rat was injected with 0.1 ml of Freund's complete adjuvant (Sigma) intradermally in the left hind metatarsal footpad for inducing inflammation (except the normal control group). On the 14th day, each group was treated with medication by intragastric administration once daily for next 14 days. The model group was treated with sodium carboxymethylcellulose and the control animals were given an equal volume of distilled water.

The ELISA of TNF-α and IL-1β

After 14 days of medication, blood samples were drawn from orbital plexus for preparation of the serum ELISA test and then killed by cervical dislocation. TNF-α, IL-1β quantitative ELISA kits were purchased from Jingmei Biotech Co. Ltd (Beijing, China). The ELISA was performed according to the manufacturer's instructions.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Experiments

Extraction of total RNA

After 14 days of medication, the rats were killed by cervical dislocation and synovial tissue of knee joints was extracted and then total RNA of synovial cells was estimated as directed.

Primer (Designed by Nanjing Sunshine Biotechnology Co. Ltd.)

TNF-α: Leading strand: 5' GCCAATGGCATGGATCTCAAAG3'
Lagging strand: 5' CAGAGCAATGACTCCAAAGT3'
Enhanced products: 357 bp
IL-1β: Leading strand: 5' GAAGCTGTGGCAGCTACCTATGTCT3'
Lagging strand: 5' CTCTGCTTGAGAGGTGCTGATGTAC3'
Enhanced products: 520 bp
GAPDH: Leading strand: 5' GGTGCTGAGTATGTCGTGGAG 3'
Lagging strand: 5' ATGCAGGGATGATGTTCTGG 3'
Enhanced products: 356 bp

Method of RT-PCR

2 μg RNA sample and 1 μg oligo dT15 were mixed in a 200 μL PCR tube without RNase, and then water was added up to 15 μL. After reacting for 5 min at 70°C, these were put into ice. The reverse transcription was conducted using Promeger products (5.0 μL, M-MLV 5× Buffer; 1.25 μL, DNTPs (10 mM); 1.25 μL, RNase inhibitor (20 U/μL); 1 μL, 200 U M-MLV RT; 1.5 μL, H2O). The sample was centrifuged quickly, and then placed at 42°C for 60 min, and amplified or frozen at -20°C. PCR was performed by adding the PCR mixture (volume of 20 μL) (2 μL, 10×B; 0.4 μL, 10 mM dNTP; 1 μL, 10 μM up and downstream primers; 1.2 μL, 25 mM MgCl2; 0.2 μL, DNA Taq polymerase; 1 μL, cDNA; 13.2 μL, H2O). The reaction mixture was then subjected to 30 cycles of 95°C for 5-min pre-apomorphosis, 94°C for 30-s apomorphosis, 60°C for 30-s anneals and 72°C for 1-min extension. After the last cycle, a final extension at 72°C for 5 min was done. Then 7 μL PCR product was added to 1.5% agarose gel electrophoresis. The gel was photographed and analyzed by JEDA. The relative content of each sample was computed and compared with internal consulting. The average OD value of the strap of amplified products was determined.

Western blot analysis

After continuous intragastric administration of drugs for 14 days, the rats were killed by cervical dislocation and synovial tissues of knee joints were extracted. After treatment with different reagents as described in the figure legends, synovial tissues were homogenised in M2 buffer (20 mM Tris (SIGMA) pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM beta-glycerol phosphate, 1 mM sodium vanadate, 1 mg/mL leupeptin). Fifty to eighty micrograms of the lysates were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel (12%) which was purchased from Ameresco and blotted onto the PVDF membrane. After blocking with 5% skimmed milk in PBST,

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the membrane was probed with the relevant antibodies, anti-caspase-3 (BD Pharmingens, San Diego, CA.) and α-Tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Then the blotted and washed membrane was visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA).

Statistical Analysis

The results were expressed as mean ± S.D. for each group. Data were assessed by one-way ANOVA followed by Tukey post hoc test, and a value of $P < 0.05$ was considered to be statistically significant.

Results

The Effect of YJB on TNF-α and IL-1β

As shown in Figure 1, serum TNF-α and IL-1β levels in AA rats were significantly elevated in the model group compared with control. However, a significant decrease was seen in these parameters in the YJB-treated groups.

The Effect of YJB on the TNF-α mRNA and IL-1β mRNA Expression

As shown in Figures 2-4, synovial TNF-α mRNA and IL-1β mRNA expression in AA rats was significantly elevated in the model group compared with control. In YJB-treated group, a significant decrease in these parameters was seen. CPA also significantly decreased these values.

