Efficacy of Zhonglun’a-decoction-containing serum on fibroblast-like synoviocyte apoptosis in rats with collagen-induced arthritis via inhibiting Janus kinase/signal transducer and activator of transcription signaling pathway

Yang Yongsheng, Dong Qiumei, Ma Chunjie, Zhang Yudi, Li Yongle, Chen Kun, Zhang Suo

Abstract

OBJECTIVE: To investigate the efficacy of Zhonglun’a-decoction-containing serum (ZHONGL-CS) on the in vitro apoptosis of fibroblast-like synoviocytes (FLS) from rats with collagen-induced arthritis (CIA) by investigating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.

METHODS: A CIA rat model was established using bovine type II collagen. FLS were isolated, cultured and identified. A cell counting kit-8 was used to detect cell activity. The half maximal inhibitory concentration (IC50) was calculated. Experimental subjects were divided into control, CIA, ZHONGL-CS, JAK2 inhibitor AG490, and ZHONGL-CS with AG490 groups. The in vitro cell cycle and apoptosis rate were detected in FLS by flow cytometry. Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), caspase-3, cyclin D1, phosphorylated JAK2, STAT1, and STAT3 protein expressions in FLS were examined by Western blotting. JAK2, STAT1 and STAT3 mRNA levels were examined by quantitative real-time polymerase chain reaction.

RESULTS: Compared with the CIA group, FLS proliferation was inhibited, the FLS G0/G1 cell cycle was arrested, and the rate of FLS apoptosis was increased in the ZHONGL-CS group. In the ZHONGL-CS group, the protein levels of Bcl-2 and cyclin D1 were reduced compared with the CIA group and the levels of Bax and caspase-3 in FLS were increased. In the ZHONGL-CS group, the expressions of JAK2, STAT1, and STAT3 mRNA and the levels of phosphorylated JAK2, STAT1, and STAT3 proteins were reduced.

CONCLUSION: ZHONGL-CS may induce FLS apoptosis in CIA rats. Activation of the JAK/STAT signaling pathway was inhibited in FLS in vitro.

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Keywords: Arthritis, rheumatoid; Synoviocytes; Apoptosis; Janus kinases; Zhonglun’a-decoction-containing serum

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic auto-
Immune disease that primarily affects the joints, and is characterized by synovitis, synovial tissue proliferation, pannus formation, cartilage and bone destruction. The incidence of RA is approximately 1% in the adult population worldwide. Currently, the exact pathogenesis of RA remains unclear. However, increasing evidence suggests that fibroblast-like synoviocytes (FLS) play an important role in the occurrence and development of RA because they are resistant to apoptosis and their proliferation is anchorage-dependent. In RA patients, defective synovial cell apoptosis is closely related to synovial tissue hyperplasia. RA FLS exhibit damage and erosion, which may lead to further synovial tissue hyperplasia and destruction of the articular cartilage. Thus, inhibition of RA FLS proliferation and the induction of apoptosis are gradually being considered a therapeutic strategy for the treatment of RA.

Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2) and caspase-3 proteins play important roles in apoptosis. Bax promotes apoptosis, Bcl-2 has a wide range of anti-apoptotic effects, and caspase-3 is a key molecule of apoptosis and a common downstream effector in many apoptosis signaling pathways. Cyclin D1 is a positive cell cycle regulator that is closely related to the transition of autoimmune diseases and tumor proliferation. Over-expressed cyclin D1 reduces the G0/G1 phase, which can lead to abnormal cell proliferation.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway plays a pivotal role in cell growth, differentiation, proliferation, and immune regulation. It is also related to the occurrence of many human diseases, such as RA and cancers. Research has indicated that activation of the JAK/STAT signaling pathway through interferon-γ induces an in situ apoptosis resistance phenotype in cells of the synovial tissue in inflammatory rheumatoid arthritis, leading to a marked increased density of synovial cells. In addition, the JAK/STAT signaling pathway induces anti-apoptotic effects via translational regulation.

Recently, JAKs have aroused great interest as a new therapeutic target, and the unique effects of these enzymes play key roles in signaling transduction processes. Activated JAKs phosphorylate STATs, which form active dimers, transfer to the nucleus, and bind to specific response elements to activate or inhibit the expression of target genes. Seven STATs have been identified, and STAT1 and STAT3 are the major activating factors. The expression and activity of STAT1 was increased in the early synovial tissues of human RA and STAT3 promoted synovial fibroblasts. Other studies reported that the activation of STAT1 and STAT3 has an important role in synovial cell proliferation. The traditional approaches of controlling RA rely on disease modifying antirheumatic drugs (DMARDs), including methotrexate (MTX), leflunomide and sulfasalazine. These drugs have certain benefits in the clinical; however, they induce a series of toxic reactions.

