Suppressive effects of Wang-Bi Tablet on adjuvant-induced arthritis in rats via NF-κB and STAT3 signaling pathways

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Abstract. Rheumatoid arthritis (RA) severely affects the quality life of patients due to its high association with disability. Traditional Chinese medicines have been reported to exert notable therapeutic effects on RA. The Chinese medicinal prescription Wang-Bi Tablet (WB) has been successfully used to clinically treat RA for many years; however, its pharmacological mechanism of action is largely unclear. In the present study, adjuvant-induced arthritis (AIA) rats were used to evaluate the anti-inflammatory effects of WB and western blotting was used to explore the molecular mechanisms. The experimental results demonstrated that WB treatment significantly reduced arthritis score and hind-paw volume. Furthermore, synovial hyperplasia, inflammatory cell infiltration and joint destruction were ameliorated by WB. The expression levels of the proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor-α and IL-6, were reduced in the joints of WB-treated rats. Western blotting revealed that WB could also inhibit excessive activation of nuclear factor (NF)-κB and Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling pathways. These results indicated that the therapeutic effects of WB on AIA may be accomplished through inhibition of the NF-κB and JAK-STAT3 signaling pathways. These findings provide experimental evidence to support WB as a therapeutic agent for the treatment of patients with RA.

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Abbreviations: RA, rheumatoid arthritis; WB, Wang-Bi Tablet; AIA, adjuvant-induced arthritis; TCM, traditional Chinese medicine; TGP, total glucosides of paeony

Key words: Wang-Bi Tablet, rheumatoid arthritis, NF-κB, JAK-STAT3, cytokines

Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune disease, which is characterized by the persistent recurrence of synovitis, and intra-articular cartilage and bone destruction. Worldwide, 0.5-1% of the population suffers from RA. The prevalence rate of RA in China is 0.32-0.34% of the population, among which the disability rate after 1 year can reach ≤20%, seriously affecting the quality of life of patients (1). RA is generally associated with numerous factors, including genetics, infection, smoking and immune dysfunction (2). Although the pathology and etiology are not yet fully distinct, numerous studies have confirmed that T-cell dysfunction, particularly imbalances in T helper (Th)1/Th2 and Th17/regulatory T cells, results in an increase in proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which have an essential role in RA occurrence and progression (3,4). Nuclear factor (NF)-κB is a transcription factor, which is considered the core of inflammation and immune responses. NF-κB can be activated by IL-1β, IL-6, transforming growth factor-β, TNF-α and other cytokines, and further promotes the transcription of various cytokines, which constitutes the feedback mechanism of NF-κB and cytokines, thus leading to persistent inflammation and joint damage in RA (5).

At present, RA remains one of the most incurable diseases. Biological agents, including TNF-α or IL-1 inhibitors, have garnered attention and are recommended for RA treatment as they are able to halt disease progression (6). However, various unfavorable elements of these drugs, such as severe adverse reactions, potential toxicity and high costs, limit their application (7). Traditional Chinese medicine (TCM) has been reported to regulate the systemic immune response (6). Long-term clinical practice and experience has indicated that TCM may have potential for the development of novel therapeutic agents that not only prevent joint damage, but also have less adverse effects and lower costs. Wang-Bi Tablet (WB) is a product of Liaoning Herbapex Pharmaceutical (Group) Co.,

Ltd. (Benxi, China); the SFDA approval number is Z20044066. It has been used to treat arthritis for several years, and has exhibited a good efficacy and few side effects. WB consists of 16 herbal medicines, including Radix Rehmanniae, Radix Rehmanniae Preparata, Radix aconiti lateralis preparata, Rhizoma Drynariae, Cassia Twig, Radix Dipsaci, Epimedium brevicornu Maxim, Rhizoma Cibotii Preparata, Carthami Flos, Radix Clematidis, Spina Gleditsiae, Radix Angelicae Pubescentis, Radix Saposhnikoviae, Radix Paeoniae Alba, Rhizoma Anemarrhenae and goat bone. It has previously been reported that WB can relieve joint swelling and pain, and improve joint motion (8); however, the pharmacological mechanism of action of WB is largely unclear. The present study aimed to evaluate the anti-inflammatory effects of WB on rat adjuvant-induced arthritis (AIA) and to explore the underlying molecular mechanism.

Materials and methods

Chemicals and reagents. Acetonitrile [high performance liquid chromatography (HPLC) grade] was purchased from Caledon Laboratories, Ltd. (Georgetown, ON, Canada). Timosaponin BII (batch no. 111839-201505), mangiferin (batch no. 111607-200402), naringin (batch no. 110722-201312), hydroxysafflor yellow A (batch no. 111637-201308), paeoniflorin (batch no. 110736-201438), prim-O-glucosylcimifugin (batch no. 111522-201511), 5-O-methylvisammioside (batch no. 111523-201509), icariin (batch no. 110737-200415), protocatechuate (batch no. 110809-200604) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Columbianadin (batch no. MUST-12072906), epimedin A (batch no. MUST-14060312), epimedin B (batch no. MUST-14062312), epimedin C (batch no. MUST-14022312), benzoylmesaconine (batch no. MUST-15012216), benzoylaconitine (batch no. MUST-14052315) and benzoylhypacoitine (batch no. MUST-14032209) were purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Alibiflorin (batch no. 130824) was purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Total glucosides of paeony (TGP) tablets were produced by Ningbo Lihua Pharmaceuticals Co., Ltd. (Ningbo, China). For animal experiments, drugs were suspended in sterilized 0.5% carboxymethylcellulose sodium (CMC-Na). Heat-killed *Mycobacterium tuberculosis* (MT) H37Ra was purchased from Difco (BD Biosciences, Franklin Lakes, NJ, USA). Antibodies against phosphorylated (p)-p65 (ab194926), p(IKK) α/β (ab194528), NF-κB inhibitor α (IκBα) (ab32518), Toll-like receptor 4 (TLR4) (ab13867) and p-signal transducer and activator of transcription 3 (STAT3) (ab76315) were from Abcam (Cambridge, UK). β-actin (A5441) primary antibody was purchased from Sigma-Aldrich (Merck KGaA). Cell Counting Kit-8 (CCK-8) kit was from Beyotime Institute of Biotechnology (Shanghai, China).

