

Original Research

# A Study on the Pharmacological Effects and Mechanism of Rhodojaponin III in Rheumatoid Arthritis

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## Abstract

**Background:** Rheumatoid arthritis (RA) is a chronic inflammatory disease with a high rate of disability accompanied by various complications. The pathogenesis of RA is complex with multiple targets and links. This study aims to investigate pharmacological effects and mechanism of Rhodojaponin III in RA. **Methods:** The bovine type II collagen-induced arthritis (CIA) rat model and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced human umbilical vein endothelial cells (HUVECs) model were constructed. Different concentrations of Rhodojaponin III were utilized for intervention. The progression of CIA was assessed by the arthritis index (AI). Pathological changes in knee joints and synovium were observed. The expressions of angiogenesis-related cytokines were detected. The proliferation, migration, invasion, and angiogenesis of HUVECs were detected. The levels of pro-inflammatory cytokines were determined. The expressions of nuclear factor kappa B-inducing kinase (NIK) pathway-related proteins were analyzed. The binding of Rhodojaponin III to NIK was simulated by molecular docking. **Results:** Rhodojaponin III suppressed cartilage damage and bone erosion in the knee joints. Rhodojaponin III inhibited expressions of platelet endothelial cell adhesion molecule-1 (CD31) and vascular endothelial cell growth factor (VEGF) to decrease vascular density. Rhodojaponin III suppressed the proliferation, migration, invasion, and angiogenesis of HUVECs, and decreased the levels of interleukin (IL)-6, IL-1 $\beta$ , and TNF- $\alpha$ . Molecular docking showed that Rhodojaponin III could spontaneously bind to NIK. Rhodojaponin III decreased the expression of NIK, p52, and C-X-C motif chemokine ligand 12 (CXCL12) and the phosphorylation level of I $\kappa$ B kinase- $\alpha$  (IKK $\alpha$ ) in the synovium of CIA rats and TNF- $\alpha$ -induced HUVECs. NIK overexpression reversed the inhibitory effect of Rhodojaponin III on activation of the NIK/NF- $\kappa$ B pathway, migration, invasion, and angiogenesis of HUVECs, and the secretion of pro-inflammatory cytokines. **Conclusions:** Rhodojaponin III affected the angiogenesis and inflammation of CIA rats and TNF- $\alpha$ -induced HUVECs by regulating the NIK/IKK $\alpha$ /CXCL12 pathway. These findings suggest that Rhodojaponin III has potential as a therapeutic agent for RA. Further studies are needed to explore its precise mechanism of action and evaluate its clinical efficacy.

**Keywords:** rheumatoid arthritis; Rhodojaponin III; nuclear factor kappa B-inducing kinase; angiogenesis; inflammation

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with a high rate of disability and is accompanied by a variety of complications [1,2]. The pathogenesis of RA is complex, with multiple targets and links, and has not been fully elucidated [3,4]. The main pathological feature of RA is synovial pannus formation caused by angiogenesis associated with inflammation, leading to cartilage and bone destruction [5]. The inflammatory state in RA is maintained by enhanced angiogenesis that delivers inflammatory cells and oxygen to the synovium [6]. Therefore, inhibition of angiogenesis and inflammation may be important targets for RA therapy [7].

The nuclear factor kappa B (NF- $\kappa$ B) is an important transcription factor in the inflammatory response, immune regulation, and cell apoptosis. It has been found that the NF- $\kappa$ B pathway is associated with the occurrence and progression of RA [8]. NF- $\kappa$ B p65 gene silencing can in-

hibit the proliferation of synovial cells in RA [9]. In addition, blocking the NF- $\kappa$ B pathway is an effective strategy to control the inflammatory response of RA [10]. For example, a serine/threonine kinase inhibitor (fasudil) inhibited interleukin-1 $\beta$ -induced NF- $\kappa$ B activation, which mitigated RA to some extent [11].

NF- $\kappa$ B-inducing kinase (NIK), as an important factor in the NF- $\kappa$ B pathway, is highly expressed in synovial endothelial cells of RA patients. It also promotes the generation of pathogenic blood vessels and synovial inflammation by inducing C-X-C motif chemokine ligand 12 (CXCL12) [12,13]. Studies have reported that NIK<sup>-/-</sup> mice are resistant to antigen-induced arthritis caused by T-cell responses [14,15]. NIK knockdown has significantly reduced the activation of synovia-induced endothelial inflammation in RA patients [16]. In RA patients, the I $\kappa$ B kinase (IKK) is highly expressed in fibroblast-like synovial cells (FLSs) [17]. It has been found that the CXCL12/C-X-C motif chemokine



receptor 4 (CXCR4) axis participates in vascular remodeling and formation in the joints of RA patients [18]. After the use of the CXCL12 blocker (AMD3100), vascular remodeling was impaired, the morphology of new endothelial cells was abnormal, and the growth of capillaries was inhibited [19]. However, the role of the NIK/IKK $\alpha$ /CXCL12 pathway in the treatment of RA has not been reported.

Traditional Chinese medicine (TCM) has many advantages, such as being multi-component, multi-target, multi-pathway, and having less toxic side effects, so it has become one of the hotspots in the development of new RA drugs [20,21]. Several plants from the *Cleome* genus have been found to have strong anti-arthritis activity. The presence of secondary bioactive metabolites such as flavonoids, glycoside triterpenes and tannins may be responsible for the antiarthritic activity. The antiarthritic mechanism of action of these active ingredients mainly involves preventing the release and destruction of lysosomes to inhibit the spread of inflammatory responses [22]. Studies have shown that diterpenoids extracted from the fruits of *Rhododendron molle* G. Don can inhibit the abnormal proliferation of T lymphocytes and B lymphocytes, and reduce the levels of pro-inflammatory cytokines such as IL-6, IL-1 $\beta$ , and tumor necrosis factor-alpha (TNF- $\alpha$ ), which has the potential to be used in RA therapy [23]. In addition, Rhodojaponin II has been found to inhibit TNF- $\alpha$ -induced inflammatory responses in FLSs by blocking the protein kinase B (Akt), NF- $\kappa$ B, and toll-like receptor 4/myeloid differentiation factor 88 (TLR4/MyD88) pathways [24]. Rhodojaponin III, as one of the diterpenoids, has many pharmacological activities, such as anti-inflammatory [25] and analgesic [26], and has been widely studied. However, the specific mechanism of Rhodojaponin III on RA remains unclear.

Therefore, the aim of this study was to investigate the therapeutic effect and mechanism of Rhodojaponin III on RA. To achieve this, a bovine type II collagen-induced arthritis (CIA) rat model and a TNF- $\alpha$ -induced human umbilical vein endothelial cells (HUVECs) model were constructed. The NIK/IKK $\alpha$ /CXCL12 pathway was selected as the focus of investigation. This study will provide a theoretical basis for the prevention and treatment of RA by analyzing the effects of Rhodojaponin III on this pathway.

## 2. Materials and Methods

### 2.1 Construction of CIA Rat Model and Intervention

A total of 48 male Wistar rats (6 weeks, 170–200 g) were bought from Changsha Tianqin Biotechnology Co., Ltd. (Changsha, China). Rats were housed under standard laboratory conditions, with a temperature of  $25 \pm 2$  °C, humidity of  $50 \pm 5\%$ , and a 12-hour light/dark cycle. They were raised in separate cages with 4 in each one, and had free access to food and water for one week of adaptive feeding before experiments. 2.0 mg/mL bovine type II collagen (20022, Chondrex, Woodinville, WA, USA) that dissolved in acetic acid solution was mixed with complete Fre-

und's adjuvant (7001, Chondrex, Woodinville, WA, USA) in the ice bath at the volume of 1:1, repeated suction by syringe, fully emulsified, to make bovine type II collagen emulsion. The emulsion was injected subcutaneously into the tail (300  $\mu$ L) of rats. 7 days later, the rats were inoculated with bovine type II collagen emulsion (300  $\mu$ L) for a second time [27]. Normal rats were injected with normal saline. Groups included Sham, CIA, TWG, Rhodojaponin III Lo, Rhodojaponin III Mi, and Rhodojaponin III Hi, with 8 rats in each group. Rats in the TWG group were given intragastric administration with Tripterygium wilfordii Glycosides (TWG, 50 mg/kg) (Z33020422, Dnd Pharmaceutical Co., Ltd., Shaoxing, China) once a day. Rats in the Rhodojaponin III Lo, Rhodojaponin III Mi, and Rhodojaponin III Hi groups were separately given intragastric administration with Rhodojaponin III (0.06 mg/kg, 0.12 mg/kg, 0.24 mg/kg) (26342-66-5, yuanye Bio-Technology Co., Ltd., Shanghai, China) once a day [23]. Rats in Sham and CIA groups were intragastrically given normal saline. After 4 weeks, all rats were anesthetized to obtain the whole blood by abdominal aorta puncture. The rats were sacrificed by the cervical dislocation method to obtain knee joints and synovium for subsequent experiments. All animal experiments followed the ARRIVE guidelines. This study has been approved by the Experimental Animal Ethics Committee of Hunan University of Chinese Medicine (No. LL2022061401).