Zhonglun’a decoction, prepared with Zhizi (Fructus Gardeniae), Hezi (Fructus Chebulae), Chuansianzi (Fructus Toosendan), Kushen (Radix Sophorae Flavescentis), and Zhihuading (Herba Violae Philippicae), has antipyretic, anti-inflammatory and analgesic effects, and is used to treat RA. However, no current published reports have reported the effect of Zhonglun a decoction on inhibiting proliferation and/or inducing apoptosis of FLS in CIA rats. Collagen-induced arthritis (CIA) is widely used as an experimental model for the research of human RA. Recently, it has been extensively used for studying the pathogenesis of RA and evaluating potential therapeutic strategies against RA. The purpose of this study was to investigate the efficacy of Zhonglun’a-decoction-containing serum (ZHONGL-CS) on FLS proliferation and apoptosis via the JAK/STAT signaling pathway in CIA rats.

**MATERIALS AND METHODS**

**Induction of collagen-induced arthritis**

Male Sprague-Dawley rats (7-8 weeks old) were purchased from the Experimental Animal Center of the Third Military Medical University (Chongqing, China) and the care and treatment processes were agreed to by the Committee of Experimental Animal Administration of the Third Military Medical University. CIA was induced by bovine type II collagen (Chondrex Inc., Redmond, WA, USA). Type II collagen (2 mg/mL) was dissolved in 0.1 M acetic acid and emulsified with an equal volume of complete Freund’s adjuvant (CFA) or incomplete Freund’s adjuvant (IFA) (MP Biomedicals, Santa Ana, CA, USA). Then, 300 μL emulsion was injected into the base of the rat tail for the first immunization on day 0 and the second immunization was performed on day 7. An equal volume of saline was administered to rats in the normal group at the same time and at the same site.

**Experimental drugs**

Zhonglun’a decoction was obtained from Inner Mongolia International Mongolian Hospital (batch number Z20121216). The Zhonglun’a decoction is composed of five different herbal ingredients, including Zhizi (Fructus Gardeniae), Hezi (Fructus Chebulae), Chuansianzi (Fructus Toosendan), Kushen (Radix Sophorae Flavescentis), and Zhihuading (Herba Violae Philippicae) at a ratio of 1.5:1:1:1:1.5:1, which are ground into a coarse powder.

**ZHONGL-CS preparation**

After three days of acclimation, sixty Sprague-Dawley rats weighing 200-220 g were divided randomly into two groups: Zhonglun’a decoction (n = 40) group and control (n = 20) group. Rats received an intragastric...
dose of Zhonglun’a decoction with normal saline in suspension (9.0 g/kg, which corresponds to 10 times the adult equivalent dose according to the human and animal equivalent dose list of the ratio of the body surface area). Control groups received an equal volume of normal saline twice a day for seven consecutive days. Approximately 2 h after the final drug administration, the rats were euthanized by 10% chloral hydrate anesthesia, and blood was retrieved from the abdominal aorta and heart under aseptic conditions. Blood samples were collected by centrifugation at 3000 × g for 20 min at 4 ℃ overnight, and then filtered through a 0.22-μm filter. Sera were carefully extracted by suction and then incubated in a 56 ℃ water bath for 30 min to inactive complement, then separated and stored at −20 ℃ in sterile centrifuge tubes.

**Culture of FLS**

Forty-two days after the initial immunization, rats from the CIA group and the control group were euthanized. Isolated synovial tissues from the knee joints were minced and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 ℃ in a humidified atmosphere with 5% CO<sub>2</sub>. After the identification of FLS by morphology and immunofluorescence, FLS were used from passages 3 to 5 in subsequent experiments. Cells were treated with or without a JAK inhibitor, AG490 (25 μmol/L) (Abmole Bioscience Inc., Houston, TX, USA), or 20% ZHONGL-CS.

**Cell proliferation assay**

Cell proliferation was analyzed using the cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) assay. Cells were seeded in three 96-well plates at a final density of 3 × 10<sup>4</sup> cells per well, after treatment with blank serum containing RPMI-1640 medium for 24, 48, or 72 h. The absorbance at 450 nm was measured using a microplate reader (Beckman Coulter Inc., Brea, CA, USA).

