

New bitongling (NBTL) ameliorates rheumatoid arthritis in rats through inhibiting JAK2/STAT3 signaling pathway

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ABSTRACT

Rheumatoid arthritis (RA) is featured by a variety of physical symptoms and fibroblast-like synoviocytes (FLSs) abnormal proliferation. Increasing evidence has demonstrated that traditional Chinese medicine exerts an important role in RA treatment. New bitongling (NBTL) as one of the traditional Chinese medicine has been reported to be involved in the progression of RA, but the exact mechanism is unclear. In our study, we intended to investigate the effect of NBTL on RA to identify the mechanisms related to JAK2/STAT3 signaling pathway. Extracts of *Tripterygium wilfordii* (TW), a traditional Chinese herbal medicine, have been widely used for treating RA in China for several decades, so, TW was used as a positive control drug for TBTL. RA rats were constructed by immunization with collagen type II to evaluate the action of NBTL *in vivo*. Body weight and arthritic index were evaluated. Hematoxylin & Eosin staining was performed to analysis the morphological changes of ankle joints tissue. TUNEL and flow cytometry were performed to examine cell apoptosis, while CCK8 and Ethynyl-2'-deoxyuridine (EdU) were performed to examine cell proliferation. In addition, the markers of inflammation were detected by Western blot, ELISA, and RT-qPCR. Firstly, we find that rats treated with NBTL or TW not only reduced swelling degree and bone destruction, but also repressed IL-1 β and IL-6 levels. In addition, NBTL and TW could increase the weight of rats, and promote the level of IL-10 and IL-4 *in vivo*. Furthermore, NBTL inhibited inflammation of FLS, induced cell apoptosis and hindered cell proliferation, which was reversed by dipeptidyl peptidase (DPP), a JAK2/STAT3 pathway activator. Taken together, NBTL potentially retarded RA *via* JAK2/STAT3 pathway, highlighting novel mechanisms associated with RA

Key words: Rheumatoid arthritis; NBTL; JAK2/STAT3; FLS; inflammation.

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Introduction

Rheumatoid arthritis (RA), a chronic disease related to inflammation and autoimmunity, mainly disturbs joints. If treated inappropriately, it may lead to joint injury and even irreversible disability.¹ The global incidence of RA is rising, affecting 0.5% to 1% of the total population, with a ratio of 3:1 (female-to-male).² There may be significant differences between affected individuals in the progress and etiology of RA, and its treatment is still challenging. Although RA treatment has improved significantly, as many as 30% of the patients did not respond to the interventions or had significant side effects related to treatment.³

Fibroblast-like synoviocytes (FLSs) are the most common cell type on pannus-cartilage junction in RA. FLS may eventually cause joint destruction by generating cytokines, chemical factors and matrix degrading molecules.⁴ In addition, FLSs with aggressive phenotype can resist apoptosis, proliferate excessively, and invade surrounding tissues like bones and cartilage, thus leading to angiogenesis, activating immune cells and arthritis.⁵ Therefore, understanding the mechanism of FLS in RA is crucial to the treatment of RA.⁶

Traditional Chinese medicine assumes that RA is caused by blood stagnation, heat and toxins.⁷ New bitongling (NBTL) prescription was derived from the experience and thoughts of L.Q. Wang (1919-1999), the late professor of Nanjing University of Traditional Chinese Medicine, a well-known Chinese medicine expert in rheumatology (composed and effective compositions of NBTL were showed in Table 1). In the preliminary study, the research group used orthogonal T-value method to analyze and study the interaction and dose of the main drugs and auxiliary drugs of the compound, simplified the compound, and finally determined to be composed of *Herba Ephedrae*, *Ramulus Cinnamomi*, *Caulis Sinomenii*, *Radix Saposhnikoviae*, *Radix Aconitii Preparata*, *Nidus Vespa*.⁸ Early research study found that the NBTL can be effective treatment of RA,⁹ significantly inhibited the overexpression of vascular endothelial growth factor (VEGF) mRNA in synovial tissue of joints,¹⁰ and inhibited the increase of soluble intercellular adhesion factor -1 (sICAM-1) in serum of C57BL mice.¹¹ In addition, the microvascular density and the overexpression of IL-17 in the synovial tissue of the joint of the model rats were decreased by NBTL.¹² It is reported that NBTL inhibits the expression of prostaglandin E2 (PGE2), have anti-inflammatory effects,¹³ can be different levels of the two aspects of nonspecific immunity and nonspecific immune inhibition,¹⁴ can effectively reduce rats serum and synovial tissues of IL-2, the content of TNF alpha,¹⁵ but the exact mechanism is unclear. Therefore, this study sought to determine whether NBTL has therapeutic effect on RA and study the therapeutic mechanism. In addition, Clinical practice guideline for the use of the extracts of *Tripterygium wilfordii* (TW) in the treatment of RA was approved on June 2020 by the Standardization Office of Chinese Association of Chinese Medicine.^{16,17} So, TW was used as a positive control drug for TBNL. This study uses a collagen-induced arthritis (CIA) rat model to assess the therapeutic effect of NBTL on RA. This study also observed the effects of NBTL on proliferation and apoptosis of FLS. This study investigated whether NBTL treatment could restore the immune balance and apoptosis of FLS in RA.