### 2.2 Arthritis Index (AI) Score

The arthritis index (AI) was evaluated according to the swelling degree of joints every 4 days during the experiment [28]. AI was utilized as the criterion to judge the successful construction of the CIA rat model. When  $AI \geq 4$ , the CIA rat model was successfully constructed. AI scoring rule: 0, no swelling; 1, slight swelling of toe joints; 2, swelling of phalangeal joints and toe joints; 3, Paw swelling below the ankle joint; 4, Swelling of all feet, including the ankle joints. Since the incidence of forepaw inflammation was very low, and the joints of the hind feet were more prone to severe swelling, we calculated the AI score of the two hind feet of rats (total scores: 8).

### 2.3 Cell Culture

HUVECs (AW-CNH488, Abiowell, Changsha, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (10099141, Gibco, Carlsbad, CA, USA) and 1% Penicillin/Streptomycin (SV30010, Beyotime, Shanghai, China) with 5% CO<sub>2</sub> at 37 °C. HUVECs can be used in experiments when the confluence reached 70–80%. The oe-NC plasmids or oe-NIK plasmids (HonorGene, Changsha, China) were transfected separately into HUVECs with Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA). The cells used in the experiment were validated and were free from bacteria, fungi, and mycoplasma infections.

In addition, the expression of von Willebrand factor (VWF) in HUVECs was evaluated using immunofluorescence (IF) staining, as shown in the **Supplementary Figure**.

#### 2.4 Cell Grouping and Treatment

Experiment 1 was divided into the following groups: Control, TNF- $\alpha$ , Rhodojaponin III Lo, Rhodojaponin III Mi, and Rhodojaponin III Hi. The cells in the Control group were not treated. Cells in the TNF- $\alpha$  group were induced with 10 ng/mL TNF- $\alpha$  (ab259410, Abcam, Cambridge, UK) for 24 h [24,29–31]. Cells in Rhodojaponin III Lo, Rhodojaponin III Mi, and Rhodojaponin III Hi groups were induced with 10 ng/mL TNF- $\alpha$  for 24 h and treated with 9  $\mu$ g/mL, 18  $\mu$ g/mL, and 36  $\mu$ g/mL Rhodojaponin III for 24 h [32]. Experiment 2 was divided into the following groups: TNF- $\alpha$ , Rhodojaponin III, Rhodojaponin III+oe-NC, and Rhodojaponin III+oe-NIK. Cells in the TNF- $\alpha$  group were treated as above. Cells in the Rhodojaponin III group were induced with 10 ng/mL TNF- $\alpha$  for 24 h and then treated with 36  $\mu$ g/mL Rhodojaponin III for 24 h. Cells in the Rhodojaponin III+oe-NC group were treated with oe-NC plasmids, induced with 10 ng/mL TNF- $\alpha$  for 24 h, and then treated with 36  $\mu$ g/mL Rhodojaponin III for 24 h. Cells in the Rhodojaponin III+oe-NIK group were transfected with oe-NIK plasmids, induced with 10 ng/mL TNF- $\alpha$  for 24 h, and then treated with 36  $\mu$ g/mL Rhodojaponin III for 24 h.

#### 2.5 Hematoxylin-Eosin (HE) Staining

The knee joint and synovium were firstly fixed with 4% paraformaldehyde (N1012, NCM Biotech, Suzhou, China), then sliced after paraffin embedding. They were dewaxed in xylene followed by dehydration with gradient ethanol (75–100%). Slices were stained with hematoxylin (AWI0001a, Abiowell, Changsha, China) and returned to blue with phosphate-buffered saline (PBS). After being stained with eosin (AWI0029a, Abiowell, Changsha, China), the slices were rinsed with distilled water, followed by dehydration with gradient ethanol (95–100%). The slices were placed in xylene for 10 min for transparency and observed by a microscope after being sealed with neutral gum (AWI0238a, Abiowell, Changsha, China).

#### 2.6 Immunohistochemistry (IHC) Staining

Platelet endothelial cell adhesion molecule-1 (CD31) and vascular endothelial cell growth factor (VEGF) expressions in the synovium were detected by IHC staining. The slices were dewaxed in xylene followed by dehydration with gradient ethanol (75–100%). Subsequently, antigen retrieval and inactivation of endogenous enzymes were performed. Slices were incubated with primary antibodies of CD31 (1:300, ab182981, Abcam, Cambridge, UK) and VEGF (1:200, 19003-1-AP, Proteintech, Chicago, IL, USA) at 4 °C overnight followed by 100  $\mu$ L of horseradish peroxidase (HRP) goat anti-rabbit IgG (1:100, AWS0005,

Abiowell, China) at 37 °C for 30 min. Then 100  $\mu$ L of DAB (ZLI-9017, ZSBG-Bio, Beijing, China) was added for color development. Slices were re-stained with hematoxylin and returned to blue with PBS. The slices were placed in xylene for 10 min for transparency and observed by a microscope after being sealed with neutral gum.

#### 2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

The whole blood, the synovial samples, and cell cultures were pretreated to obtain the supernatant for detection. IL-6 (CSB-E04638h, CUSABIO, Wuhan, China), IL-1 $\beta$  (CB-E08053h, CUSABIO, Wuhan, China), and TNF- $\alpha$  (CB-E04740h, CUSABIO, Wuhan, China) ELISA kits were utilized to detect the levels of cytokines (IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ).