Preparation of herbs. WB was prepared by Liaoning Herbapex Pharmaceutical (Group) Co., Ltd. It contains 16 herbal medicines and one animal medicine, including Radix Paeoniae Alba and Rhizoma Anemarrhenae, which were crushed into a fine powder; and Radix Rehmanniae, Radix Rehmanniae Preparata, Rhizoma Drynariae, Rhizoma Cibotii and goat bone, which were decocted with water two times, after which the decoction was mixed with the alcohol precipitate from the decoction of the remaining 10 herbs and was concentrated to acquire a thick paste. A total of 1 g paste is equivalent to 8.92 g crude medicinal herbs.

HPLC analysis. WB (1.0 g) was weighed and subjected to ultrasonic extraction with 25 ml alcohol solution (529 ml ethanol diluted in 1 l water) for 60 min at 25˚C. The extract solution was then filtered through a 0.45-μm filter membrane prior to HPLC analysis. The separation was carried out at 30˚C, with a flow rate of 1.0 ml/min, on an Agilent ZORBAX SB-C18 (880975-902) 1100 HPLC system (4.6x250 mm, 5 μm) (Agilent Technologies, Inc., Santa Clara, CA, USA). The mobile phase consisted of solvent A (100% acetonitrile) and solvent B (water and phosphoric acid, 100:0.1, v/v), and 10 ml sample was injected onto the column. The elution program was optimized and conducted as follows: 0-40 min, linear gradient was increased from 5 to 18% solvent A; 40-60 min, linear gradient was increased from 18 to 25% solvent A; 60-80 min, linear gradient was increased from 25 to 40% solvent A; 80-90 min, linear gradient was increased from 40 to 80% solvent A; and 90-95 min, linear gradient was maintained at 80% solvent A. Monitoring was performed at 235 nm using a VWD G1314A detector (Agilent Technologies, Inc.).

The compounds were analyzed by comparing sample retention times with those of authentic standards, including paeoniflorin, benzoylaconine, epimedin C and icariin. Data analysis of chromatographic fingerprints was performed using a similarity evaluation system. OpenLAB CDS ChemStation software (Edition Revision C. 01. 07; Agilent Technologies, Inc.) was used to evaluate the similarities between different chromatograms and to calculate the correlation coefficient of different patterns.

Preparation of medicated sera. Sprague-Dawley male rats (n=30; age, 6-7; weight, ~200 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were housed in a specific pathogen-free animal room under the following conditions: 50±5% humidity and 23±1˚C ambient temperature. Rats were maintained under a 12-h light/dark cycle, and food and water were provided *ad libitum* during all experiments. The rats were divided into the following groups: Normal group, WB group and TGP group. WB or TGP were suspended in 0.5% CMC-Na solution and administered at a dose of 2.8 g and 0.93 g/kg/day, respectively for 7 days. The dosage was five times the equivalent clinical dose. The rats in the normal group were administered the same volume of 0.5% CMC-Na solution. On day 8, whole blood was obtained from the abdominal aorta after an overnight fast (9). The medicated serum samples were collected by centrifugation of whole blood at 2,000 x g for 20 min at room temperature. Dulbeccos's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to the corresponding serum, and the final concentration of medicated serum in the medium was 10%.

Animals and cells. Sprague-Dawley female rats (n=30; age, 6-7 weeks; weight, 200 g) were purchased from Shanghai

SLAC Laboratory Animal Co., Ltd. The rats were housed in a controlled environment: Temperature, 25±1˚C; humidity, 50±5% under a 12-h light/dark cycle with free access to sterilized food and water. All animal procedures were performed following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA) (10), and the present study was approved by the ethics committee of Experimental Research, Shanghai Medical College, Fudan University (Shanghai, China). Raw264.7 cells, purchased from the cell repository of Chinese Academy of Sciences (Shanghai, China), were cultured in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were treated with medium containing 10% normal serum, or TGP-(1:4 dilution with normal rat serum), WBL-(1:4 dilution with normal rat serum), WBM-(4:6 dilution with normal rat serum) or WBH-(6:4 dilution with normal rat serum) medicated sera for 48 h, followed by CCK-8 assay or western blotting. Cell viability was determined using the CCK-8 assay based on water-soluble tetrazolium salt-8 as described in our previous study (11).