Materials and Methods

Rheumatoid arthritis induction

Adult female Sprague-Dawley (SD) rats (200-250 g) were

used, 7-10 weeks. SD rats were acquired from Zhejiang Academy of Medical Sciences (Zhejiang, China). Rats were kept in standard animal rooms with temperatures of 18-22°C, where food and water are freely available. Rats were treated humanely using approved procedures granted by the local Animal Ethics Committee. Collagen-induced arthritis (CIA) was caused in SD rats by immunization with collagen type II (CII, 234184-M, Sigma-Aldrich, St. Louis, MO, USA).¹⁸ In short, collagen was dissolved in 0.1 M acetic acid (2 mg/mL) and gently stirred overnight at 4°C. Emulsification contained the same volume of Complete Freund's Adjuvant (CFA, F5881, Sigma-Aldrich) containing mycobacteria to produce inducers. Each rat was injected subcutaneously with 100 µL emulsion at the bottom of the tail. The second injection was performed 14 days after immunization to stabilize the disease symptoms.

Grouping of animals

There were 6 groups (6 rats per group): Control group; RA group (Collagen induced arthritis); TW group (RA+treated with TW 8 mg/kg body weight oral daily); RA+NBTL (low concentration) group (RA+treated with NBTL 2.75 g/kg body weight oral daily); RA+NBTL (medium concentration) group (RA+treated with NBTL 5.5 g/kg body weight oral daily); RA+NBTL (high concentration) group (RA+treated with NBTL 11 g/kg body weight oral daily). The dosage was selected according to the preliminary research conducted in laboratory and literature. The arthritis index of these animals was evaluated using a scale of 0 to 4 points, as follows: 0, normal; 1, slight erythema and swelling; 2, moderate erythema and swelling; 3, severe erythema and swelling, limited joint use; 4, deformed claws.¹⁹ The two hind legs both were scored in each rat, with the highest possible score being 8 points.¹⁹

ELISA

The level of Interleukin-6 (IL-6), Interleukin-1β (IL-1β), Interleukin-10 (IL-10), and Interleukin-4, (IL-4) were measured according to the instructions of a commercial ELISA kit (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China).

H&E staining

After anesthesia and sacrifice on the 33rd day after immunization, ankle joints of rats were collected, fixed in 4% paraformaldehyde for 24 h, dehydrated with graded alcohol and cleared in xylene, paraffin embedded, and sectioned (5 µm). Sections were dewaxed and rehydrated. Then, sections were stained with hematoxylin (474-07-7, Jisskang Biotechnology Co., Ltd., Qingdao, China), and then eosin (RY0648, Jisskang), followed by dehydration and mounting. The morphological changes of FLS in the rat ankle joints sections were subjected to a morphological image analysis system (JD801; Jeda Technology Co. Ltd., Nanjing, China).

Table 1. Components and the effective compositions of NBTL.

Component	Weight (g)
<i>Herba Ephedrae</i>	6
<i>Caulis Sinomenii</i>	15
<i>Ramulus Cinnamomi</i>	10
<i>Nidus Vespa</i>	10
<i>Radix Saposhnikoviae</i>	10
<i>Radix Aconiti</i>	10

qRT-PCR

TRIzol reagent (Takara, Liaoning, China) was used to extract the total RNA. M-MLV Reverse Transcriptase (RNase H) kit (Takara, Liaoning, China) was performed to synthesize cDNA. qPCR was performed with SYBR Green qPCR kit (Life Technologies, Shanghai, China). β -actin²⁰ serves as an endogenous control for MCM7,²¹ PCNA,²² Ki-67,²³ Bcl-2, Bax,²⁴ caspase-3 and caspase-9.²⁵ The primers are listed in Table 2.

Western blot

Tissues or cell lysates were isolated using cell lysis buffer (Beyotime, Nanjing, China). The membrane was transferred and blocked at 37°C for 1 h, followed by primary antibodies incubation, rabbit anti-MCM7 (1:1000, ab52489), PCNA (1:2000, ab92552) and Ki-67 (1:1000, ab16667), Bax (1:5000; ab32503), Bcl-2 (1:1000, ab59348), Cleaved caspase-3 (1:1000, ab49822), Cleaved caspase-9 (1:1000, ab2324), JAK2 (1:5000, ab108596), p-JAK2 (1:5000, ab32101), STAT3 (1:2000, ab109085), p-STAT3 (1:2000, ab76315) and rabbit anti- β -actin (1:2000, ab8227) were purchased from Abcam (Cambridge, UK), and then re-probed with immunoglobulin G (IgG) complexed to horseradish peroxidase (1:2000, ab6721) antibody. The chemiluminescence kit (Millipore, Germany) was adopted to observe the immune response zone and Image J software (ImageJ Software Inc., USA) was used to quantify the integrated density of each band. Antibodies mentioned before were supplied by Abcam (Cambridge, UK).

Primary FLS isolation

Discarded isolated synovial tissue were obtained. Tissue was cut into small pieces. Samples were then digested with 0.4% type I collagenase for 2 h, 0.25% trypsin for 30 min. Digestion were performed in a 37°C. Filtered the digested cells using a 70 mm strainer and washed three times with PBS. Using immunocytochemistry staining with Anti-Vimentin antibody and flow cytometry with Anti-CD55 antibody for cell purity test.^{19,24}

CCK8 assay

CCK8 assay kit (CK04, Dojindo, Japan) was utilized to analyze cell viability. Cells were incubated with different dosages of NBTL (10, 20, 30 μ M)²⁶ and TW (50 nM) concentrations for 48 h. 1×10^4 cells/well were seeded for 24 h of pre-culture, followed by 48 h treatment. After NBTL or TW treatment, 10 μ L CCK8 was added for reaction at 37°C for 3 h. After that, optical density at 450 nm was checked on a microplate reader. A cell growth curve was drawn.