#### 2.8 Cell Counting Kit-8 (CCK-8) Assay

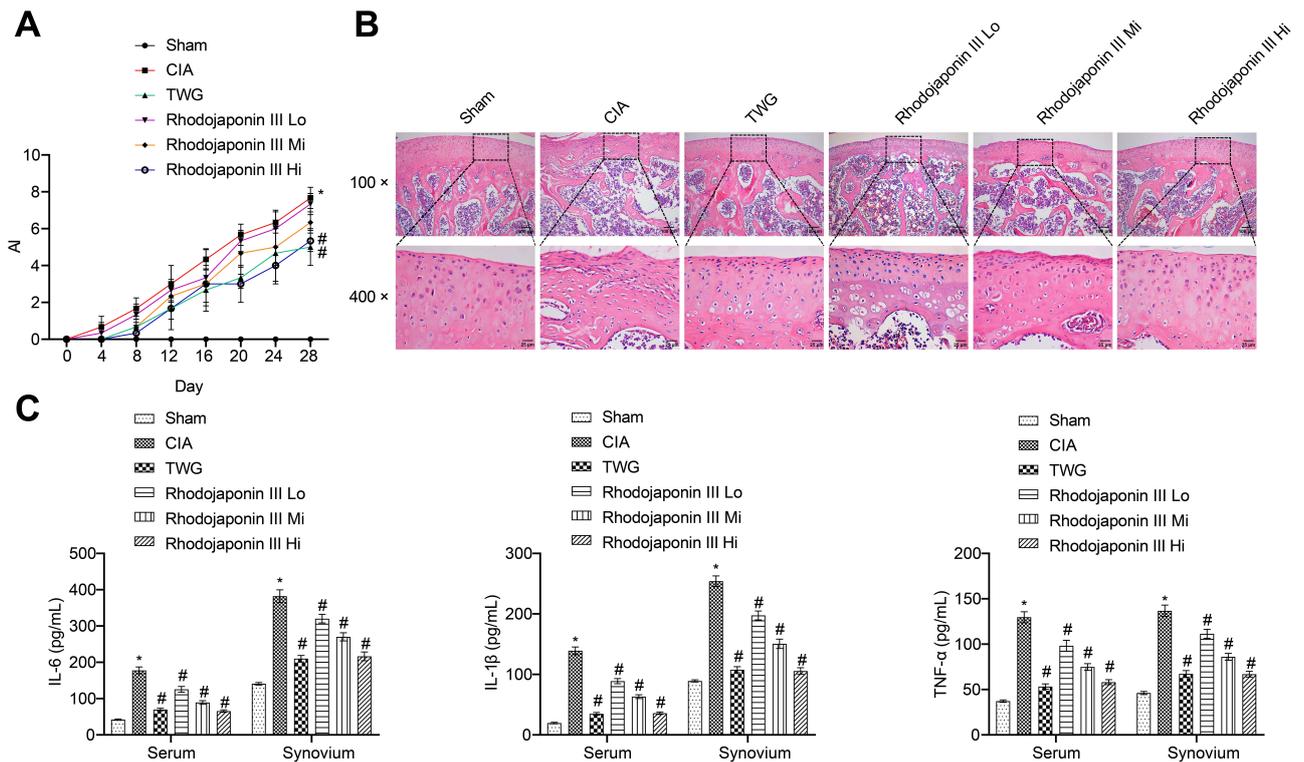
Logarithmic growth of HUVECs was digested by trypsin (AWC0232, Abiowell, Changsha, China).  $1 \times 10^4$  cells were inoculated in 96-well plates, 100  $\mu$ L per well. After cell adhesion, each group was treated with the corresponding drug for 24 h and 48 h, respectively. Then, 10  $\mu$ L of CCK-8 reagent (NU679, Dojindo, Kumamoto, Japan) was added and incubated at 37 °C for 4 h. Optical density (OD) at 450 nm was analyzed with a multifunctional microplate reader (MB-530, HEALES, Shenzhen, China).

#### 2.9 Scratch Assay

The scratch assay was applied to detect the migration ability of HUVECs. HUVECs from different treatment groups were digested to make cell suspension.  $5 \times 10^5$  cells were uniformly inoculated in 6-well plates. When the cells had filled the plates, lines were drawn and viewed under a microscope to measure the initial scratch width. After 24 h and 48 h, the scratch widths were measured and photographed.

#### 2.10 Transwell Invasion Assay

All reagents and equipment were pre-chilled one day in advance. 100  $\mu$ L of Matrigel (354262, Corning Inc. Corning, NY, USA) was added to the upper transwell chamber (3428, Corning Inc. Corning, NY, USA) and incubated at 37 °C for 30 min for solidification. HUVECs from different treatment groups were digested to make cell suspension. DMEM without FBS was used to suspend the cells to  $2 \times 10^6$  cells/mL. 100  $\mu$ L of cell suspension was added to the upper chamber, and 500  $\mu$ L of DMEM with 10% FBS was added to the lower chamber followed by incubation at 37 °C for 48 h. The upper chamber was washed with PBS, and the cells not penetrating the membrane were gently wiped off with sterile cotton balls. After fixation with 4% paraformaldehyde and staining with 0.1% crystal violet (G1062, Solarbio, Beijing, China), cells on the outer surface of the upper chamber were observed and counted.



**Fig. 1. Rhodojaponin III alleviated rheumatoid arthritis (RA) in collagen-induced arthritis (CIA) rats.** (A) Arthritis index (AI) score. (B) Hematoxylin-eosin (HE) staining analyzed the pathological changes in the knee joints (n = 8). (C) Enzyme-linked immunosorbent assay (ELISA) detected cytokine levels in serum and synovium (n = 8). \*  $p < 0.05$  vs. Sham, #  $p < 0.05$  vs. CIA. TWG, Tripterygium wilfordii Glycosides.

### 2.11 Tube Formation Assay

All reagents and equipment were pre-chilled one day in advance. 70  $\mu$ L of Matrigel was evenly spread in the 96-well plates at 37  $^{\circ}$ C for 30 min for solidification. HUVECs from different treatment groups were digested to make cell suspension.  $1 \times 10^4$  cells were inoculated in 96-well plates for 24 h. The formation of blood vessels was evaluated by observing and counting closed-loop or pro-angiogenic structures with an inverted microscope.

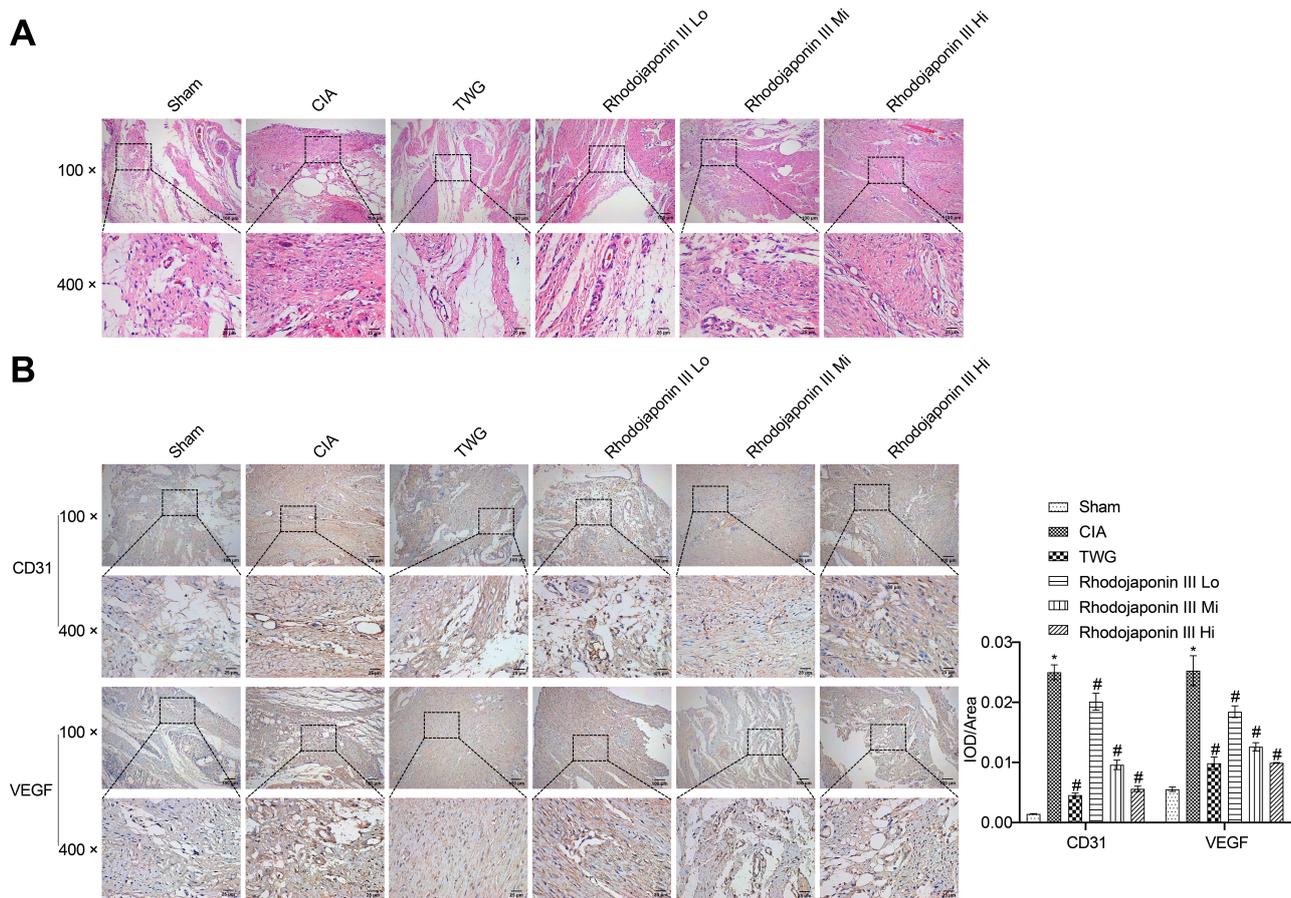
### 2.12 Molecular Docking Verification of Rhodojaponin III and NIK

The three-dimensional structure diagram of NIK was searched from the PDB database (<https://www.rcsb.org/>), and imported into PyMOL (ver.2.3.1, Schrödinger, LLC., New York, NY, USA) to remove water molecules. Then, it was imported into Autodock tools (ver.1.5.6, The Scripps Research Institute, La Jolla, CA, USA) to add hydrogen atoms and then saved in PDBQT format after calculation of the total charge and set of the atomic type. The Chemical Abstracts Service (CAS) number was searched from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>), and the 3D structure diagram of Rhodojaponin III was downloaded. Its energy was minimized with Chem3D pro and was saved in mol2 format. VINA software (Ver. 1.1.2,

The Scripps Research Institute, La Jolla, CA, USA) was used for docking, and the results were visualized with Discovery Studio software (ver. 18.0, BIOVIA, San Diego, CA, USA). Finally, the 3D molecular docking model was output.