Induction of AIA and WB treatment. Heat-killed MT H37Ra was ground in a roughened mortar until its color changed to white, and mineral oil was added gradually to make a paste. Each female rat was subcutaneously injected at the base of the tail with 0.1 ml MT suspension containing 62.5 μg MT (12). From day 0 after adjuvant injection, the rats were treated by gavage once a day with 0.5% CMC-Na solvent in the model group (n=8), WB (0.56 g/kg/day, clinical equivalent dose) in the WB group (n=7), and TGP (0.186 g/kg/day) in the positive control TGP group (n=7). In addition, rats (n=8) in the normal control group (without MT injection) were treated with an equal volume of 0.5% CMC-Na. After 30 days of treatment, all rats in each group were sacrificed following sodium pentobarbital anesthesia.

Assessment of arthritis severity. Arthritis score was evaluated every 3 days from the onset of arthritis according to the previously described method (13). The highest score for each rat was 16. Hind-limb volume was measured using a volumetric meter. Radiological and histological evaluation was processed as described in our previous study (13).

Hematoxylin and eosin staining. The left hind paw from each rat was fixed in 4% phosphate-buffered formaldehyde for 3 days at room temperature, then decalcified in 5% nitric acid solution for 48 h at room temperature. The metatarsophalangeal joint from the third toe was cut and embedded in paraffin. Longitudinal sections (6 μm) were prepared for routine hematoxylin and eosin staining. After routine deparaffinization, the sections were stained with hematoxylin for 15 min at room temperature, followed by counterstaining with eosin for 1 min. Images were captured using a light microscope (Zeiss GmbH, Jena, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Total RNA was isolated from the paws of rats in each group using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized using a Takara reverse transcriptase kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Finally, fluorescent qPCR experiments (procedure: 95˚C for 2 min, 39 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec; melt curve, 60 to 95˚C, increment 0.5˚C) were conducted using a Bio-Rad iQ5 Real Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Green (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol and results were quantified using the $2^{-\Delta\Delta Cq}$ method (14). The primer sequences used were as follows: TNF-α, forward (F) 5'-ATGGGCTCCCTCTCATCAGT-3', reverse (R) 5'-GCTTGGTGGTTTGCTACGAC-3'; IL-6, F 5'-ATGAACAGCGATGATGCACT-3', R 5'-ACAAACTCCAGGTAGAAACGG-3'; IL-1β, F 5'-GAGCTTCAGGAAGGCAGTGT-3', R 5'-TCACACACTAGCAGGTCGTC-3'; and β-actin, F 5'-ATCTATGAGGGTTACGCGCTCC-3' and R 5'-CAGCTGTGGTGGTGAAGCTG-3'.

For western blotting, rat paw lysates were prepared using Radioimmunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology), and protein concentrations were quantified using the bicinchoninic acid method. Equal amounts of protein (20 μg) were separated in different concentrations of polyacrylamide gel (8-10%), according to the molecular weight of the proteins. Proteins were transferred to polyvinylidene difluoride membranes, which were blocked in 5% milk for 30 min at room temperature. Subsequently, membranes were incubated with various primary antibodies using the dilutions recommended by the manufacturer's protocol, for 24 h at 4˚C. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G secondary antibodies (1:5,000; cat. nos. W4011 and W4021; Promega Corporation, Madison, USA) at room temperature for 1 h and were detected using GeneGnomeXRQ (Syngene, Frederick, MD, USA). The intensity of each lane was semi-quantified using ImageJ 1.48 (National Institutes of Health).

Statistical analysis. Data are presented as the means ± standard error of values from three experiments. Student's t-test was used to compare two groups, and one-way analysis of variance followed by Bonferroni post hoc test was used for multiple comparisons. All statistical analyses were performed using SPSS 13.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Quality control of WB. The HPLC profile of WB contained numerous visible peaks. The compounds were identified by comparing the retention times of the samples with authentic standards. When compared with the standard references, 17 peaks, corresponding to relative retention time and relative peak area of protocatechuate, mangiferin, hydroxysafflor yellow A, timosaponin BII, albiflorin, paeoniflorin, prim-O-glucosylcimifugi, naringin, 4'-O-β-glucopyranosyl-5O-methylvisamminol, benzoylmesaconine, benzoylaconine, benzoylhypacoitine, epimedin A, epimedin B, epimedin C, icariin and columbianadin were identified (Fig. 1A). The