**Cell cycle analysis**

FLS within the logarithmic growth phase were collected and seeded into culture flasks at a density of 2 × 10<sup>5</sup> cells/mL. After cell synchronization for 24 h, FLS were divided into control, CIA, ZHONGL-CS, AG490, or ZHONGL-CS + AG490 groups, incubated with the corresponding drugs for 24 h, collected, suspended in cold phosphate-buffered saline (PBS), centrifuged, fixed with 75% cold ethanol and stored at 4 ℃ for 24 h. The cells were stained with propidium iodide staining solution (Cell Cycle and Apoptosis Analysis Kit; Beyotime Institute of Biotechnology, Nanjing, China) for 30 min at 37 ℃ in the dark. The cell cycle analysis was performed by flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

**Apoptosis analysis**

Apoptosis of FLS was measured by flow cytometry with the Annexin V-FITC/PI double labeling method. Briefly, cells were seeded at a density of 2 × 10<sup>5</sup> cells/ml in five culture flasks and incubated for 24 h. After exposure to drugs for 24 h, the cells were collected and re-suspended with PBS, counted, centrifuged, discarded the Supernatant, and re-suspended in 195 μL binding buffer. Suspended cells were incubated with 5 μL of Annexin V-FITC and 10 μL of Propidium Iodide (Annexin V-FITC Apoptosis Detection Kit, Beyotime) for 20 min at room temperature in the dark. Apoptotic cells were analyzed by flow cytometry.

**Western blot analysis**

Cells were incubated in RPMI-1640 medium for 24 h with the corresponding drugs and collected in radio immunoprecipitation assay (RIPA) lysis buffer (Chongqing Golden Wheat Biotechnology Co., Ltd., Chongqing, China). The protein concentration was determined by bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Nanjing, China). Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% skim milk for 2 h at room temperature. After three washes with Tris-buffered saline containing 0.05% Tween-20 (TBST), they were incubated overnight at 4 ℃ with antibodies against β-actin (diluted to 1:1000) (Santa Cruz Biotechnology, Inc., CA, USA), caspase-3, phosphorylated-JAK2 (p-JAK2) (1:1000; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), Bax, Bcl-2, cyclin D1, phosphorylated-STAT1 (p-STAT1), and phosphorylated-STAT3 (p-STAT3) (diluted to 1:1000) (BBI Life Science Corporation, Shanghai, China). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Beyotime Institute of Biotechnology, Nanjing, China) at a dilution of 1:1000 for 2 h at room temperature after washing the membrane with TBST. Protein bands were visualized with enhanced chemiluminescence (ECL) reagents (Beyotime Institute of Biotechnology, Nanjing, China).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cultured FLS with
TRIZol reagent according to the manufacturer’s method (Sangon Biotech Co., Ltd., Shanghai, China). RNA was reverse-transcribed with an AMV First Strand cDNA Synthesis Kit (Sangon Biotech Co., Ltd., Shanghai, China) in the presence of random primers and RNase-free ddH2O. The primers were designed by Sangon Biotech Co., Ltd., (Shanghai) and β-actin was used as an endogenous control (Table 1). The conditions for amplification were as follows: 3 min hold at 95 °C, followed by 7 s denaturation at 95 °C, 10 s annealing at 57 °C and 15 s extension at 72 °C until the end with 40 cycles. The PCR products were analyzed using StepOne Plus™ Real-Time PCR System (Applied Biosystems, Inc., USA). The q RT-PCR data were analyzed using the 2^-∆∆CT method.

Statistical analysis
Quantitative data are presented as the mean ± standard deviation (x ± s). All data were analyzed with SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA). One-way analysis of variance (ANOVA) and the Games-Howell test were conducted to test the differences between groups. P < 0.05 indicated statistical significance.

RESULTS
Identification of FLS
The cellular morphology of cultured rat FLS from passage three exhibited a spindle shape under an inverted microscope (Figure 1A). In addition, 100% of the cultured cells demonstrated the positive expression of vimentin under immunofluorescence staining; thus confirming them as FLS (Figure 1B).