TUNEL assay

Tissue samples were fixed at 37° for 30 min, then incubated with 0.2% Triton X-100 for 5 min. In order to improve the permeability, samples were incubated with 50 μ L reaction mixture (the ratio of concentrated enzyme solution to label solution is 1:9) at 37°C for 1 h and observed using a fluorescence microscope. Randomly selected five fields to determine the apoptosis rate, apoptosis rate = (apoptotic cells/total cells) \times 100.

For cell apoptosis *in vitro*, 48 h after NBTL or TW treatment, FLSs were fixed using 2% formalin at 37°C for 1 h, using 0.1% Triton X-100 to permeate on ice for 2 min, followed by 1-h-incubation with 50 μ L TUNEL reaction mixture at 37°C under dark conditions. The results were observed under fluorescence microscope (LSM880, Zeiss, Germany).²⁴

Ethynyl-2'-deoxyUridine assay

Cell proliferation after NBTL or TW treatment was detected using an EdU kit (CA1170, Solarbio, China). In brief, cells were

incubated with 50 μ M EdU for 18 h firstly. Cells were fixed with 4% formaldehyde for 30 min, followed by 0.5% Triton X-100 for ten minutes. Then, incubated 30 min with $1 \times$ Apollo reagent. Finally, DAPI was used for nuclear staining.²⁷

Flow cytometry

1.00×10^6 cells/mL were applied for propidium iodide staining for 10 min and subjected to cell apoptosis analysis using FITC and FACSCalibur flow cytometer (342975, BD, New Jersey, USA). The number of cells was acquired at 488 nm wavelength and analyzed using Cell Quest software.

Statistical analysis

The mean \pm SD represents data from three independent experiments. SPSS 21.0 (IBM Corp., NY, USA) is used for statistical analysis of all data. Data within multiple groups is analyzed using one-way ANOVA and Tukey's post-tests. Time-based multi-comparison was tested by repeated measures ANOVA; $p < 0.05$ indicated that the difference is statistically significant.

Results

NBTL impacts inflammation in RA rats

In the third week after the induction of RA rats, we analyzed the degree of swelling of the hind paws of rats. Clinical symptoms of RA can be seen in RA rats, including swelling and redness of the hind paws. Compared to rats in the RA group, rats treated with NBTL or TW reduced swelling degree and exhibited NBTL concentration dependence (Figure 1A). NBTL and TW also significantly increased the weight of rats (Figure 1B) while reducing the arthritis index value (Figure 1C), which also showed NBTL concentration dependence.

The serum levels of IL-1 β , IL-6, IL-10, IL-4 were analyzed by ELISA, which indicated a significant increase in the expression of IL-1 β and IL-6 in rats of RA group, relative to the control group, while NBTL treatment was able to suppress IL-1 β and IL-6 levels with concentration dependence compared to the RA group. In contrast, NBTL and TW treatment significantly promoted the level of IL-10 and IL-4 (Figure 1D). Taken together, NBTL could prevent inflammation in RA rats.

Table 2. Primer sequences used for qRT-PCR.

Genes		Primer sequences (5'-3')
MCM7	Forward	GATAAGAGGAAACATCCACATCTG
	Reverse	AGACGGTCAATGTAAGACAGG
PCNA	Forward	TGACTGGTTATCGTCCCTCCT
	Reverse	CTCAAACATGTTGGCGGAGT
Ki-67	Forward	GGGTTTCCAGACACCAGAC
	Reverse	CCAGGAAGACCAGTTAGAACC
Bcl-2	Forward	GACTGAGTACCTGAACCGGC
	Reverse	TAGTTCACAAAGGCATCCAG
Bax	Forward	CCAGTTGAAGTTGCCGTCAGAA
	Reverse	GCGAGTGTCTCAAGCGCATC
Caspase-3	Forward	GCTTGTCCGCATACCTGTTTCAG
	Reverse	AGAAGTGGACTGTGGCAITGAG
Caspase-9	Forward	ATTGGCGACCCTGAGAAG
	Reverse	CCAGATGCTGTCCCATTACC
β -actin	Forward	GAGAGGGAATCGTGCCTGAC
	Reverse	CATCTGCTGGAAGGTGGACA

NBTL inhibits synovioocyte proliferation in RA rats

To discover the effect of NBTL in the RA model system, histopathology of each group of rats was assessed. The rats in the control group showed normal joint structure, while the RA model

rats exhibited substantial inflammatory cells penetration, synovial hyperplasia, bone destruction. TW and NBTL-mediated dose-dependent reduce bone destruction, and synovial hyperplasia of rats (Figure 2A). The mRNA of MCM7, PCNA and Ki-67 was

