

## Original Research Article

# Effect of Wu-Wei-Gui-Shao decoction on complete Freund's adjuvant-induced arthritis rats

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### Abstract

**Purpose:** To investigate the anti-rheumatic potential of Wu-Wei-Gui-Shao decoction (WGD) and its possible mechanism of action.

**Methods:** Adjuvant arthritis (AA) rats were established using complete Freund's adjuvant. The rats were then given different doses of WGD (100, 200, and 400 mg/kg, for 28 days). The anti-arthritic effects of WGD were evaluated. Furthermore, the in vitro anti-arthritic effects of WGD and its related mechanisms were also determined in MHTA cells.

**Results:** WGD (100 - 400 mg/kg) exhibited significant anti-rheumatic properties after 28 days of treatment, inhibiting paw edema in AA rats, reducing arthritis score and thymus and spleen index, and inhibiting the tumor necrosis factor (TNF)- $\alpha$  and the interleukin (IL)-6. In addition, the results of in vitro cell experiments also confirmed that WGD reduced the release of cytokines, as well as mRNA levels of matrix metalloproteinase (MMP) -2, -3, and -9.

**Conclusion:** These findings suggest that WGD can be further developed as a traditional Chinese medicine to treat rheumatic arthritis.

**Keywords:** Wu-Wei-Gui-Shao decoction, Complete Freund's adjuvant, Arthritis, Molecular mechanism, Traditional Chinese Medicine

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## INTRODUCTION

Rheumatic arthritis (RA) is a common acute or chronic connective tissue inflammation, mainly manifested by floating sores, and red, painful joints and muscles, with a wide incidence, long course of disease, high deformity and disability [1]. The results of epidemiological analysis showed that RA occurs worldwide, with an average incidence of up to 0.5 - 1 % [2]. In addition, RA could occur at any age, and the

incidence in women is 2 - 3 times higher than that in men.

Presently, the etiology of RA is unclear, and no specific treatment exists for RA [3,4]. The commonly used therapeutic drugs include NSAIDs, glucocorticoids, and biological agents [5-8]. However, taking these drugs has many side effects, toxicity and other problems, such as infection risk, hepatorenal toxicity, bone marrow suppression, and gastrointestinal reactions [9-

12], and will bring heavy economic burden to patients. Therefore, it is very important to identify more affordable RA drugs with fewer side effects.

*Wu-Wei-Gui-Shao decoction* (WGD) comprises of five traditional Chinese medicines (TCMs), Cassia twig, peony, *Achyranthes*, *Atractylodes* and Licorice (Table 1). Clinically, it is used to treat conditions such as RA, sciatica, cervical spondylosis, and peri-arthritis of the shoulder. However, thus far, no report has investigated the mechanism of WGD in the treatment of RA. Therefore, this study explored the therapeutic effect of WGD on AA model rats and an arthritis cell model, as well as to uncover its possible pharmacological mechanism, which will be of great reference value for the clinical use of WGD to cure RA.

## EXPERIMENTAL

### Herbal preparation and extraction

All the five herbal medicines listed in Table 1 were obtained from the Pharmacy for Chinese medicine of the Affiliated Qingdao Hiser Hospital of Qingdao University. Decoction of the above five herbs was performed by adding eight volumes of water for each decoction for 1h by three times. The decoction was decompressed and evaporated to dryness, and the residue was called WGD extract.

### Animals

Sixty Wistar rats, (aged  $7 \pm 1$  weeks, weighing  $170 \pm 10$  g), were obtained from the Animal Center of Qingdao University. Before the animal experiments, the rats were adaptive fed for a week, and were allowed free access to food and water. At the same time, they were maintained in a light-dark alternate environment for 12 h, with a relative humidity is 40 – 70 %, and at a room temperature of  $25 \pm 2^\circ\text{C}$ . The animal study was approved by the committee of the Affiliated Qingdao Hiser Hospital of Qingdao University and Qingdao Hospital of Traditional Chinese Medicine (approval no.20180911A), and conducted in accordance with international animal studies guidelines [13].