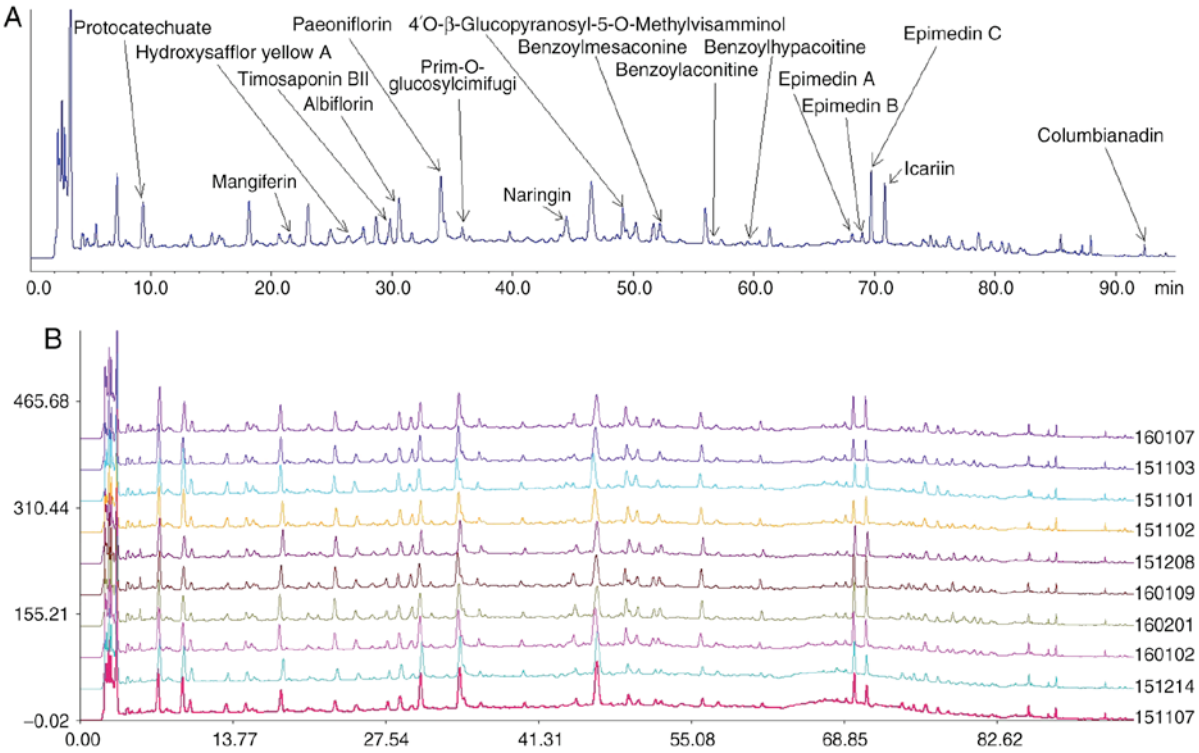

Figure 1. Quality control of WB. (A) High-performance liquid chromatography profile of WB at 235 nm. (B) Chromatogram of 10 batches of WB, used to conduct a similarity evaluation. WB, Wang-Bi Tablet.

similarity indices of 10 batches of WB samples were calculated using a similarity evaluation system (Fig. 1B). The results demonstrated that the samples had good correlation and shared a similar chromatographic pattern with similarity indices at >0.900.

Inhibitory effects of WB on the symptoms of AIA in rats. The onset of AIA occurred ~12 days following immunization. Symptoms, such as swollen red paws and functional joint impairment, were observed and the incidence rate was 100%. The arthritis score and hind-paw volume were typically increased and reached a peak 2 weeks following the onset of arthritis. Treatment with WB or TGP decreased arthritis score and hind-paw volume (Fig. 2A and B). The arthritis score of WB and TGP groups began to decrease at day 10 following drug administration compared with the model group. In addition, between days 10 and 30, arthritis scores were continuously decreased and inflammatory symptoms were obviously improved (Fig. 2A). Hind-paw volumes were measured to evaluate the severity of swelling. Following WB treatment for 16 days, hind-paw volume was significantly decreased compared with the model group (Fig. 2B). A significant inhibition of hind-paw volume was also observed in the TGP-treated group between days 16 and day 30 compared with the model group (Fig. 2B). The inhibitory effects of TGP on arthritis score and hind-paw volume were stronger than WB.

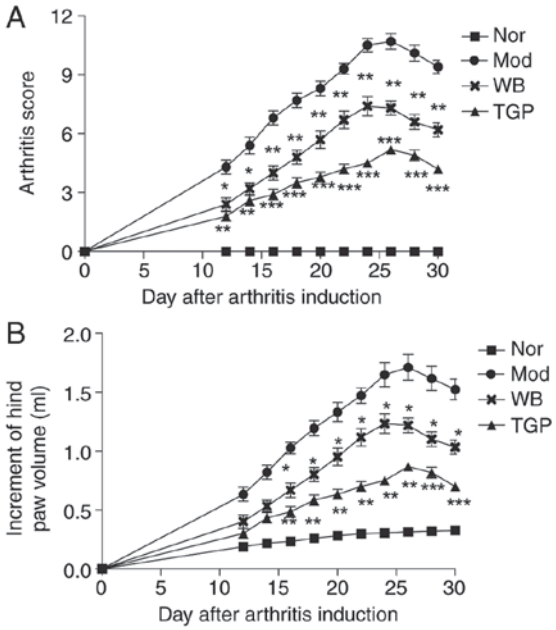

Figure 2. Therapeutic effects of WB treatment on the symptoms of rat adjuvant-induced arthritis. Into the stomachs of Sprague-Dawley female rats, WB (0.56 g/kg/day, n=7) and TGP (0.186 g/kg/day, n=7) were infused from the day of adjuvant injection for 30 days. Nor (n=8) and Mod (n=8) groups were orally treated with 0.5% carboxymethylcellulose sodium. (A) Arthritic score of each group. (B) Hind-paw volume of each group. Data are expressed as the means ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001. vs. the Mod group. Mod, model; Nor, normal; TGP, total glycosides of paeony; WB, Wang-Bi Tablet.