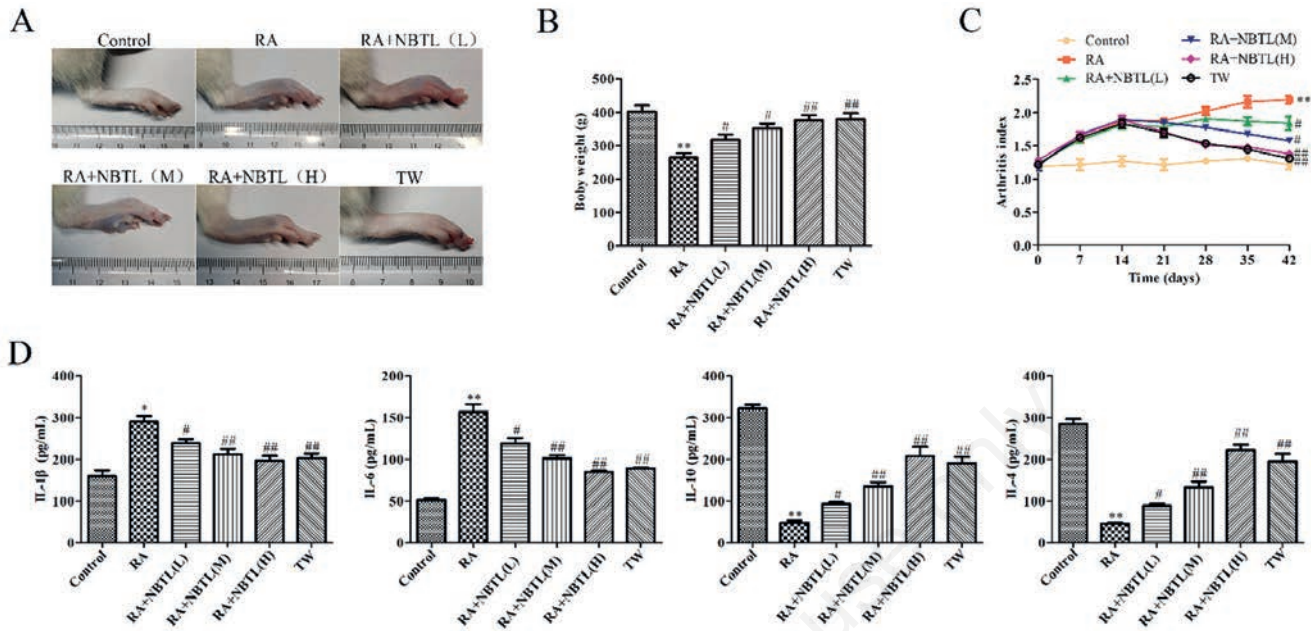


Figure 1. Inflammation inhibitory effects of NBTL on rheumatoid arthritis (RA) *in vivo*. RA rats were constructed by immunization with collagen type II, then we treated RA rats with different concentrations of NBTL, and TW (positive control drug for NBTL). A) Observation of the paw swelling. B) Body weight of rats in treated groups. C) Arthritic scores of rats were used to assess the severity of the disease. D) ELISA was performed to analyze the serum levels of IL-1 β , IL-6, IL-10, and IL-4. Data were shown as mean \pm SD (n = 10 for all groups). *p<0.05, **p<0.01 vs Control, #p<0.05, ##p<0.01 vs RA.