Effect of ZHONGL-CS on FLS proliferation in CIA rats
To determine the effect of ZHONGL-CS treatment on rat-derived FLS proliferation, the CCK-8 assay was performed on FLS treated with blank serum containing 1540 medium and different concentrations (5%, 10%, 15%, 20%, and 25%) of normal rat serum and ZHONGL-CS for 24, 48, or 72 h. Compared with the normal rat serum group, 5%, 10%, 15%, 20%, and 25% different concentrations of ZHONGL-CS significantly inhibited the proliferation of rat FLS (P < 0.05, P < 0.01) (Table 2). The IC50 value of the ZHONGL-CS group for FLS was calculated according to the inhibition rates of ZHONGL-CS. The IC50 of the ZHONGL-CS group was estimated to be 20% at 24 h; therefore, this concentration and time point were used in subsequent experiments (Figure 2).

ZHONGL-CS induces cell cycle arrest of FLS in CIA rats
Compared with the control group, the percentage of S phase FLS in the CIA group was increased (P < 0.05) and the percentage of G0/G1 phase FLS was decreased (P < 0.01). However, the percentages of FLS treated with ZHONGL-CS, AG490, or ZHONGL-CS + AG490 at the G2/M phase were significantly decreased compared with the CIA group (all P < 0.01). Simultaneously, those at the G0/G1 phase were increased (P < 0.01). Furthermore, compared with the CIA group, the percentage of S phase FLS were decreased in the ZHONGL-CS + AG490 group (P < 0.05). Compared with the AG490 group, the percentage of cells at the G0/G1 and G2/M phases in the ZHONGL-CS or ZHONGL-CS + AG490 groups were significantly different (P < 0.05) (Figure 3). Cell cycle arrest was a major cause of cell proliferation inhibition.

ZHONGL-CS induces FLS apoptosis in CIA rats
The apoptosis rates (19.8% ± 0.9%) of the control group FLS were significantly lower (14.5% ± 1.1%) compared with the CIA group (P < 0.05). The apoptosis rates of FLS treated with ZHONGL-CS, AG490, or ZHONGL-CS + AG490 were 18.5% ± 0.3%, 21.4% ± 0.7%, and 23.6% ± 1.7%, respectively, and there was no significant difference between them (P < 0.05). However, the apoptosis rates were significantly different between the ZHONGL-CS and AG490 groups (P < 0.01).

### Table 1 Primers used for qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product length (bp)</th>
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<tbody>
<tr>
<td>JAK2</td>
<td>Forward: 5’AGACTCCCTCCGCTGTCTATAAAC 3’</td>
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<td></td>
<td>Reverse: 5’ACCTTACCCGGCTTCCGAAGT 3’</td>
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<tr>
<td>STAT1</td>
<td>Forward: 5’TATCAGCAAGGAGCGAGAAGC 3’</td>
<td>130</td>
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<tr>
<td></td>
<td>Reverse: 5’ACCTCCATTTTGGGACCGT 3’</td>
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<tr>
<td>STAT3</td>
<td>Forward: 5’GAGAAGGACATCGTGGCAAGA 3’</td>
<td>162</td>
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<td></td>
<td>Reverse: 5’GGGAATGTCAGGGTATACAGG 3’</td>
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<tr>
<td>β-actin</td>
<td>Forward: 5’CGTAAAGACCCCTATGGCCCAACA 3’</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’AGCCACCAATCCACACAGG 3’</td>
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</table>

Notes: qRT-PCR: quantitative real-time polymerase chain reaction; JAK2: Janus kinase 2; STAT1/3: signal transducer and activator of transcription 1/3.
ZHONGL-CS on FLS in CIA rats was related to modulation of the JAK/STAT signal pathway. FLS were incubated with drugs for 24 h. Total RNA was extracted from cultured FLS and the mRNA expression levels of JAK2, STAT1, and STAT3 were detected in the cell extracts by qRT-PCR (Figure 6), and proteins levels of p-JAK2, p-STAT1, p-STAT3 were detected by Western blotting (Figure 7). The expression levels of JAK2, STAT1 and STAT3 mRNA and the levels of the phosphorylated proteins were low in the control group. The expression levels of JAK2, STAT1 and STAT3 mRNA and the levels of the phosphorylated proteins were significantly increased in the CIA group compared with the control group \((P < 0.01)\). The expression levels of JAK2, STAT1 and STAT3 mRNA and the levels of the phosphorylated proteins were significantly decreased when FLS were treated with ZHONGL-CS, AG490, ZHONGL-CS + AG490 when compared with the CIA group \((P < 0.05, P < 0.01)\). In addition, qRT-PCR analysis revealed that the expression levels of STAT3 mRNA were inhibited to a greater extent by treatment with AG490 than with ZHONGL-CS \((P < 0.01)\) (Figure 6). Moreover, Western blotting analysis demonstrated that levels of p-JAK2 and p-STAT3 were decreased by treatment with AG490 compared with ZHONGL-CS \((P < 0.05, P < 0.01)\) (Figure 7).