WB suppresses pathological alterations to joints. Histological alterations in the metatarsophalangeal joint of the third toe were evaluated. As shown in Fig. 3A, in the model group, inflammatory cells extensively infiltrated into the joints, and pannus formation, synovial tissue proliferation, cartilage destruction and subchondral bone erosion were detected. Compared with in the model group, the histological lesions of the TGP and

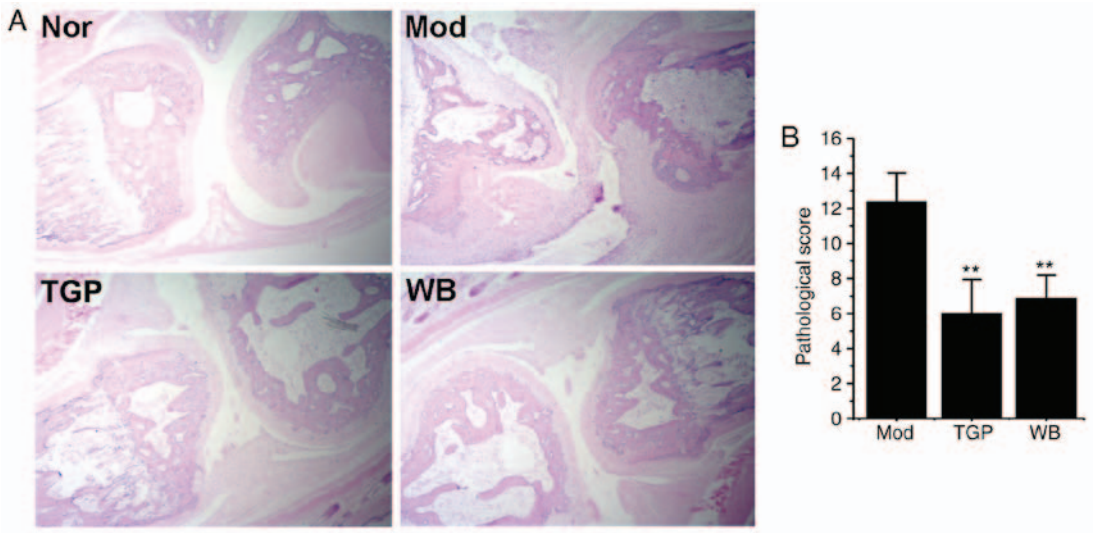

Figure 3. Effects of WB on pathological alterations associated with rat adjuvant-induced arthritis. The stomachs of rats were infused daily with WB or TGP, from the day of adjuvant injection for 30 days. (A) Representative images and (B) pathological scores of arthritic alterations in each group after 30 days of treatment (hematoxylin and eosin staining; magnification, x100). Data are expressed as the means ± standard error of the mean. **P<0.01. Mod, model (n=8); Nor, normal (n=8); TGP, total glycosides of paeony (n=7); WB, Wang-Bi Tablet (n=7).

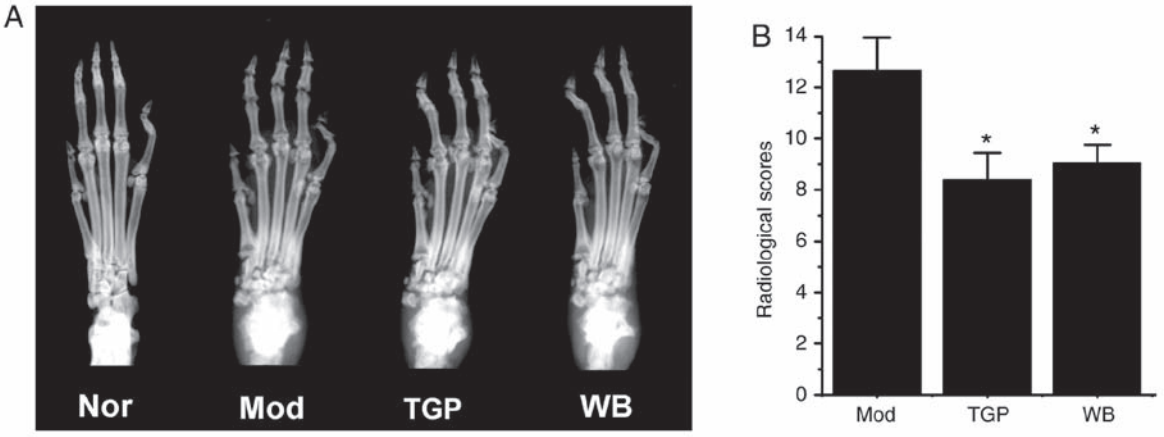

Figure 4. Protective effects of WB on joint damage in a rat model of adjuvant-induced arthritis. The stomachs of rats were infused daily with WB or TGP, from the day of adjuvant injection for 30 days. (A) Left hind paws then underwent radiological inspection and (B) radiological scores were evaluated. Data are expressed as the means ± standard error of the mean. *P<0.05.

WB groups were markedly reduced; in addition, synovial hyperplasia, inflammatory cell infiltration and joint destruction were moderate, and the pathological score was significantly reduced (Fig. 3B, P<0.05). These findings indicated that WB may improve the pathological alterations of AIA.

Protective effects of WB on joint destruction. A total of 30 days from the onset of arthritis, the left hind paws of rats were subjected to radiological examination. As shown in Fig. 4A, the paws of the model group exhibited severe bone resorption, enlarged joint spaces and joint destruction. However, paws from rats in the WB- or TGP-treated groups exhibited reduced joint destruction and increased bone density. The radiological scores of the WB and TGP groups were markedly decreased compared with in the model group (Fig. 4B, P<0.05).

WB suppresses proinflammatory cytokine expression. After 30 days of treatment, the rats were sacrificed and total RNA was extracted from the right hind paws in each group. The mRNA expression levels of proinflammatory cytokines, TNF-α, IL-1β and IL-6, were examined by RT-qPCR. WB treatment significantly decreased the mRNA expression levels of TNF-α, IL-1β and IL-6 in paw tissues compared with in the model group. TGP exhibited a stronger inhibitory effect than WB (Fig. 5).