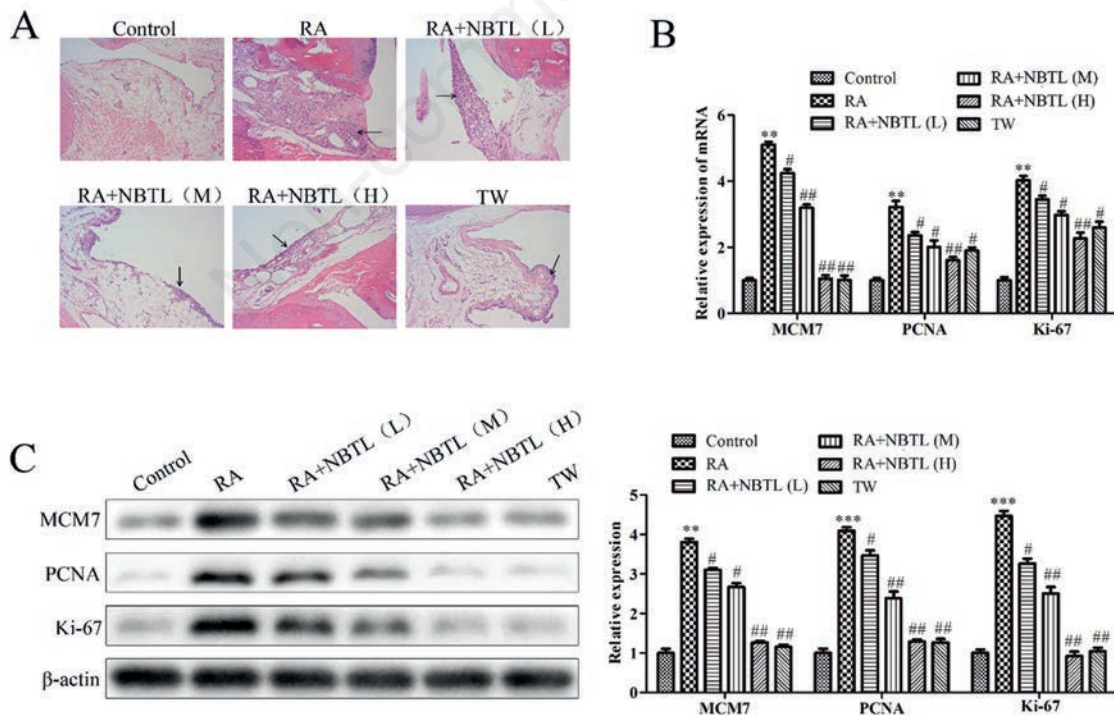


Figure 2. NBTL inhibits cell proliferation in RA *in vivo*. A) Damage to joint tissues determined using H&E staining (200 \times). B) The mRNA expression levels of MCM7, PCNA and Ki-67 were measured by qRT-PCR. C) The protein expression of proliferation-associated proteins MCM7, PCNA and Ki-67 were measured by Western blot. Data were shown as mean \pm SD (n=10 for all groups). **p<0.01, ***p<0.001 vs Control, #p<0.05, ##p<0.01 vs RA.

assessed using qRT-PCR. The results exhibited that mRNA of MCM7, PCNA and Ki-67 were significantly promoted in RA group, respectively, but were significantly repressed by TW and NBTL (Figure 2B). Finally, the expression of proliferation-associated proteins MCM7, PCNA and Ki-67 were detected using Western blot. The results exhibited that MCM7, PCNA, Ki-67 in RA group were significantly promoted. Conversely, MCM7, PCNA and Ki-67 were remarkably diminished due to TW and NBTL treatment (Figure 2C). Collectively, NBTL inhibited cell proliferation in RA rats.

NBTL inhibits inflammation of fibroblast-like synoviocytes (FLS) *in vitro*

For *in vitro* experiments, FLS was initially characterized by immunofluorescent staining. These cells were Vimentin-positive (Figure 3A). The expression of CD55 in cells was also determined using flow cytometry assay (Figure 3B), which clarified that FLS purity >90%, making it suitable for subsequent experiments. Then, after treating FLS with NBTL, the level of inflammation-related factors after NBTL treatment were measured by ELISA. The result

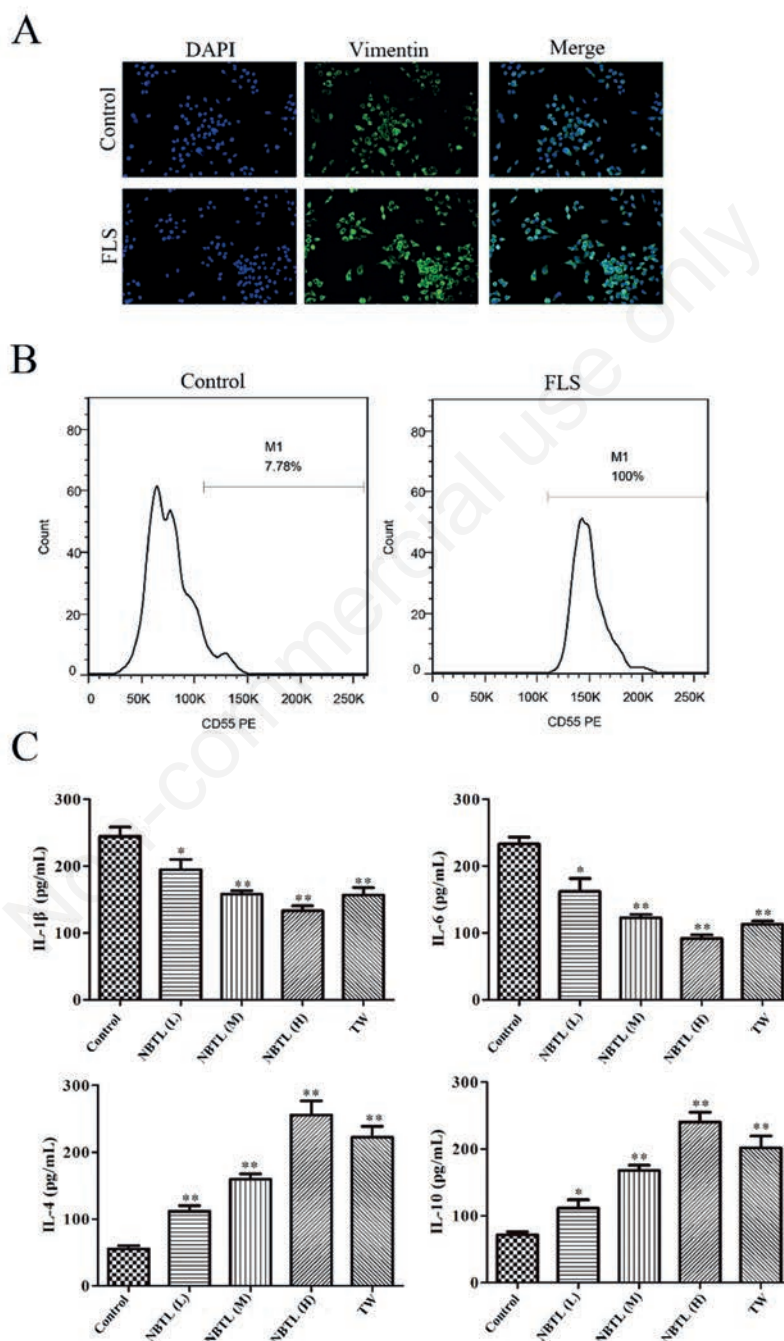


Figure 3. The validation and inflammation of fibroblast-like synovial (FLS) cells isolated from RA rats treated with NBTL. A) The expression levels of v were determined using immunocytochemistry (200 \times). B) The positive cell rate of CD55 were determined using flow cytometry. C) ELISA was performed to analyze the supernatant levels of IL-1 β , IL-6, IL-10, and IL-4 from FLS cells. Data were shown as mean \pm SD (n=3). *p<0.05, **p<0.01 *vs* Control.

indicated that IL-1 beta and IL-6 were significantly reduced after NBTL treatment, while the level of IL-10 and IL-4 were significantly promoted, in a concentration-dependent manner (Figure 3C). Collectively, NBTL might inhibit the inflammation of FLS.