### 2.13 Western Blot

Different samples were treated with RIPA lysate (AWB0136, Abiowell, Changsha, China) to extract total proteins. The proteins were transferred to nitrocellulose (NC) membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking in 5% bovine serum albumin (BSA) or 5% skimmed milk (AWB0004, Abiowell, Changsha, China), the membranes were incubated with the primary antibodies at 4  $^{\circ}$ C overnight, including NIK (15  $\mu$ g/mL, ab203568, Abcam, Cambridge, UK), IKK $\alpha$  (1:1000, ab32041, Abcam, Cambridge, UK), p-IKK $\alpha$  (1:1000, ab38515, Abcam, Cambridge, UK), p52 (1:5000, 10655-1-AP, Proteintech, Chicago, IL, USA), CXCL12 (1:20,000, ab155090, Abcam, Cambridge, UK), and  $\beta$ -actin (1:5000, 66009-1-Ig, Proteintech, Chicago, IL, USA). The membranes were incubated with HRP-labeled secondary antibodies for 1.5 h. Finally, the membranes were incubated with ECL reagent (AWB0005, Abiowell, Changsha, China) and followed by



**Fig. 2. Rhodojaponin III decreased vascular density in the synovium of inflammatory joints in CIA rats.** (A) HE staining in the synovium (n = 8). (B) Platelet endothelial cell adhesion molecule-1 (CD31) and vascular endothelial cell growth factor (VEGF) expressions in the synovium were assessed by immunohistochemistry (IHC) staining (n = 8). \*  $p < 0.05$  vs. Sham, #  $p < 0.05$  vs. CIA.

imaging. The protein expressions were analyzed by Quantity One 4.6.6 (Bio-Rad Inc., Hercules, CA, USA) with  $\beta$ -actin as the reference protein.

#### 2.14 Data Analysis

All data were analyzed by GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) and presented as mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post-hoc test were conducted in comparison between groups. Two-way ANOVA was performed in groups at different time points. The difference was significant as  $p < 0.05$ . All the experiments followed randomization and blind analysis to avoid experimental bias.

### 3. Results

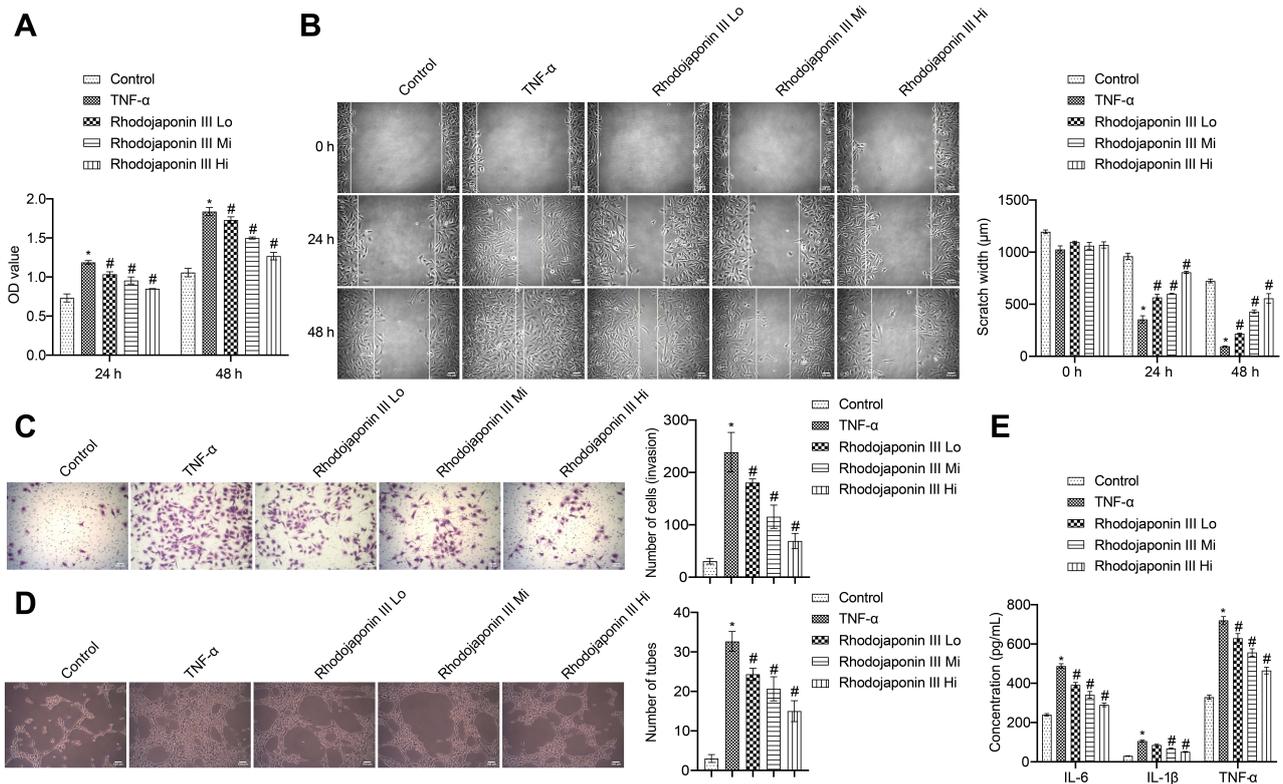
#### 3.1 Rhodojaponin III Alleviated Arthritis Progression and Disease Severity in CIA Rats

To explore the effect of Rhodojaponin III on RA, rats were induced by bovine type II collagen and then given intragastric administration with different concentrations of Rhodojaponin III with TWG as a positive control drug.

High AI score in the CIA group indicated that the CIA rat model was successfully constructed. The AI scores in the TWG group and different concentrations of Rhodojaponin III groups were decreased compared with the CIA group (Fig. 1A). HE staining in knee joints showed that inflammatory infiltration and cartilage damage were significant in the CIA group. However, these lesions were significantly alleviated in the TWG group or different concentrations of Rhodojaponin III groups (Fig. 1B). It was further found by ELISA that IL-6, IL-1 $\beta$ , and TNF- $\alpha$  levels in serum and synovium of rats in the TWG group or different concentrations of Rhodojaponin III groups were decreased compared with the CIA group (Fig. 1C). Together, the above results proved that Rhodojaponin III alleviated arthritis progression and disease severity in CIA rats.

#### 3.2 Rhodojaponin III Decreased Vascular Density in the Synovium of Inflammatory Joints of CIA Rats

Then, we studied the effect of Rhodojaponin III on the vascular density in the synovium of inflammatory joints. HE staining showed a large number of synovial hyperplasia and pannus formation in the synovium. However, these



**Fig. 3. Rhodojaponin III inhibited migration, invasion, angiogenesis, and inflammation of tumor necrosis factor-alpha (TNF- $\alpha$ )-induced human umbilical vein endothelial cells (HUVECs).**(A) Cell proliferation was assessed by CCK-8 (n = 3). (B) Cell migration was detected by scratch assay (n = 3). (C) Cell invasion was detected by Transwell invasion assay (n = 3). (D) Tube formation assay (n = 3). (E) Cytokine levels in the cell supernatant were determined by ELISA (n = 3). \*  $p < 0.05$  vs. Control, #  $p < 0.05$  vs. TNF- $\alpha$ .

were significantly reduced after intervention with TWG or different concentrations of Rhodojaponin III (Fig. 2A). In addition, IHC staining analysis displayed that CD31 and VEGF were highly expressed in the synovium. After intervention with different concentrations of Rhodojaponin III, CD31 and VEGF expressions in the synovium significantly decreased, and the expression level in the Rhodojaponin III Hi group reached in the TWG group (Fig. 2B). These results proved that Rhodojaponin III decreased vascular density in the synovium of inflammatory joints in CIA rats.