### DISCUSSION

In this study, we investigated the effect of ZHONGL-CS on the proliferation or apoptosis of FLS from CIA rats and whether this involved the JAK/STAT signaling pathway. We found that ZHONGL-CS inhibited the proliferation of FLS and induced cell apoptosis *in vitro* by partially downregulating JAK/STAT activation.

### Table 2 Effect of different concentrations of ZHONGL-CS on FLS proliferation in CIA rats (OD values, \(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.1141 ± 0.0020</td>
<td>0.1240 ± 0.0031</td>
<td>0.1225 ± 0.0021</td>
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<tr>
<td>5% Normal serum</td>
<td>5</td>
<td>1.3175 ± 0.0541</td>
<td>2.9018 ± 0.0970</td>
<td>3.3636 ± 0.1059</td>
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<tr>
<td>10% Normal serum</td>
<td>5</td>
<td>1.4025 ± 0.0779</td>
<td>2.9416 ± 0.0867</td>
<td>3.3846 ± 0.0611</td>
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<tr>
<td>15% Normal serum</td>
<td>5</td>
<td>1.1638 ± 0.0918</td>
<td>2.8238 ± 0.1541</td>
<td>3.4018 ± 0.1093</td>
</tr>
<tr>
<td>20% Normal serum</td>
<td>5</td>
<td>1.1128 ± 0.1243</td>
<td>2.8615 ± 0.2511</td>
<td>3.4606 ± 0.1288</td>
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<tr>
<td>25% Normal serum</td>
<td>5</td>
<td>1.1175 ± 0.1594</td>
<td>2.7896 ± 0.0775</td>
<td>3.4101 ± 0.0734</td>
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<tr>
<td>5% ZHONGL-CS</td>
<td>5</td>
<td>1.1530 ± 0.0330</td>
<td>2.4084 ± 0.0507</td>
<td>2.6067 ± 0.1155</td>
</tr>
<tr>
<td>10% ZHONGL-CS</td>
<td>5</td>
<td>1.0809 ± 0.0400</td>
<td>2.1736 ± 0.0674</td>
<td>2.0935 ± 0.1404</td>
</tr>
<tr>
<td>15% ZHONGL-CS</td>
<td>5</td>
<td>0.7545 ± 0.0289</td>
<td>1.7091 ± 0.0999</td>
<td>1.7175 ± 0.1223</td>
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<tr>
<td>20% ZHONGL-CS</td>
<td>5</td>
<td>0.5932 ± 0.0191</td>
<td>1.3334 ± 0.0702</td>
<td>1.4286 ± 0.0652</td>
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<tr>
<td>25% ZHONGL-CS</td>
<td>5</td>
<td>0.5460 ± 0.0608</td>
<td>1.2051 ± 0.1210</td>
<td>1.3206 ± 0.0711</td>
</tr>
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</table>

Notes: control: treated with blank serum containing RPMI-1640 medium; FLS were cultured with 5%, 10%, 15%, 20%, or 25% normal serum, ZHONGL-CS respectively for 24, 48 and 72 h at 37 °C in a humidified atmosphere under 5% CO2, ZHONGL-CS: Zhongln’s decoction-containing serum; FLS: fibroblast-like synoviocytes; CIA: collagen-induced arthritis; OD: optical density; RPMI: Roswell Park Memorial Institute. \(n\): the number of wells. Compared with 5%, 10%, 15%, 20%, or 25% normal serum group at same time point, \(P < 0.05, P < 0.01\).
The treatment of RA includes the administration of drugs including DMARDs, non-steroidal anti-inflammatory drugs, steroid hormones, and biologics, which are associated with severe adverse effects, including gastrointestinal lesions, cardiovascular complications, and reproductive toxicity. Modern pharmacological studies reported that Zhonglun’s decoction had anti-inflammatory and analgesic effects that reduced the swelling of joints, improved blood rheology, regulated immune functions, and reduced inflammation induced by cytokines in CIA rats. Although some of the effects of Zhonglun’s decoction are related to the etiological factors of RA, the detailed features of this compound remain to be characterized.