**Table 1:** Composition of WGD

Herbal name	Medicinal used part	Amount (g)
<i>Cinnamomum cassia</i> Presl.	Twigs	15
<i>Paeonia lactiflora</i> Pall	Roots	15
<i>Achyranthes bidentata</i> Blume.	Roots	10
<i>Atractylodes macrocephala</i> Koidz	Roots	15
<i>Glycyrrhiza uralensis</i> Fisch.	Roots	10

### Cell culture

MH7A cells were cultured in RPMI - 1640 complete cell culture medium containing 10 % fetal bovine serum (FBS) at  $37^\circ\text{C}$  (5 %  $\text{CO}_2$ ).

### Establishment of the AA model, and intervention treatment

Before modeling, the animals were divided into six groups randomly: normal group, adjuvant arthritis (AA) model group, positive group, WGD treatment group (100, 200, and 400 mg/kg), and a group of eight animals. Following the method introduced by Perera *et al* [14], the AA rat model was established by injecting complete Freund's adjuvant into the feet.

Briefly, *Bacillus Calmette-Guerin* was added to the mixture of liquid paraffin (10 mg/mL) and lanolin to prepare complete Freund's adjuvant (CFA). All the rats in the other groups received subcutaneous injection of 0.1 mL of CFA into the left toe, except the normal rats. The intervention was started on the first day after immunization, and methotrexate (MTX) (3 mg/kg) was administered to the positive group by intragastric administration (i.g.) twice a week. The rats in the WGD group were administered different doses of WGD (i.g.; 100, 200, and 400 mg/kg). Normal and model rats were given the same amount of normal saline for 28 consecutive days.

### Determination of toe swelling index and arthritis score

Arthritis index: the posterior foot volume of each rat was recorded with electronic water plethysmometer (TECHMAN Co., Chengdu, China) before modeling, and then measured every 3 days after modeling. In addition, to observe the symptoms and severity of arthritis in the rats, they were evaluated every 3 days from day 1 to day 28 after modeling. The paw volumes were detected and classified based on the erythema severity and swelling of the paw according to a 5-point rating scale: 0 points, no signs of change; 1-points, red and swelling in the ankle/wrist;

2-points, red and swelling in the ankle plus tarsals of the hind paw and/or wrist plus carpals of the forepaw; 3-points, extension to the metatarsals or metacarpals; 4-points, severe red and swelling involving the entire hind or fore paw [15].

#### Assessment of spleen and thymus index

At the end of the experiment, the rats were anesthetized (pentobarbital sodium, 40 mg/kg, i.p.), and blood was taken from the abdominal aorta. Finally, the thymus and spleen were collected, the wet weight volume was recorded, and the thymus and spleen indices (D) were calculated as in Eq 1 [16].

$$D = (Ww/Aw) \dots\dots\dots (1)$$

where Ww and Aw are the wet weight of the (spleen or kidney) and animal weight, respectively.

#### Evaluation of serum proinflammatory cytokines

Blood was coagulated at 25 °C for 1h and stored at -20 °C. Before the analysis, the serum was separated by centrifugation. The level of proinflammatory cytokines in the serum were measured by commercial ELISA kits using the standard protocol described in manufacturer's instructions.

#### Cell viability assay

CCK-8 method was used to study the effect of WGD on cell viability following the manufacturer's protocol. MH7A cells were cultured in 96-well plates ( $5 \times 10^3$  cells/well), and then were co-incubated with WGD (20 – 300 µg/mL) for 24 h. Next, the CCK-8 reagent was added. Later, the absorbance values were read at 450 nm to calculate the cell inhibition rate for different concentrations of WGD.

#### Effect of WGD on levels of inflammatory cytokines

Inflammatory cytokines in the cells were determined by ELISA. MH7A cells were cultured into 6-well plates ( $2 \times 10^5$  cells/well), and then were treated for 12 h with TNF- $\alpha$  (10 ng/mL). Thereafter, incubation with WGD (40 - 80 µg/mL) was given for 24 h. Next, the cell supernatant was collected, and the ELISA kits were used to determine the inflammatory cytokines (IL-1 $\beta$ , -6, -8, -10 and -17A).