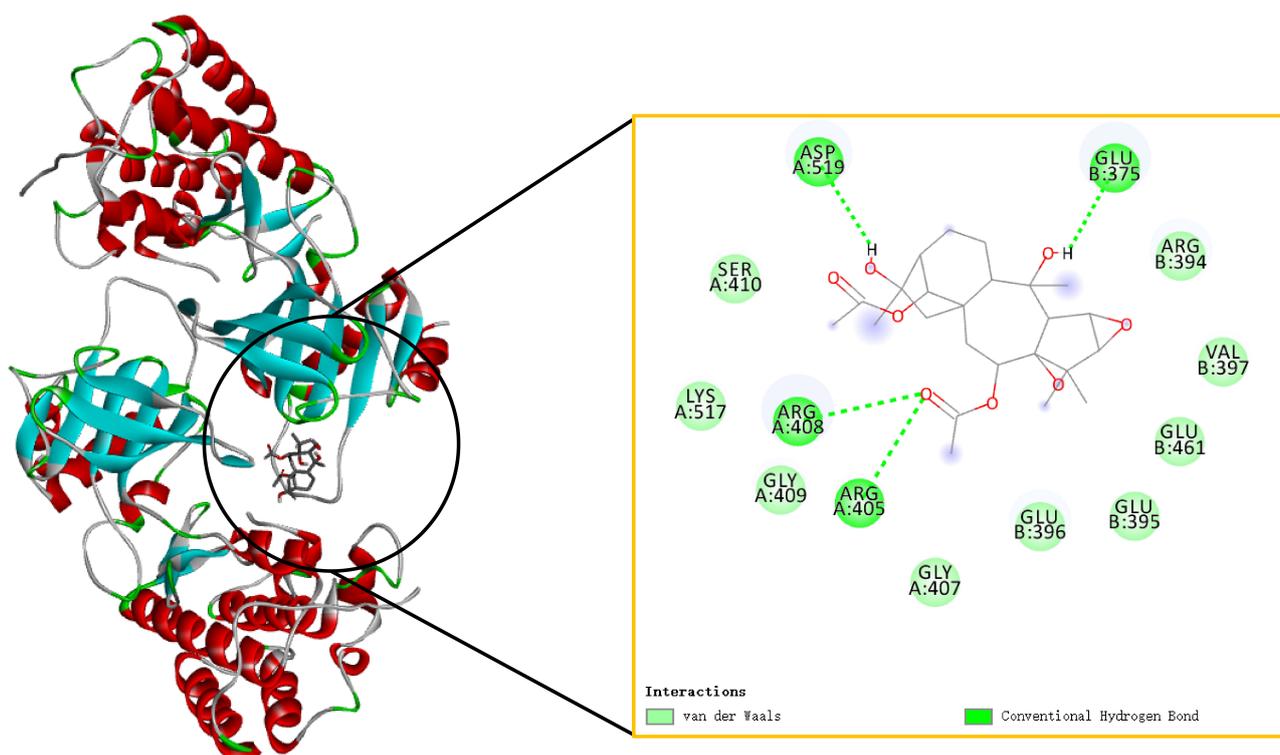
### 3.3 Rhodojaponin III Inhibited Migration, Invasion, Angiogenesis, and Inflammation of TNF- $\alpha$ -Induced HUVECs

To determine the specific therapeutic effect of Rhodojaponin III on RA, a cell model of HUVECs was constructed by TNF- $\alpha$  induction. The proliferation ability of HUVECs increased after TNF- $\alpha$  induction and decreased in a concentration-dependent manner after intervention with different concentrations of Rhodojaponin III (Fig. 3A). The migration ability of HUVECs enhanced after TNF- $\alpha$  induction. However, after intervention with different concentrations of Rhodojaponin III, the migration ability of HUVECs markedly decreased (Fig. 3B). The number of HUVECs passing through the membrane increased after TNF- $\alpha$  in-

duction. After intervention with different concentrations of Rhodojaponin III, the number of cells passing through the membrane considerably decreased (Fig. 3C). In addition, TNF- $\alpha$  induced the formation of numerous tubes from HUVECs. After intervention with different concentrations of Rhodojaponin III, the number of tubes decreased significantly (Fig. 3D). Next, ELISA illustrated that IL-6, IL-1 $\beta$ , and TNF- $\alpha$  levels in the supernatant of HUVECs increased after TNF- $\alpha$  induction, while they were significantly reduced after intervention with different concentrations of Rhodojaponin III (Fig. 3E). These results showed that Rhodojaponin III inhibited migration, invasion, angiogenesis, and inflammation of TNF- $\alpha$ -induced HUVECs.

### 3.4 Rhodojaponin III Interacted with NIK

The binding of Rhodojaponin III and NIK was simulated by molecular docking. In Fig. 4, the binding energy of Rhodojaponin III to NIK was -7.2 kcal/mol, indicating that Rhodojaponin III could spontaneously bind to NIK. The optimal docking conformation between Rhodojaponin III and NIK was shown on the left. Rhodojaponin III bound to the hydrophobic cavity of NIK and interacted with surrounding amino acid residues. Amino acid residues around Rhodojaponin III were shown in the truncated part on the right, including 13 amino acid residues: ASP A:519, SER A410,



**Fig. 4. Rhodojaponin III interacted with NF- $\kappa$ B-inducing kinase (NIK).** Molecular docking model of Rhodojaponin III and NIK (Binding energy:  $-7.2$  kcal/mol).

LYS A:517, ARG A:408, GLY A:409, ARG A:405, GLY A:407, GLU B:396, GLU B:395, GLU B:461, VAL B:397, ARG B:394, and GLU B:375. Rhodojaponin III was bound to ASP A:519, ARG A:408, ARG A:405, and GLU B:375 by conventional hydrogen bonds. Rhodojaponin III was bound to SER A410, LYS A:517, GLY A:409, GLY A:407, GLU B:396, GLU B:395, GLU B:461, VAL B:397, and ARG B:394 by van der Waals force. These results proved that Rhodojaponin III interacted with NIK.

### 3.5 Rhodojaponin III Inhibited the Activation of the NIK Pathway in CIA Rats and TNF- $\alpha$ -Induced HUVECs

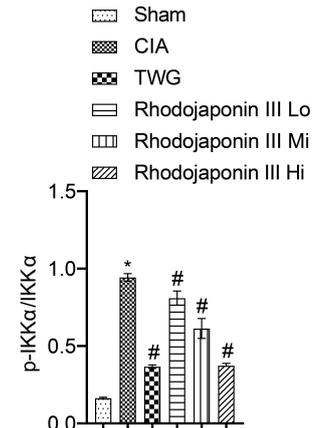
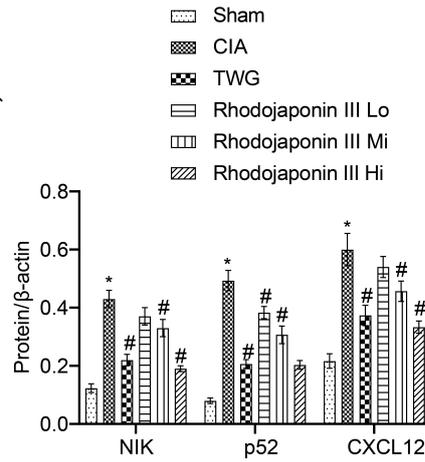
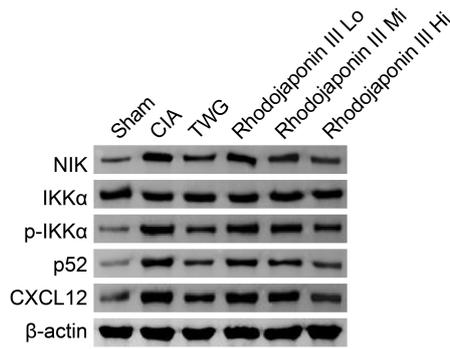
To elucidate the specific molecular mechanism of Rhodojaponin III on RA, Western blot was applied to detect the expressions of NIK pathway-associated proteins in CIA rats and TNF- $\alpha$ -induced HUVECs. Compared with the Sham group, NIK, p52, and CXCL12 expressions in the synovium of rats in the CIA group were up-regulated, and the phosphorylation level of IKK $\alpha$  was increased. Compared with the CIA group, NIK, p52, and CXCL12 expressions in the synovium of rats in the TWG group or different concentrations of Rhodojaponin III groups were significantly down-regulated, and the phosphorylation level of IKK $\alpha$  was decreased (Fig. 5A). NIK, p52, and CXCL12 expressions were up-regulated, and the phosphorylation level of IKK $\alpha$  was increased in HUVECs after TNF- $\alpha$  induction. Compared with the TNF- $\alpha$  group, NIK, p52, and CXCL12 expressions in HUVECs intervened with differ-