Inhibitory effects of WB on activation of the STAT3 and NF-κB signaling pathways in AIA rats. It has previously been reported that excessive generation of proinflammatory cytokines is closely associated with activation of the NF-κB signaling pathway (3). In order to further study the molecular pharmacological mechanism underlying the anti-AIA effects of WB, total proteins were extracted from rat paws in each group, and the expression levels of proteins crucial for the NF-κB signaling pathway were detected by western blotting. As shown in Fig. 6, treatment with WB significantly

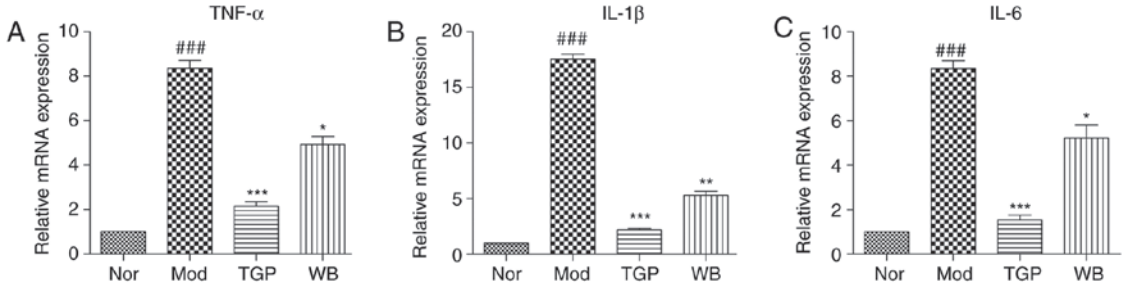

Figure 5. Effects of WB on the mRNA expression levels of proinflammatory cytokines in the paws of adjuvant-induced arthritis rats. The stomachs of rats were infused daily with WB or TGP, from the day of adjuvant injection for 30 days. The mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 were detected. Data are expressed as the means ± standard error of the mean. $^{###}$P<0.001 vs. the Nor group; *P<0.05, **P<0.01, ***P<0.001 vs. the Mod group. IL, interleukin; Mod, model (n=8); Nor, normal (n=8); TGP, total glycosides of paeony (n=7); TNF-α, tumor necrosis factor-α; WB, Wang-Bi Tablet (n=7).

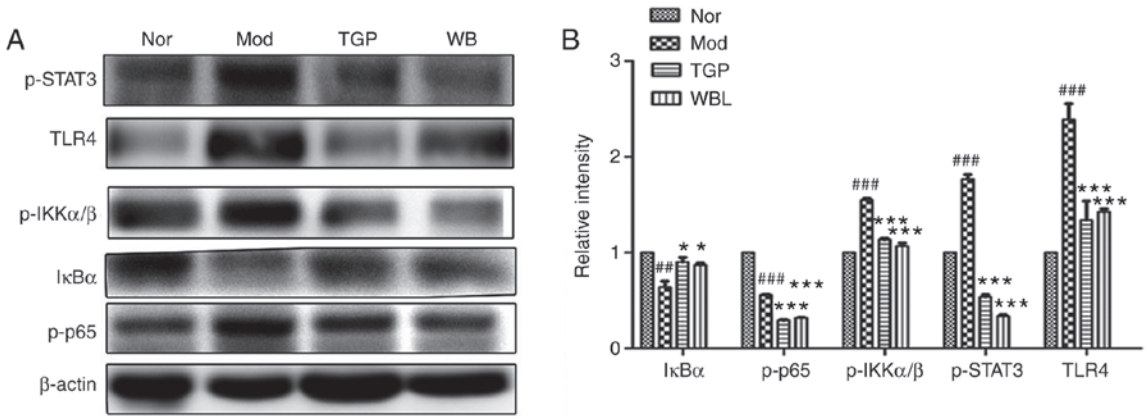

Figure 6. Effects of WB on activation of the STAT3 and NF-κB signaling pathways in the paws of adjuvant-induced arthritis rats. The stomachs of rats were infused daily with WB or TGP, from the day of adjuvant injection for 30 days. (A) Post-treatment, equal amounts of protein from the hind paws in each group were merged into one sample, and immunoblotting was conducted using the indicated antibodies. (B) Densitometric semi-quantification of western blots. Data are expressed as the means ± standard error of the mean (n=7-8 rats). $^{##}$P<0.05, $^{###}$P<0.01 vs. the Nor group; *P<0.05, **P<0.01, ***P<0.001 vs. the Mod group. IκBα, NF-κB inhibitor α; IKK, IκB kinase; Mod, model (n=8); NF-κB, nuclear factor-κB; Nor, normal (n=8); p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; TGP, total glycosides of paeony (n=7); TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; WB, Wang-Bi Tablet (n=7).

decreased the expression levels of p-STAT3, p-IKK and p-p65 compared with in the model group. In addition, TLR4 was reduced and IκBα was increased by WB or TGP treatment (Fig. 6A). Densitometric semi-quantification of western blotting confirmed the effects of WB on activation of the STAT3 and NF-κB signaling pathways (Fig. 6B).