The Effect of YJB on the Expression of caspase-3 precursor (procaspase-3)

As shown in Figures 5 and 6, synovial level of caspase-3 precursor (procaspase-3) expression was significantly decreased in the model group compared to control. Compared with the model group, YJB group showed a significant decrease in synovial levels of caspase-3 precursor (procaspase-3) expression. Positive drug, CPA, also significantly decreased it.

Discussion

The contribution made by proinflammatory cytokines in RA, such as tumor necrosis factor TNF-α and IL-1 has been validated in preclinical animal models and in humans.[6] It is also well documented that IL-1 and TNF-α synergistically mediate synovitis and destruction of cartilage and bone.[7] TNF-α is an important factor in inflammation and immunity that can stimulate the synoviocyte and cartilage cells to synthesize the PGE_2 and collagenase causing synovium and cartilage destruction as well as those of IL-1, IL-6, and IL-8.[8] Therefore, it is one of the most important factors in the cytokine network.

In RA patients, IL-1β is overexpressed in inflamed synovial tissue, in particular in the lining layer and in sublining cells[9] and it is elevated in draining lymph from affected joints.[10] Furthermore, cartilage from arthritis patients exhibits upregulation of IL-1β mRNA as compared with normal.[11,12] In addition, increased production of IL-1β in fibroblast-like synoviocytes of susceptible individuals may lead to a higher risk of developing severe joint damage even in the absence of systemic inflammation. In general, TNF-α causes early joint swelling in RA, while IL-1β-combined the immune complex leads

Figure 1: The effect of YJB on TNF-α and IL-1β in serum of AA rats (n = 8, mean ± S.D.). ##*P*<0.01 vs. Control; **P*<0.05, ***P*<0.01 vs. Model group

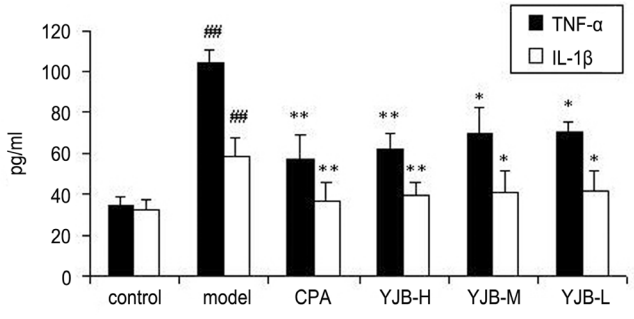

Figure 2: Detection of GAPDH mRNA, TNF-α mRNA and IL-1βmRNA expressions in rat synovium (n = 3, lanes 1-7). Lane1: 1control, Lane 2: model, Lane 3: CPA, Lane 4: YJB-H, Lane 5: YJB-M, Lane 6: YJB-L.

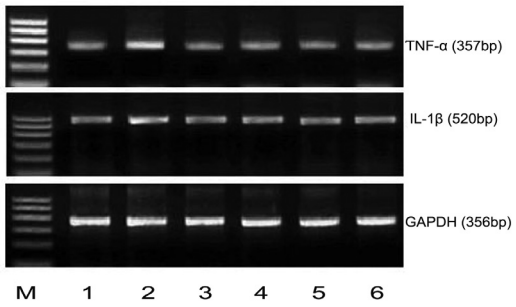

Figure 3: The effect of YJB on the expression of TNF-α mRNA of AA rats (n = 3, mean ± S.D.). ##*P*<0.01 vs. Control; **P*<0.05; ***P*<0.01 vs. Model

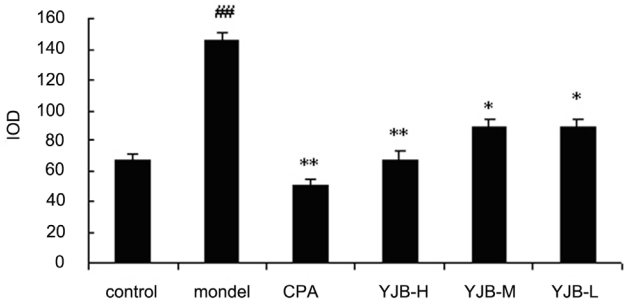

Figure 4: The effect of YJB on the expression of IL-1β mRNA of AA rats (n = 3, mean ± S.D.). ##*P*<0.01 vs. Control; **P*<0.05; ***P*<0.01 vs. Model

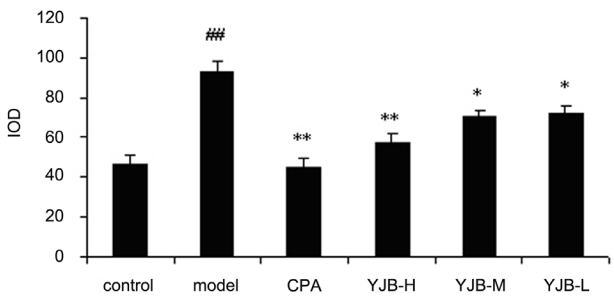

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Figure 5: Western blots of rat synovium from control and drugs treated rats probed using the relevant antibodies, anti-caspase 3 and α-Tubulin (n=3).Lane 1: control, Lane 2: model, Lane 3: YJB-L, Lane 4: YJB-M, Lane 5: YJB-H, Lane 6: CPA.