#### mRNA expressions of MMPs

The effect of WGD on the mRNA levels of MMP-2, -3, -9 in MH7A cells was detected by RT-PCR. MH7A cells ( $2 \times 10^5$  cells/wells) were cultured in 6-well plates and incubated for 24 h with WGD (40, 60 and 80 µg/mL) and TNF- $\alpha$  (10 ng/mL). Next, total RNA of the cells was extracted by the RNAiso kit (Legumes Biotech. Co., Dalian, China), and PrimeScript™ RT kit (Beans Biol Tech. Co., Tokyo, Japan) to synthesize cDNA. cDNA was amplified according to the manufacturer's instructions of the SYBR Green RT-PCR reaction kit, and the amplification conditions were described as above [17]. The primers used are shown in Table 2. The mRNA levels of MMP-2, -3, and -9 and  $\beta$ -actin in each sample were determined by relative quantitative analysis of  $2^{-\Delta\Delta CT}$  [18]. All the samples are analyzed in triplicate.

**Table 2:** Primers used for reverse transcription-quantitative polymerase chain reaction analysis

Gene name	Primer sequence (5'-3')
MMP-2	Forward: TGGCAAGTACGGCTTCTGTC Reverse: TTCTTGTCGCGGTCTAGTC
MMP-3	Forward: CAGGCTTCCCAAGCAAATA Reverse: TTGCATTTGGGTCAAACCTCC
MMP-9	Forward: TGCGCTACCACCTCGAACTT Reverse: GATGCCATTGACGTCGTCTCT
$\beta$ -actin	Forward: GGGAAATCGTGCGTGACATCAAAG Reverse: CATACCCAAGAAGGAAGGCTGGAA

#### Statistical analysis

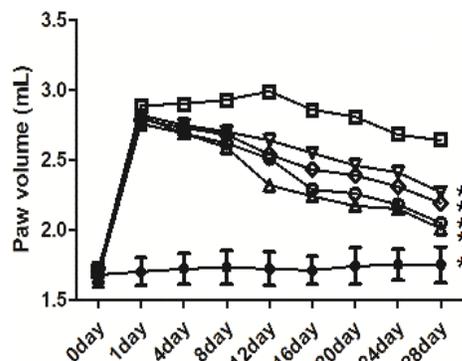
The data were expressed as mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was applied to analyze the means between two groups using the IBM SPSS 19.0.  $P < 0.05$  was recognized as statistical significance.

## RESULTS

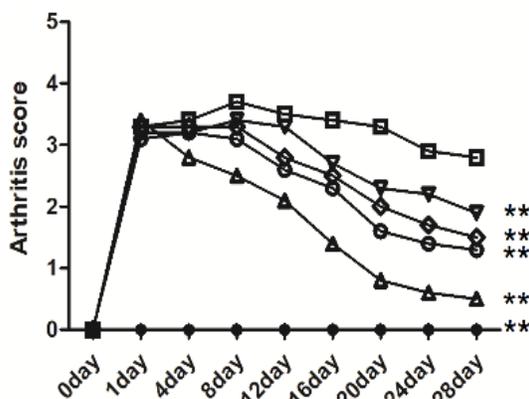
#### WGD reduces paw volume and arthritis score of AA rats

Compared with control rats, WGD (100 - 400 mg/kg) could significantly reduce the paw volume of the AA rats ( $p < 0.01$ ). In addition, the high doses showed the same effect as that of the positive group. This finding confirmed that the paw volume of AA rats decreased after WGD treatment, and WGD could significantly improve the symptoms of the AA mice. Similarly, we recorded the arthritis score of AA rats to assess the therapeutic efficacy of WGD. WGD (100 - mg/kg) reduced the arthritis score of AA rats

compare with control group ( $p < 0.01$ ), and it has the does manage. Besides, WGD was less effective in reducing the arthritis score than the positive-control rats (Figure 2).



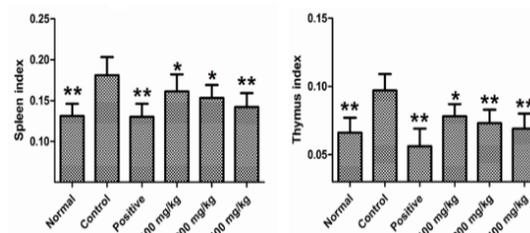
**Figure 1:** Effect of WGD on paw volume of AA rats. Data are presented as mean  $\pm$  SD ( $n = 10$ ). MTX used as the positive drugs; \* $p < 0.05$ , \*\* $p < 0.01$ , versus control. **Key:** ● Normal, □ Control, ▲ Positive, ▽ 100mg/kg, ◆ 200mg/kg, ○ 400mg/kg