ent concentrations of Rhodojaponin III was significantly down-regulated, and IKK $\alpha$  phosphorylation was decreased (Fig. 5B). These results displayed that Rhodojaponin III inhibited the NIK pathway activation in CIA rats and TNF- $\alpha$ -induced HUVECs.

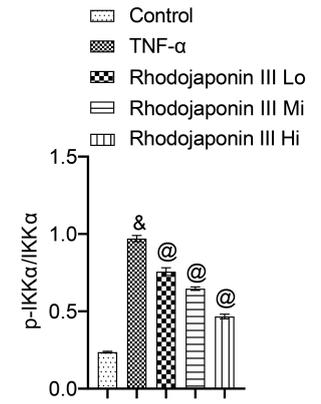
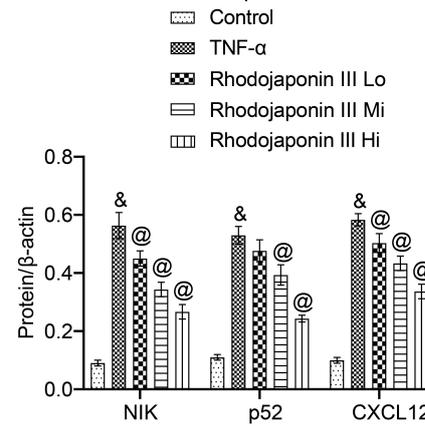
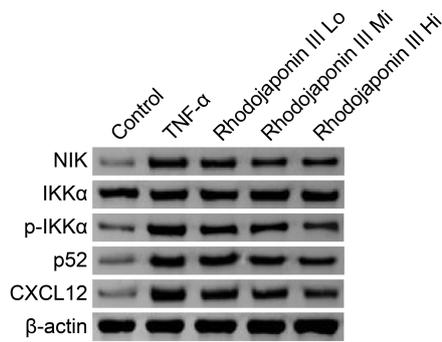
### 3.6 Rhodojaponin III Inhibited the Migration, Invasion, Angiogenesis, and Inflammation of HUVECs by Inhibiting the NIK/NF- $\kappa$ B Pathway

To verify the role of the NIK/NF- $\kappa$ B pathway in RA, HUVECs were transfected with oe-NIK plasmids, induced by TNF- $\alpha$ , and then intervened with Rhodojaponin III. Compared with the TNF- $\alpha$  group, NIK, p52, and CXCL12 expressions in HUVECs in the Rhodojaponin III group were down-regulated, and the phosphorylation level of IKK $\alpha$  was decreased. Compared with the Rhodojaponin III+oe-NC group, NIK, p52, and CXCL12 expressions in HUVECs in the Rhodojaponin III+oe-NIK group were significantly up-regulated, and the phosphorylation level of IKK $\alpha$  was increased (Fig. 6A). The migration ability of HUVECs in the Rhodojaponin III group was decreased compared with the TNF- $\alpha$  group. The migration ability of HUVECs in the Rhodojaponin III+oe-NIK group was increased compared with the Rhodojaponin III+oe-NC group (Fig. 6B). The proliferation ability of HUVECs in the Rhodojaponin III group decreased compared with the TNF- $\alpha$  group. The proliferation ability of HUVECs in the Rhodojaponin III+oe-NIK group was increased compared

**A**



**B**



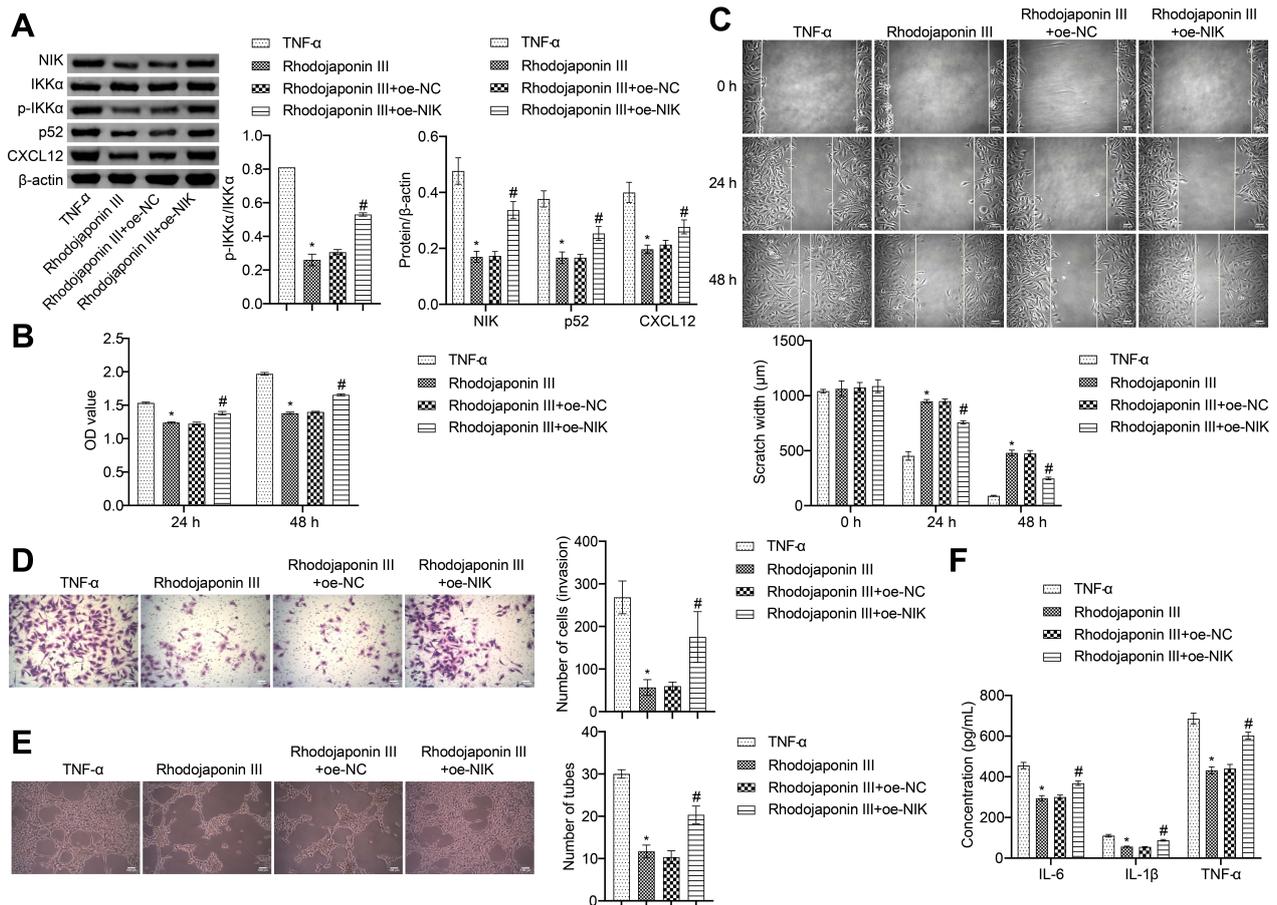
**Fig. 5. Rhodojaponin III inhibited the NIK pathway activation in CIA rats and TNF- $\alpha$ -induced HUVECs.** (A,B) NIK, I $\kappa$ B kinase alpha (IKK $\alpha$ ), p-IKK $\alpha$ , p52, and C-X-C motif chemokine ligand 12 (CXCL12) expressions in the synovium of rats (n = 8) and HUVECs (n = 3) were detected by Western blot. \*  $p < 0.05$  vs. Sham, #  $p < 0.05$  vs. CIA, &  $p < 0.05$  vs. Control, @  $p < 0.05$  vs. TNF- $\alpha$ .