Suppression of LPS-induced STAT3 and NF-κB activation in Raw264.7 cells following treatment with WB-medicated sera. In order to confirm the inhibitory effects of WB on the STAT3 and NF-κB pathways at the cellular level, the present study detected the toxicity of WB-medicated sera on Raw264.7 cells. The results demonstrated that WB-medicated sera exerted no toxic effect on Raw264.7 cells in the tested concentration range (Fig. 7A). WB-medicated sera hindered degradation of IκB and reduced the expression levels of p-STAT3, p-IKK and p-p65 (Fig. 7B). Densitometric semi-quantification of western blotting confirmed the effects of WB-medicated sera on STAT3 and NF-κB activation (Fig. 7C).

Discussion

WB has been applied in the clinical treatment of RA for several years in China, due to its efficacy and few side effects. The HPLC profile of WB confirmed that it contained several main compounds that possess anti-inflammatory effects. Protocatechuate has been reported to possess anti-inflammatory and antioxidant activities via the inhibition of inflammatory cytokines, including TNF-α, IL-1β and IL-6, through the NF-κB pathway (15). Mangiferin has been demonstrated to act as an effective inhibitor of the NF-κB signaling pathway; its ability to regulate various transcription factors, such as NF-κB and nuclear factor (erythroid-derived 2)-like 2, and modulate the expression of numerous proinflammatory signaling intermediates, such as TNF-α, cyclooxygenase 2 (COX-2), contributes to its anti-inflammatory potential (16). Hydroxysafflor-yellow A has been reported to exert inhibitory effects on NF-κB, ultimately suppressing abnormal proliferation of vascular endothelial cells (17). Timosaponin B-II has also been revealed to inhibit proinflammatory cytokine production by attenuating increases in TNF-α, IL-1β and IL-6 (18). In addition, icariin may exert therapeutic effects against osteoporosis; it was reported to be able to synergistically increase osteoblast proliferation, alkaline phosphatase activity and mineralized nodule formation, and to promote bone matrix formation by upregulating bone morphogenetic protein and the Wnt/β-catenin signaling pathway in osteoblasts (19). Icariin may also stimulate

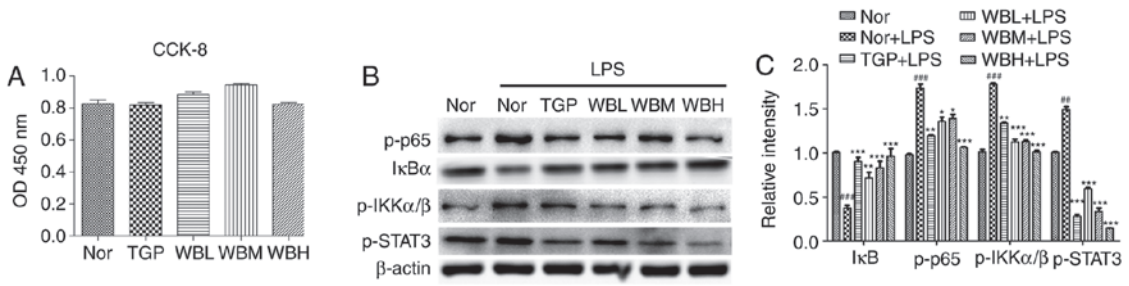

Figure 7. Suppression of LPS-induced STAT3 and NF-κB activation in Raw264.7 macrophages following treatment with WB medicated sera. (A) Raw264.7 cells were treated with medium containing normal serum, or TGP-, WBL-, WBM- or WBH-medicated sera for 48 h, followed by CCK-8 assay. (B) Raw264.7 cells were pretreated, as described in (A), and were then treated with 100 ng/ml LPS for 15 min. Cell lysates were immunoblotted with the indicated antibodies. (C) Densitometric semi-quantification of western blots. Data are expressed as the means ± standard error of the mean. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the Nor group; *P<0.05, **P<0.01, ***P<0.001 vs. the Nor + LPS group. CCK-8, Cell Counting Kit-8; IκBα, NF-κB inhibitor α; IKK, IκB kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; Nor, normal; OD, optical density; p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; TGP, total glycosides of paeony TLR4, Toll-like receptor 4; WB, Wang-Bi Tablet; WBH, high-dose WB; WBL, low-dose WB; WBM, medium-dose WB.

osteogenic differentiation of rat bone marrow stromal stem cells by increasing tafazzin expression (20). Albiflorin and paeoniflorin are often applied in RA treatment due to their anti-inflammatory effects. It has been reported that they may inhibit LPS-induced inducible nitric oxide synthase and COX-2 gene expression, as well as the subsequent production of nitric oxide, prostaglandin E2 (PGE2) and COX-2. Furthermore, they both reduce the production of cytokines (TNF-α and IL-6) induced by LPS in Raw264.7 macrophages (21). Naringin has been revealed to stimulate angiogenesis in the process of bone healing by regulating the vascular endothelial growth factor (VEGF)/VEGF receptor 2 signaling pathway in osteoporosis (22). Prim-O-glucosylcimifugin and 4'-O-β-Glucopyranosyl-5-O-Methylvisamminol both exert inhibitory effects on the proliferation of smooth muscle cells stimulated by TNF-α (23). Benzoylmesaconine is known for its analgesic effects (24). Furthermore, benzoylhypacoitine, benzoylaconitine and benzoylmesaconine have all been reported to possess anti-inflammatory and immunosuppressive effects (25). Epimedin A, epimedin B and epimedin C have been demonstrated to possess potential activity against osteoporosis (26). In addition, columbianadin is an active constituent of Radix Angelicae Pubescentis, which exerts anti-inflammatory and analgesic effects (27). Therefore, the numerous herbal components of WB may exert effects that vary from one another, and even a single component may initiate various actions. Therefore, the overall pharmacology of WB may be achieved through numerous pathways, factors and targets.