NBTL induces FLS cell apoptosis and hinders proliferation *in vitro*

In the following experiments, we detected FLS apoptosis by TUNEL assay, which indicated that apoptosis rate was significantly promoted after NBTL treatment with concentration dependence (Figure 4A). Moreover, we detected proliferation of FLS cells through CCK8 (Figure 4B) and EdU staining (Figure 4C), and both showed that NBTL significantly restrained cell proliferation. In contrast to the proliferation results, subsequent results of flow cytometry analysis were consistent with the TUNEL assay results (Figure 4D).

Next, the mRNA of Bcl-2, Bax, caspase-3 and caspase-9 were detected by qRT-PCR, which demonstrated that the mRNA of Bax, caspase-3 and caspase-9 were significantly improved after NBTL

treatment, while the results of Bcl-2 were the opposite (Figure 4E). Same as mRNA, the result of Western blot indicated that in the group treated with NBTL, Bax, Cleaved caspase-3, and Cleaved caspase-9 increased significantly, while Bcl-2 was suppressed (Figure 4F). Taken together, NBTL might stimulate FLS apoptosis, while restraining proliferation *in vitro*.

NBTL's effect on FLS cell proliferation and apoptosis was via JAK2/STAT3 pathway

Recent studies have validated the significant roles of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathways in RA.²⁸ So, we assumed that NBTL modulated FLS cells *via* the JAK/STAT pathway and this hypothesis was validated. First, we used Western blot to measure the level of JAK2/STAT3 axis-related proteins and found that NBTL was able to significantly inhibit p-JAK2 and p-STAT3 level. Then we treated FLS with both NBTL and DPP (JAK2/STAT3 pathway activator) simultaneously and found that p-JAK2 and p-STAT3 level were rescued by DPP (Figure 5A). Above results revealed