with the Rhodojaponin III+oe-NC group (Fig. 6C). The number of HUVECs passing through the membrane in the Rhodojaponin III group decreased compared with the TNF- $\alpha$  group. The number of HUVECs passing through the membrane in the Rhodojaponin III+oe-NIK group was increased compared with the Rhodojaponin III+oe-NC group (Fig. 6D). The number of tubes in HUVECs in the Rhodojaponin III group decreased compared with the TNF- $\alpha$  group. The number of tubes in HUVECs in the Rhodojaponin III+oe-NIK group increased compared with the Rhodojaponin III+oe-NC group (Fig. 6E). It was found by ELISA that compared with the TNF- $\alpha$  group, IL-6, IL-1 $\beta$  and, TNF- $\alpha$  levels in the supernatant of HUVECs in the Rhodojaponin III group were decreased. Compared with the Rhodojaponin III+oe-NC group, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  levels in the supernatant of HUVECs in the Rhodojaponin III+oe-NIK group were increased (Fig. 6F). These results indicated that Rhodojaponin III inhibited the migration, invasion, angiogenesis, and inflammation of HUVECs by inhibiting the NIK/NF- $\kappa$ B pathway.

#### 4. Discussion

Currently, the therapeutic drugs of RA can be divided into three categories: chemical drugs, biological products, and TCM [33]. Chemical drugs and biological products mainly treat RA by inhibiting the activity of target enzymes, the release of cytokines, and the activation of inflammatory pathways, but these drugs have certain toxic side effects and limitations [34,35]. TCM can regulate the immune system, induce the apoptosis of inflammatory cells and reduce angiogenesis in the treatment of RA [36–38]. TCM has many advantages in the treatment of RA, and many effective ingredients and their mechanisms of action have been confirmed in clinical and experimental studies. Here, the effect and mechanism of Rhodojaponin III on CIA rats and TNF- $\alpha$  induced HUVECs were investigated, which provided a theoretical foundation for the further application of Rhodojaponin III in the clinical treatment of RA (Fig. 7).

Oxidative stress in RA patients can induce inflammation in the body and produce many inflammatory cytokines, which have an important impact on the occurrence and development of RA [39]. Pathologically, inflammatory cytokines are the main mediators of the inflammatory response of RA and the initial factors to regulate inflam-

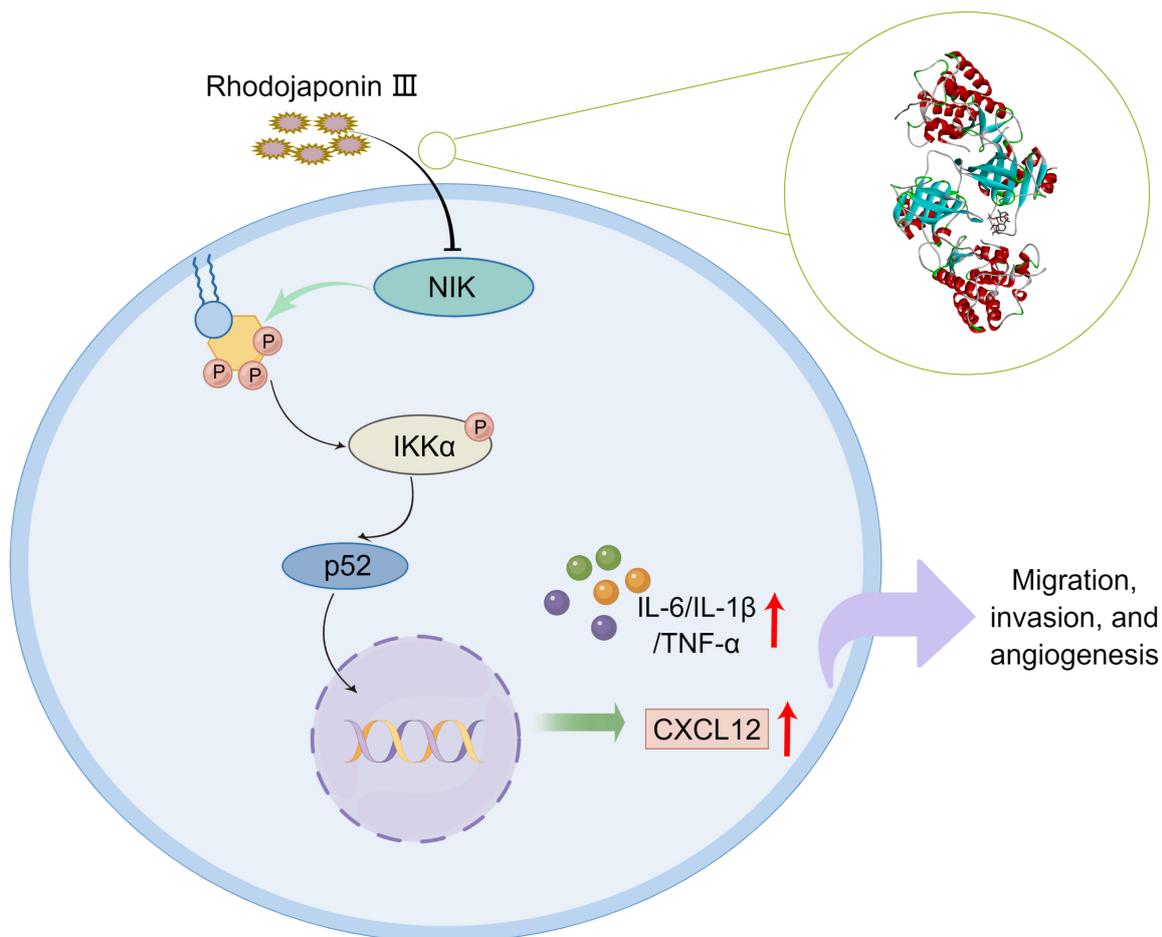


**Fig. 6. Rhodojaponin III inhibited the migration, invasion, angiogenesis, and inflammation of HUVECs by inhibiting the NIK/nuclear factor kappa B (NF-κB) pathway.** (A) NIK, IKKα, p-IKKα, p52, and CXCL12 expressions in HUVECs were detected by Western blot (n = 3). (B) Cell proliferation was detected by CCK-8. (C) Cell migration was detected by scratch assay (n = 3). (D) Cell invasion was detected by Transwell invasion assay (n = 3). (E) Tube formation assay (n = 3). (F) Cytokine levels in the cell supernatant were determined by ELISA (n = 3). \*  $p < 0.05$  vs. TNF-α, #  $p < 0.05$  vs. Rhodojaponin III+oe-NC.

mation. In RA, IL-6, IL-1β, and TNF-α can activate and prolong the lifespan of osteoclasts and promote bone resorption [40]. IL-6 plays a key role in the immune activation and inflammatory response of RA. Therefore, inhibition of IL-6 can effectively control the progression of RA [41]. Excessive secretion of IL-1β can lead to synovial inflammation, breakdown of cartilage matrix, aggregation of inflammatory cells, and aggravation of joint inflammation [42]. TNF-α is one of the earliest cytokines produced during the course of RA. TNF-α and its receptors widely exist in the synovial fluid of RA patients to promote inflammation. In addition, TNF-α inhibitors are also used clinically to block the binding of TNF-α and receptors to treat RA [43]. Here, the knee joint of CIA rats suffered serious cartilage injury, inflammatory infiltration, and bone erosion, which were significantly improved after intervention with TWG and different concentrations of Rhodojaponin III. In addition, high concentrations of IL-6, IL-1β, and TNF-α were detected in the serum and synovium of CIA rats, but the concentrations decreased significantly after intervention

with TWG and different concentrations of Rhodojaponin III. These results indicated that Rhodojaponin III could affect other cells in joints by regulating the secretion of pro-inflammatory cytokines, such as inhibiting the activation of osteoclasts and reducing the aggregation of inflammatory cells, to delay the development of RA disease.