NF-κB is an important drug target for the treatment of RA, which regulates the transcription of various inflammatory cytokines, including TNF-α, IL-1β and IL-6 (28). Furthermore, the accumulation of these inflammatory cytokines contributes to further activation of the NF-κB signaling pathway (29). In the resting state, IκBα is contained within the IκBα/p50/p65 inactive complex in the cytoplasm; however, once cells are stimulated by external stimuli, IκBα is rapidly phosphorylated by activation of p-IKKα/β, and p-IκBα is then rapidly degraded. The p50/p65 complex subsequently translocates into the nucleus, rapidly initiating the transcription of NF-κB-associated genes (30-33). Clinical studies have revealed that NF-κB signaling molecules are overactive in the synovial tissues of patients with RA. Therefore, inhibition of the NF-κB signaling pathway is a key point in RA therapy. In order to clarify the pharmacological mechanism underlying the effects of WB on RA, the therapeutic effects of WB were evaluated on AIA rats. The results demonstrated that WB may significantly reduce the progression of AIA in rats. As determined by western blotting, the expression levels of NF-κB signal molecules were detected in rat paws and Raw264.7 cells; the results confirmed that WB may inhibit activation of the NF-κB signaling pathway, thereby inhibiting the progression of inflammation.

Under normal conditions, proinflammatory and anti-inflammatory cytokines are maintained in a dynamic balance. In RA, proinflammatory cytokines are overly transcribed and expressed, thus resulting in destruction of the normal cytokine balance (34-36). Excessive IL-1β and TNF-α can stimulate the production of vascular endothelial cells and induce overexpression of cell adhesion factors. In addition, they stimulate synovial cells and cartilage cells to release collagenase, PGE2 and other inflammatory mediators, which have a synergistic effect in arthritis. IL-6 amplifies the biological effects of cytokines during the inflammatory response. It can induce the production and release of other inflammatory cytokines, including TNF-α and IL-1β, further enhancing inflammatory effects. Therefore, reducing the production of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, has a pivotal role in RA treatment (37). In the present study, the mRNA expression levels of TNF-α, IL-1β IL-6 were detected in AIA; the results confirmed that WB significantly decreased the mRNA expression levels of TNF-α, IL-1β and IL-6, and therefore reduced RA-associated inflammation.

Various inflammatory cytokines and growth factors may activate the Janus kinase (JAK)-STAT3 signaling pathway. Firstly, inflammatory cytokines bind to their corresponding receptors to induce receptor dimerization, after which, phosphorylation of the upstream JAKs, as a result of tyrosine phosphorylation, catalyzes phosphorylation of downstream STAT3. Subsequently, p-STAT3 dissociates from the receptor and forms a dimer that combines with the DNA target sequence in the nucleus, thus participating in specific transcription of downstream genes and completing the whole process of cytokine transduction (38-41). Finally, the concentrations of various inflammatory cytokines are elevated in RA, which are transduced via the JAK-STAT3

signaling pathway. Therefore, blocking the JAK-STAT3 signaling pathway may inhibit the transduction of inflammatory cytokines. The present data indicated that p-STAT3 is decreased in a rat model of AIA in response to WB treatment, thus suggesting that WB may reduce the inflammatory reaction of AIA through suppressing activation of STAT3.

RA is a chronic systemic autoimmune disease, which is associated with chronic inflammation and destructive events, including joint pain and swelling, synovial hyperplasia, pannus formation, joint deformity, and articular cartilage and bone destruction. Articular cartilage and bone destruction poses a particular problem. The present results demonstrated that WB not only had an inhibitory effect on AIA-associated inflammation, but also significantly improved joint pathology and bone destruction. This may be associated with inhibition of the production of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. Although the molecular mechanisms underlying the pathological effects of RA on joints are not completely understood, TNF-α, IL-1β and IL-6 are known to have key roles in the pathological process of RA. It has previously been suggested that an increased response of T cells to pathogenic antigens may be an underlying reason for the formation of RA via cell contact and the production of a large number of proinflammatory cytokines (4). Stimulated T cells can activate monocytes, macrophages and synovial cells to further produce substantial proinflammatory cytokines, mainly TNF-α, IL-1β and IL-6. Once bound to their receptors, these soluble molecules trigger signal transduction, resulting in matrix metalloproteinase production, periarticular connective tissue injury and joint damage. In addition, TNF-α and IL-1β also induce the production of receptor activator of NF-κB (RANK) in osteoblasts, which mediates osteoclast differentiation and directly leads to joint bone resorption and destruction (39).

In conclusion, the present study examined the effects and mechanism of WB on AIA rats and in a Raw264.7 cell model. The results demonstrated that WB may not only inhibit inflammation, but may also prevent joint damage. The inhibitory effects of WB on AIA might be achieved by inhibiting activation of the NF-κB and STAT3 signaling pathways.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XS and GY conceived and designed the experiments. Y-YG, L-XL, W-LB and H-DL performed the experiments. Y-YG, YZ and DX analyzed the data. Y-YG, YZ, GY and XS wrote the paper.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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