**Figure 2:** Effects of WGD on arthritis score of AA rats. Data are presented as mean  $\pm$  SD ( $n = 10$ ). MTX used as the positive drugs, \* $p < 0.05$ , \*\* $p < 0.01$ , versus control. **Key:** ● Normal, □ Control, ▲ Positive, ▽ 100mg/kg, ◆ 200mg/kg, ○ 400mg/kg

### WGD reduces spleen and thymus indices in AA rats

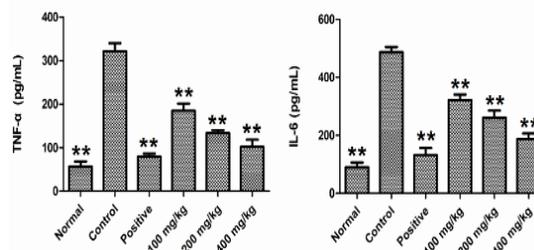
Figure 4 shows the effect of WGD on immune organ index in AA rats. The spleen and thymus indices of control AA rats were significantly higher than those of the normal rats ( $p < 0.01$ ). Although the effect of WGD was not as significant as that of MTX, the thymus and spleen indices of the rats in WG were significantly lower than those of AA mice ( $p < 0.01$ ). Thus, WGD could regulate the immune function of AA rats (Figure 3).



**Figure 3:** Effects of WGD on spleen index and thymus index of AA rats. Data are presented as mean  $\pm$  SD ( $n = 10$ ). MTX is used as the positive drugs; \* $p < 0.05$ , \*\* $p < 0.01$ , versus control

### WGD reduces levels of inflammatory cytokines in AA rats

AA rats have higher levels of TNF- $\alpha$  and IL-6 ( $p < 0.01$ ) than normal rats. After treatment with MTX (3 mg/kg), the TNF- $\alpha$  and IL-6 were significantly decreased ( $p < 0.01$ ). Similar to MTX treatment, WGD treatment (100, 200, and 400 mg/kg) also reduced the TNF- $\alpha$  and IL-6 in the serum ( $p < 0.01$ ), confirming that WGD exerts certain anti-inflammatory effects on AA rats (Figure 4).



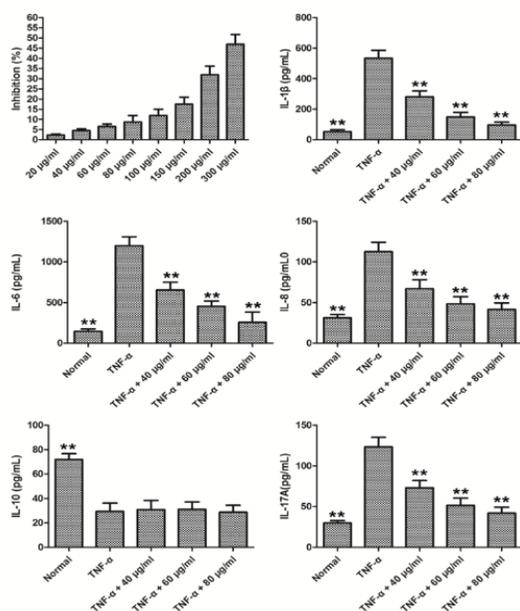
**Figure 4:** Effect of WGD on TNF- $\alpha$  and IL-6 on spleen index and thymus index of AA rats. Data are presented as mean  $\pm$  SD ( $n = 10$ ). MTX were used as the positive drugs; \* $p < 0.05$ , \*\* $p < 0.01$ , versus control

### Effect of WGD on MH7A cell viability

The effect of WGD on MH7A cell viability was shown in Figure 5, WGD showed no obvious inhibitory potential on MH7A cells at 150  $\mu$ g/mL (the inhibition ratio was lower than 20 %); thus we selected the concentrations of 40, 60 and 80  $\mu$ g/mL to carry out further experiments.