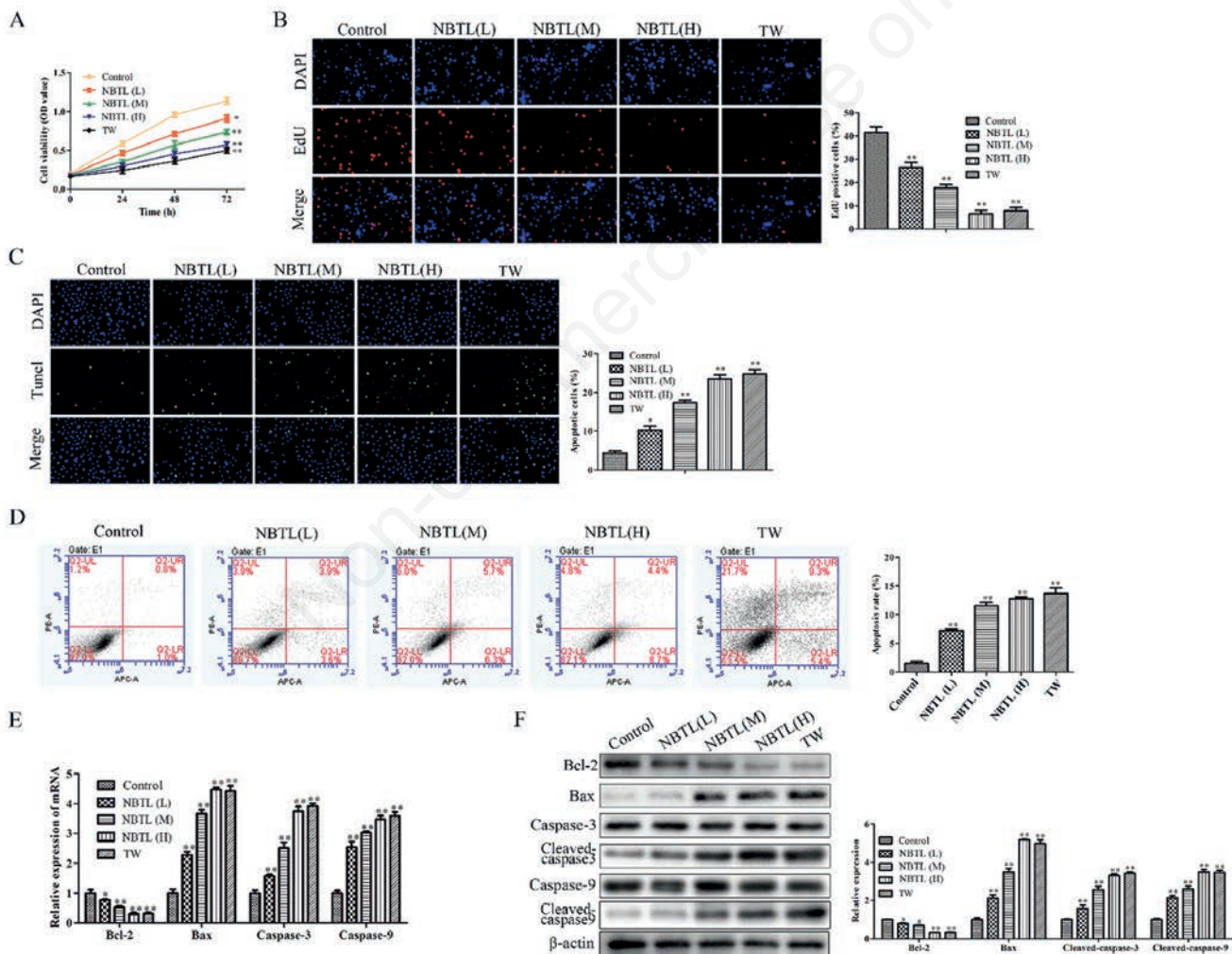


Figure 4. NBTL inhibits cell proliferation and promotes apoptosis of FLS *in vitro*. A) The cell apoptosis determined using TUNEL staining (400 ×). B) The cell viability was determined using CCK8 assay. C) The cell proliferation was determined using EdU staining (400 ×). D) The cell apoptosis determined using flow cytometry. E) The mRNA expression levels of Bcl-2, Bax, caspase-3 and caspase-9 were measured by qRT-PCR. F) The expression of apoptosis-associated proteins, including Bcl-2, Bax, Cleaved caspase-3, Cleaved caspase-9 were measured by Western blot. Data were shown as mean ± SD (n=10 for all groups). *p<0.05, **p<0.01 vs Control.

that NBTL can suppress the JAK2/STAT3 pathway.

In order to further confirm that NBTL's effect on FLS cell proliferation and apoptosis is through the JAK/STAT pathway, we detected cell proliferation and apoptosis by CCK8 (Figure 5B), EdU staining (Figure 5C), TUNEL staining (Figure 5D), and flow cytometry (Figure 5E), respectively. It was found that NBTL significantly promoted FLS apoptosis and inhibited proliferation, while DPP can reverse NBTL's effects. Finally, the level of Bcl-2, Bax, Cleaved caspase-3 and Cleaved caspase-9 were measured via Western blot. NBTL resulted in enhanced the expression of Bax, Cleaved caspase-3 and Cleaved caspase-9, and restrained the level of Bcl-2. More importantly, the DPP treatment reversed such activation effects of NBTL on the cell apoptosis (Figure 5F).

Discussion

RA is a systemic immune disease, which is chronic and progressive and will reduce the patient's life expectancy by 3 to 20 years. The pathogenesis of RA is very complex, so treatment is usually aimed at improving life quality by slowing down disease progression.

CIA is an autoimmune model, which is helpful to study possible therapeutic strategies for injury and inflammation in RA.^{18,29} In this research, the effects of NBTL on RA rats were studied by

inducing a CIA model. Animal research showed that compared with RA group, the severity of arthritis decreased significantly after NBTL treatment. Histopathology also indicated that the articular cartilage was intact after NBTL treatment, indicating that arthritis of RA rats was significantly relieved after NBTL treatment. It is reported that inflammation plays a major role in the progression of RA, and IL-1 β and IL-6 plays a proinflammatory role in RA.^{30,31} While IL-10 and IL-4 are anti-inflammatory cytokines.³² So, we tested IL-1 β , IL-6, IL-10 and IL-4 to check for the anti-inflammatory effects of NBTL. Our study indicated that the contents of IL-1 β and IL-6 in RA group were upregulated compared to control group, while repressed after NBTL treatment. In contrast, IL-10 and IL-4 were upregulated after NBTL treatment. Our results showed that NBTL can ameliorate RA by inhibiting the level of inflammatory cytokines. These results confirmed that NBTL had anti-inflammation effect.

At present, it is now well-known that occurrence of RA may be due to the imbalance between proliferation and apoptosis.³³ FLS is synovial cell which produces synovial fluid. FLS is crucial to the regulation of cartilage and joint damage, deformation and destruction in rheumatoid arthritis.³⁴ FLS undergo significant changes in the development of RA, overcoming contact inhibition, and experiencing abnormal proliferation, which lead to tissue damage, even tissue degradation.³⁵ Therefore, repressing of FLS proliferation and inducing of cell apoptosis are considered to be a useful strate-