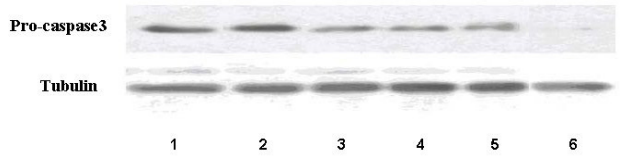
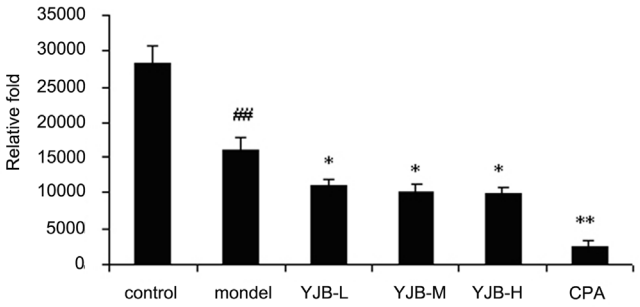


Figure 6: The effect of YJB on the expression of pro caspase-3 of AA rats (n = 3, mean ± S.D.). ##*P*<0.01 vs. Control; **P*<0.05; ***P*<0.01 vs. Model group



to the cartilage erosion.[13] Considering these investigations it can be concluded that TNF-α and IL-1β have a pivotal role in the pathogenesis of RA. It can also be pointed out that blocking of both TNF-α and IL-1β is necessary in the treatment of RA.

The study revealed that serum levels of TNF-α and IL-1β in the model group were significantly higher than that of normal rats. In addition, TNF-α mRNA and IL-1β mRNA expression in synovial cells of the model group were significantly higher than that of normal rats. These observations correlate well with the above-mentioned research findings in RA. Pro-inflammatory cytokines, such as tumor necrosis factor α and interleukin-1β, are expressed in the arthritic joints in both AA rats and human rheumatoid arthritis, and blockade of these molecules results in amelioration of disease.[14] Our results confirm that YJB could significantly reduce the level of TNF-α and IL-1β levels in serum, while they decrease the TNF-α mRNA and IL-1β mRNA expression in synovial cells. This may be one of the underlying mechanisms of amelioration of inflammation in RA by YJB. Therefore it seems to be a promising drug for the treatment of cytokine expression *in vivo*.

Currently it is believed that the injuries of osteochondral tissues in RA are closely associated with activation and proliferation of synovial cells. Apoptosis may play a significant role in its pathogenesis.[15] Caspase belongs to the protease family which plays an important role in cell apoptosis. Apoptotic effects of caspases include two pathways.[16,17] The inductors are different in these two, but they both gather at the downstream caspases inducing cell apoptosis. In general, caspase-3 is a key enzyme for apoptotic cell death and is recognized as a terminator for cells to be killed.[18] We have used purified mouse anti-caspase-3 antibody for detecting caspase-3 levels. This antibody recognizes the mouse 32 kDa pro-caspase-3, which is related to active caspase 3. Active caspase-3 is a homodimer of heterodimers, produced by cleavage of procaspase-3.[19,20] Our results showed that, pro-caspase-3 in synovial tissue of AA rats in the model group was lower than the normal control group. This suggests that there is a certain degree of apoptosis in synoviocytes in the model group. Compared to the drug-treated groups, there may be an excessive proliferation and inadequate apoptosis leading to the inflammatory response[21] in AA rats. After the treatment with YJB in AA rats, procaspase-3 decreased significantly. It indicates that the increase of apoptosis cells improved the imbalance of excessive proliferation and apoptosis. The agent that induces apoptosis of synoviocytes provides a novel and effective treatment for RA.[22] These findings suggest that YJB induces apoptosis through activation of caspase-3 in AA and may represent a potent drug in RA therapy.

In conclusion, our results have confirmed that YJB possesses an immunomodulatory activity by regulating the key cytokines and also it potently induces the apoptosis of synoviocytes of AA rats, which may be mediated by up-regulating the expression of apoptosis-associated caspase-3. This may be one of the underlying mechanisms that explain how YJB treats the rheumatoid arthritis. However, we believe that further understanding of the mechanism of YJB action should be beneficial for the development of an effective drug for RA.

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