### Effect of WGD on the cytokine levels in MH7A cells

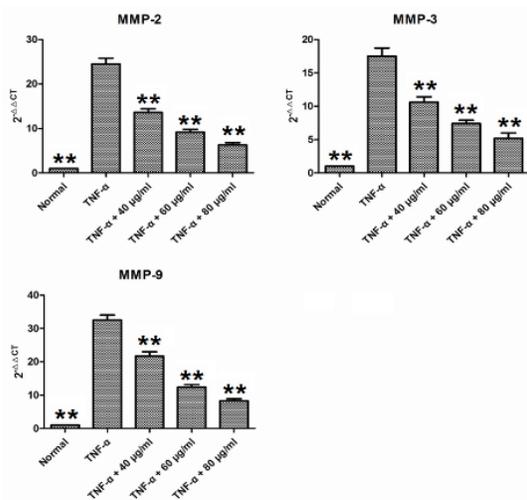
The levels of inflammatory cytokines (IL-1 $\beta$ , -6, -8, -10 and -17A) in TNF- $\alpha$ -induced MH7A cells were determined by ELISA. In addition to IL-10, the other inflammatory cytokines were decreased ( $p < 0.01$ , versus TNF- $\alpha$  group) by WGD treatment (40, 60, 80  $\mu$ g/mL) (Figure 5).



**Figure 5:** Inhibitory effect of WGD on levels of IL-1 $\beta$ , IL-6, IL-8, IL-10 and IL-17A in TNF- $\alpha$  stimulated MH7A cells. Data are presented as mean  $\pm$  SD (n = 4), \*\*p < 0.01, versus control (TNF- $\alpha$  group)

#### Effects of WGD on the mRNA of MMP-2,-3, and -9 in TNF- $\alpha$ -induced MH7A cells

RT-qPCR analysis was applied to determine the mRNA levels of MMP-2, -3, and -9 of TNF- $\alpha$ -induced MH7A cells, and the expression levels of mRNA were significantly increase compare with the normal group. After 24 hs' WGD treatment, the mRNA levels of MMP-2,-3,-9 were decreased (P < 0.01, versus TNF- $\alpha$  group; Figure 6).



**Figure 6:** Inhibitory effects of WGD on mRNA expressions of MMP-2, -3, -9 in TNF- $\alpha$  stimulated MH7A cells. Data are presented as mean  $\pm$  SD (n = 4), \*\*p < 0.01, versus control (TNF- $\alpha$  group)

## DISCUSSION

*Wu-Wei-Gui-Shao* decoction (WGD) is a traditional Chinese medicine decoction for RA, which has shown good efficacy in clinical treatment, but its therapeutic mechanism is still unknown. Adjuvant arthritis (AA) rat model has clinical and pathological characteristics similar to human RA, is a commonly applied animal models in RA research. In this study, the AA rat model was successfully established, and WGD was proven to have a therapeutic effect on the AA rat model for the first time by reducing the degree of toe swelling, arthritis score and immune organ index. In addition, it found that the TNF- $\alpha$  and IL-6 in the serum were reduced by WGD, suggesting that WGD can be used as a natural alternative drug for RA.

Through literature review, it found that excessive proliferation of synovial cells is a basic pathological manifestation of RA patients [19]. Excessive proliferation of synovial fibroblasts thickens the synovial membrane, gradually encroaching on the adjacent cartilage, blocking the contact between cartilage and synovial fluid, affecting its nutrient supply. At the same time, certain released secretions can erode articular cartilage, eventually leading to joint destruction and loss of joint function [20].

Therefore, inhibiting the proliferation of MH7A cells and reducing the release of inflammatory cytokines can provide an effective method for RA treatment. The results showed that WGD significantly inhibited the growth of TNF- $\alpha$  - induce MH7A cells *in vitro* and reduced the interleukin (IL)-1 $\beta$ , -6, -8 and -17A. IL-10 is a cytokine that negatively controls RA progression and plays a strong role in protecting bone and joint and improving RA symptoms [21-23]. *In vitro* experiments, it found that after WGD treatment, the IL-10 level of MH7A cells was significantly increased. Besides, the mRNA of MMP-2, -3, -9 were lower than those of the TNF- $\alpha$  group. These effects play an important role in alleviating synovial hyperplasia, inflammation and joint destruction.

## CONCLUSION

WGD improves the symptoms of arthritis in AA rats *in vivo* and also significantly inhibits MH7A cell growth, while reducing inflammatory cytokines and mRNA expression. Thus, WGD may exert a significant therapeutic effect on RA in humans possibly by reducing the release of inflammatory factors and inhibiting the

proliferation of synovial fibroblasts and the expression of related genes.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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