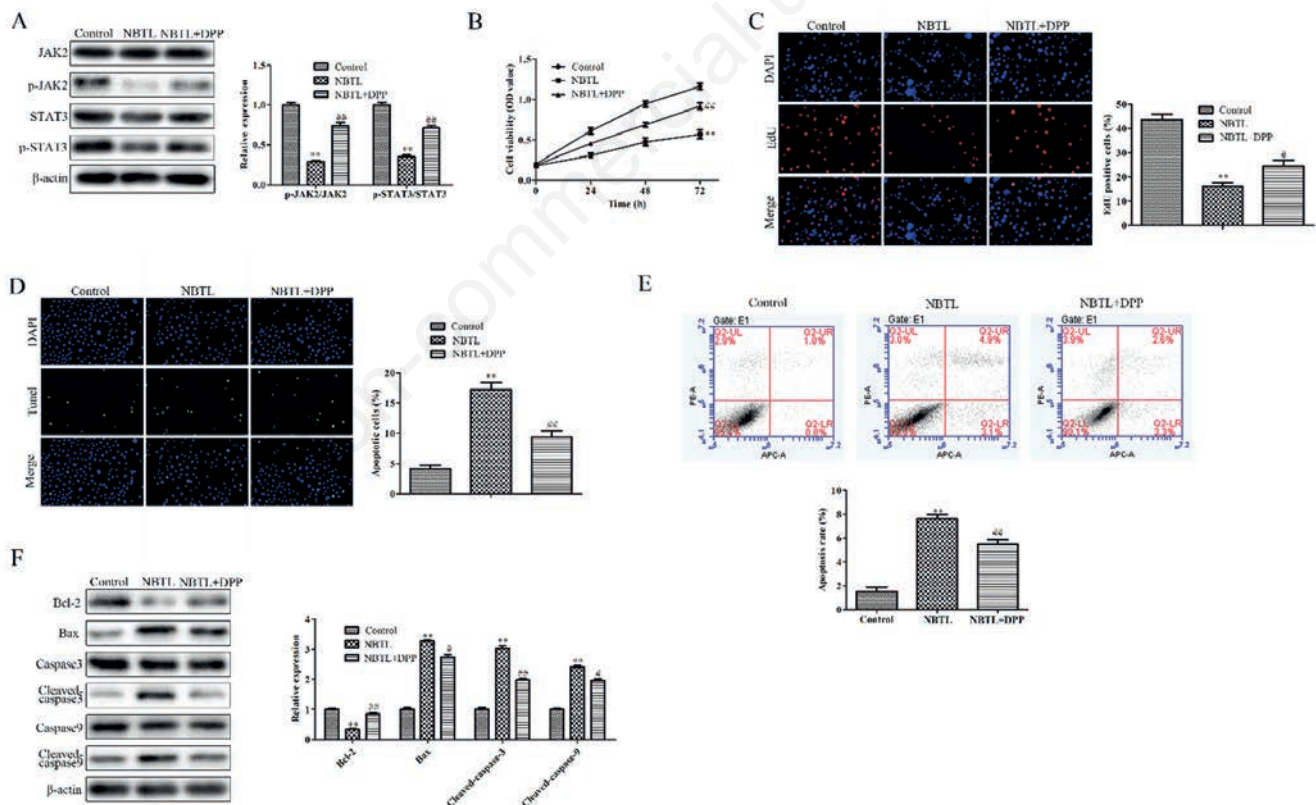


Figure 5. The effect of JAK2/STAT3 pathway on NBTL impacts on cell proliferation and apoptosis of FLS *in vitro*. We treated FLS with both NBTL and DPP (JAK2/STAT3 pathway activator) simultaneously. A) The protein expression levels of JAK2/STAT3 pathway were measured by Western blot. B) The cell viability was determined using CCK8 assay. C) The cell proliferation was determined using EdU staining (400 ×). D) The cell apoptosis was determined using the TUNEL assay (400 ×). E) The cell apoptosis was determined using flow cytometry. F) The protein expression levels of apoptosis-associated proteins, including Bcl-2, Bax, Cleaved caspase-3, Cleaved caspase-9, were measured by Western blot. Data were shown as mean ± SD (n=10 for all groups). **p<0.01 vs Control, #p<0.05, ##p<0.01 vs C.

gy for the treatment of RA.^{36,37} In present study, our *in vitro* experiments results showed that NBTL could significantly induced cell apoptosis and hindered proliferation of FLS isolated from RA rat, which is consistent with the results of animal experiments *in vivo*. It also suggested that NBTL might play its therapeutic effect on rheumatoid arthritis through facilitating apoptosis and restraining proliferation of FLS.

JAK/STAT pathway plays important biological mechanisms, such as cell proliferation and inflammation. JAK/STAT pathway is provoked by receptor binding of cytokines, like IL-6.³⁸ Cells initiate intracellular signal transduction cascade through JAK/STAT pathway stimulated by IL-6, resulting in STAT3 phosphorylation.³⁹ Stimulating signals like IL-6 can activate JAK/STAT signaling pathways, and activation of JAK/STAT pathways lead to promoting downstream inflammatory cytokines expression. Elevated level of IL-6 has a positive regulatory effect on the JAK/STAT pathway activation, and this positive feedback results in continuous and expanded inflammatory response in RA.⁴⁰ JAK2 plays a vital role in many immune responses mediated by cytokine, and JAK2 inhibitors have been proved to have significant clinical effects on RA.⁴¹ STAT3 can be activated by cytokines such as IL-6, and keep activated in RA synovium.⁴² JAK2 and STAT3 are closely associated with RA pathogenesis. Anti-RA drugs usually clarify their mechanism by inhibiting JAK2/STAT3 signaling pathway.²⁸ In present study, our results showed that NBTL could significantly inhibit JAK2/STAT3 pathway, and our *in vitro* experiments further proved that NBTL's effect on FLS cell proliferation and apoptosis was *via* JAK2/STAT3 pathway.

In summary, *in vivo* and *in vitro* experiments carried out in this research all verified that NBTL has therapeutic effects on RA by promoting the apoptosis of FLS and repressing the proliferation of FLS by inhibition of JAK2/STAT3 pathway. In this study, immunology and animal studies are used to support NBTL as a potential drug for RA.

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