To fulfill our objective, the effect of ZHONGL-CS was investigated in CIA rat FLS by CCK-8 and flow cytometry analysis. The results clearly demonstrated that ZHONGL-CS dramatically inhibited CIA FLS proliferation in a dose-dependent manner. Previous studies found that FLS were highly proliferative in RA, and that G1 phase cells were reduced, and G2 and S phase cells were increased. Cell cycle regulation disorders can cause the loss of cell growth control, which is important in the development of RA. In this study, ZHONGL-CS blocked cell proliferation at the G0/G1 phase, decreased the proportion of G2/M phase cells, and increased the apoptosis rates of CIA FLS. These findings show that ZHONGL-CS induces...
Apoptosis requires the activation of a series of caspases, FLS.

ZHONGL-CS downregulated the expression of cyclin mRNA was relevant to the inhibition of synovial cell membrane, and the decreased expression of cyclin D1 related to the abnormal proliferation of synovial membrane. The apoptosis of in vitro FLS from CIA rats.

Bax was increased, suggesting ZHONGL-CS promotes the expression of Bcl-2 was decreased, while that of RA patients. As demonstrated in the present study, the expression of Bcl-2 was decreased, while that of Bax was increased, suggesting ZHONGL-CS promotes the apoptosis of in vitro FLS from CIA rats. A significant downregulation of pro-caspase-3 and -9 expressions and a significant upregulation of caspase-3 and -9 expressions were observed when RA-FLS apoptosis occurred. Our findings are consistent with these results, which showed that ZHONGL-CS significantly induced the activation of caspases-3 in FLS from CIA rats.

The persistent activation of STAT1 and STAT3 in FLS in RA, as well as the expression of STAT1 and STAT3 in FLS in CIA, indicates that the JAK/STAT signaling pathway is involved in the regulation of FLS apoptosis in CIA. The activation of caspase-3 plays a crucial role in initiating apoptosis. A significant downregulation of pro-caspase-3 and -9 expressions and a significant upregulation of caspase-3 and -9 expressions were observed when RA-FLS apoptosis occurred. Our findings are consistent with these results, which showed that ZHONGL-CS significantly induced the activation of caspases-3 in FLS from CIA rats.

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STAT3 protein levels were suppressed by ZHONGL-CS in FLS from CIA rats. ZHONGL-CS induced apoptosis in FLS from CIA rats by suppressing activation of the JAK2/STAT1/3 signaling pathway. In conclusion, we demonstrated that ZHONGL-CS has an anti-proliferative effect and induces the apoptosis of FLS from CIA rats, most likely through inactivation of the JAK2/STAT1/3 pathway, downregulation of cyclin D1 and the antiapoptotic protein Bcl-2, and upregulation of the proapoptotic proteins Bax and caspase-3. Therefore, morin may be a novel therapeutic agent for RA, especially with the counter activation of JAK2/STAT1/3 signaling. However, only the JAK/STAT apoptosis signaling pathway was examined in vitro in this study, so further investigations are needed to determine the role of other apoptotic pathways and the induction of apoptosis mechanisms underpinning the therapeutic efficacy of ZHONGL-CS in RA.

REFERENCES


2. Sultanah F, Raoul M. A novel therapeutic approach targeting rheumatoid arthritis by combined administration of morin, a dietary flavanol and non-steroidal anti-inflammatory drug indomethacin with reference to pro-inflammatory-
Figure 7 Protein levels of p-JAK2, p-STAT1, and p-STAT3 in FLS from CIA rats were detected by Western blotting. A: p-JAK2, p-STAT1, and p-STAT3 protein levels were evaluated by Western blot analysis after FLS had been incubated with drugs for 24 h. B-D: Histogram shows the levels of p-JAK2, p-STAT1, and p-STAT3. 1: control group; 2: CIA group; 3: ZHONGL-CS group; 4: AG490 group; 5: ZHONGL-CS + AG490 group. CIA: collagen-induced arthritis; FLS: fibroblast-like synoviocytes; ZHONGL-CS: Zhonglun’a-decoction-containing serum. P < 0.01 vs the control group; P < 0.01, P < 0.05 vs the CIA group; P < 0.01, P < 0.05 vs the AG490 group. p-JAK2: phosphorylation of Janus kinase 2; p-STAT1/3: phosphorylation of signal transducer and activator of transcription 1/3.

Yang YS et al. / Research Article
21 Li L, Gao ZL, Qu AT, Dong QM. Effect of Zhonglun’e decoction on anti-inflammatory, analgesic and paw swelling in rats with collagen induced arthritis. Zhong Guo Shi Yan Fang Ji Xue Za Zhi 2011; 17(03): 184-186.