Studies have shown that the activated synovium in RA further expands into the pannus and invades bone to destroy cartilage. Synovial angiogenesis often contributes to the formation and maintenance of RA pannus [44]. CD31 is frequently expressed in HUVECs and RA synovium, suggesting that CD31 may be an important marker of synovium inflammation and pannus formation [45]. VEGF contributes to promoting the growth of vascular endothelial cells, enhancing vascular permeability, and promoting angiogenesis [46]. VEGF, which is mainly secreted by fibroblasts and neutrophils, is essential in the destruction of articular cartilage and joint deformity. Studies have shown that VEGF is highly expressed in the synovium of joints in RA patients, and its content increases gradually with the



**Fig. 7. Rhodojaponin III inhibited migration, invasion, angiogenesis and inflammation of endothelial cells in rheumatoid arthritis through inhibiting the NIK/NF- $\kappa$ B pathway by targeted binding to NIK.**

development of the disease [47]. Blocking VEGF and its regulatory pathways and inhibiting angiogenesis can play an effective role in treating RA [48]. Here, synovial hyperplasia, increased pannus formation, and high expressions of CD31 and VEGF in synovium were found in CIA rats. However, these lesions were improved by different concentrations of Rhodojaponin III. Vascular endothelial cells are the main effector cells of angiogenesis, and their proliferation, migration and invasion and tube formation play an important role in promoting angiogenesis [49]. Here, Rhodojaponin III significantly decreased the abilities of proliferation, migration, invasion, and tube formation of TNF- $\alpha$ -induced HUVECs. These results indicated that Rhodojaponin III could reduce pannus formation by regulating different stages of angiogenesis to reduce the degree of joint damage.

The IKK/NF- $\kappa$ B pathway consists of NF- $\kappa$ B, I $\kappa$ B, and IKK. NF- $\kappa$ B is a transcription factor that regulates the transcription of multiple genes and is involved in cell inflammation, immunity and proliferation. NF- $\kappa$ B signaling is one of the key transcriptional pathways in RA [50]. Activation of the NF- $\kappa$ B pathway leads to the release of many

cytokines, chemokines, and other pro-inflammatory mediators. Therefore, blocking the NF- $\kappa$ B pathway is considered an important strategy to control the inflammatory response in RA [51]. The role of the IKK/NF- $\kappa$ B pathway in RA has already been studied. For example, an inhibitor of IKK prevented NF- $\kappa$ B activation and bone destruction, thus effectively treating RA [52]. NIK is a kinase that controls the expressions of some cytokines and chemokines by activating the nonclassical NF- $\kappa$ B pathway [13]. NIK and IKK $\alpha$  are activated and phosphorylated to p100, which is then ubiquitinated to p52 [53]. High expression of NIK can induce NF- $\kappa$ B activation, and NIK can also act as the upstream regulatory kinase of IKK [54]. The expression of CXCL12 is regulated by the nonclassical NF- $\kappa$ B pathway [55]. It was significantly highly expressed in vascular and lymphatic endothelial cells [56]. Here, NIK, p52, and CXCL12 expressions were up-regulated, and the phosphorylation level of IKK $\alpha$  was increased in the synovium of CIA rats and TNF- $\alpha$ -induced HUVECs. After intervention with different concentrations of Rhodojaponin III, the expressions of these proteins were observed to be down-regulated. Studies have shown that NIK-specific inhibitor Cpd33 inhibits

p52 production and bone resorption activity of mature osteoclasts [57]. In the mechanism study, Rhodojaponin III and NIK were found to be combined by conventional hydrogen bonds and van der Waals force. In addition, oe-NIK transfection reversed the inhibitory effects of Rhodojaponin III on the expressions of NIK/NF- $\kappa$ B pathway-related proteins, proliferation, migration, invasion, and angiogenesis in TNF- $\alpha$ -induced HUVECs. All these proved that Rhodojaponin III inhibited the progression of RA by inhibiting the activation of the NIK/IKK $\alpha$ /CXCL12 pathway.

This study has the advantages of establishing *in vivo* and *in vitro* models, multiple indexes evaluation and practical significance, and elucidates the possible mechanism of Rhodojaponin III on RA, providing strong support for the application of Rhodojaponin III in the treatment of RA. However, there are certain limitations to this study. It mainly focuses on animal experiments and *in vitro* cell culture, which may not fully reflect the complex pathological processes and therapeutic responses in human RA patients. Moreover, the study does not investigate the long-term effects and safety profile of Rhodojaponin III, especially considering its potential use as a therapeutic agent in clinical cases. In terms of future research directions, this study suggests several areas that can be further investigated. Firstly, further research involving larger sample sizes and long-term follow-up can be conducted to evaluate the safety and efficacy of Rhodojaponin III in human subjects with RA. Clinical trials can be designed to assess its therapeutic effects, optimal dosage, and potential side effects. Secondly, the underlying molecular mechanisms of Rhodojaponin III in regulating the NIK/IKK $\alpha$ /CXCL12 pathway can be further elucidated. Thirdly, the combination of Rhodojaponin III with other therapeutic agents or treatment approaches can be explored to enhance its therapeutic effects and broaden its clinical applications. Lastly, the non-targeted site distribution of RA therapeutic drugs has inspired a comprehensive study of nanomedicine for the treatment of RA [58]. Based on nanomedicine, the development of Rhodojaponin III-loaded nanodrugs for targeted therapy of RA has a bright prospect.

## 5. Conclusions

In summary, this study highlighted the potential therapeutic effects of Rhodojaponin III in the treatment of RA. Rhodojaponin III effectively suppressed cartilage damage, bone erosion, and angiogenesis in the joints of CIA rats. Additionally, Rhodojaponin III inhibited the proliferation, migration, invasion, and angiogenesis of TNF- $\alpha$ -induced HUVECs. Mechanically, these therapeutic effects were mediated by the regulation of the NIK/IKK $\alpha$ /CXCL12 pathway, leading to the suppression of inflammation and angiogenesis. The results of this study provide a foundation for future investigations into the clinical application of Rhodojaponin III as a treatment option for RA patients.

## Abbreviations

RA, rheumatoid arthritis; CIA, collagen-induced arthritis; AI, arthritis index; NF- $\kappa$ B, nuclear factor kappa B; NIK, NF- $\kappa$ B-inducing kinase; IL-1 $\beta$ , interleukin-1beta; HUVECs, human umbilical vein endothelial cells; FLSs, fibroblast-like synovial cells; TNF- $\alpha$ , tumor necrosis factor-alpha; CXCL12, C-X-C motif chemokine ligand 12; IKK, I $\kappa$ B kinase; CXCR4, C-X-C motif chemokine receptor 4; Akt, protein kinase B; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TWG, Tripterygium wilfordii Glycosides; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HE, hematoxylin-eosin; IHC, immunohistochemistry; CD31, platelet endothelial cell adhesion molecule-1; VEGF, vascular endothelial cell growth factor; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; CCK-8, cell counting kit-8; CAS, Chemical Abstracts Service; NC, nitrocellulose; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD, standard deviation; ANOVA, analysis of variance; TCM, traditional Chinese medicine; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

## Availability of Data and Materials

The datasets used during the current study available from the corresponding author on reasonable request.

## Author Contributions

XL contributed to conceptualization, data curation, investigation, methodology, funding acquisition, and writing of the original draft. SL, YX and WM, contributed to conceptualization, formal analysis, validation, investigation, software, and validation. RZ contributed to conceptualization, project administration, supervision, and review. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

This study was approved by the animal experiment ethics committee of Hunan University of Chinese Medicine and conducted in strict accordance with the national institutes of health guidelines for the care and use of experimental animals (LL2022061401).

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### Conflict of Interest

The authors declare no conflict of interest.

### Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2812337>.

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