Effects of Huatan Tongluo decoction on vascular endothelial growth factor receptor 2 expression in synovial tissues of rats with collagen-induced arthritis

Chen Jinchun, Qiu Mingshan, Li Yihan, Zhang Qian, Zhang Yiyian, Lin Shuangjie, Zhang Shaohong, Qian Lixia, Gao Hai, Li Liang-cheng

OBJECTIVE: To determine the therapeutic effect and potential mechanism of Huatan Tongluo decoction on rats with collagen-induced arthritis.

METHODS: Forty specific pathogen-free Wistar rats were selected, and 10 were randomly selected as the control (group 1). The remaining rats were injected intradermally with emulsified type II bovine collagen at the tail base and back, followed by a booster 7 d post first immunization. After establishing collagen-induced arthritis (CIA), rats were randomly divided into three groups (n = 10). The rats were treated orally for 30 d as follows: group 1, saline; group 2, model (saline); group 3, tripterygium polyglycoside (TP; 7.81 mg/kg, positive control); group 4, Huatan Tongluo decoction (HTTL; 7.5 g/ kg). Body weight, ankle swelling and arthritis index were measured over the course of the study. The rats were sacrificed 30 d after treatment. Morphological changes in the synovium were observed by hematoxylin and eosin staining. Pannus formation and synovial thickness in the left ankle were observed by color Doppler ultrasound. Vascular endothelial growth factor (VEGF) and VEGFR2 protein levels were measured by immunohistochemistry. VEGF/VEGFR2 mRNA levels were measured by real-time quantitative polymerase chain reaction.

RESULTS: Compared with the model group, a significantly lower arthritis index was observed in the positive control group (P < 0.05) and HTTL group (P < 0.01), after treatment. Both positive control and HTTL reduced intra-articular pannus formation and synovial thickening. Furthermore, VEGF mRNA, and VEGFR2 protein and mRNA levels were significantly downregulated (P < 0.05) in the treatment groups.

CONCLUSION: Inhibition of the expression of VEGF
and VEGFR2 in synovial tissues and the formation of pannus and synovial hyperplasia may be part of the mechanism of HTTL for relieving the symptoms of rheumatoid arthritis in CIA rats.

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**Keywords:** Arthritis, experimental; Tripterygium; Vascular endothelial growth factor A; Vascular endothelial growth factor receptor-2; Huatan Tongluo decoction

**INTRODUCTION**

Rheumatoid arthritis (RA) is an autoimmune disease with progressive synovitis and chronic joint damage, inflammatory cell infiltration and pannus formation. Advanced RA leads to joint destruction, resulting in joint stiffness, deformity and disability. It is believed that abnormal vascular endothelial cell proliferation, which leads to angiogenesis, is the basis for the chronic proliferative synovitis and pannus formation in RA. The most important factor in promoting angiogenesis is vascular endothelial growth factor (VEGF), which promotes vascular endothelial cell proliferation within the joint by acting on the capillaries in the synovium and basal tissue. In our previous experimental and clinical studies, Huatan Tongluo decoction (HTTL), which is composed of Dannanxing (Rhizoma Ariaena Resum Bile), Taoren (Semen Persicae), Jiangcan (Bombbyx Batrictacatu), Baijiexi (Semen Sinapis) and Shancigu (Pseudobulbus Cremastrae), showed therapeutic effects on RA. Dannanxing is good for treating phlegm and heat retention, and Taoren (Semen Persicae) activates blood circulation and suppresses blood stasis. Hence, Dannanxing and Taoren (Semen Persicae) have generally been used as monarch herbs, key components of HTTL, to reduce phlegm and dispel wind, eliminate swelling and dissipate knots, activate blood circulation and suppress blood stasis, and dredge collaterals to relieve pain. Baijiexi (Semen Sinapis) warms the lungs, expels phlegm and regulates Qi, opens the meridians and collaterals, dissipates nodules and alleviates pain. Baijiexi (Semen Sinapis) is treated as an adjuvant drug to enhance activity of Dannanxing (Arisaena cum Bile) and Taoren (Semen Persicae). Jiangcan (Bombbyx Batrictacatu) can eliminate phlegm, reduce wind and stop tremors. Sancigu (Pseudobulbus Cremastrae) can reduce heat and phlegm, and eliminate swelling through anti-inflammatory effects and angiogenesis inhibition. These five medicines, should work as key component, as an adjuvants or as an envoy ingredients, assist the Dannanxing’s effect of eliminating phlegm, reducing wind and dredging collaterals. Taking together, based on our animal experimental and clinical observations, and the literature review, we concluded that the combination of phlegm and blood stasis is a key factor in the development of RA, and blood stasis-suppressing therapy is a promising approach for treating RA. However, the efficacy and mechanism of phlegm-resolving therapy for treating RA, especially by HTTL, have barely been reported.

To this end, in this study, the most representative phlegm-resolving formula, HTTL, was investigated and its effect on the changes in vascular proliferation in the synovium, and the expression of VEGF and VEGFR2 were examined in rats with CIA to further understand the efficacy and possible mechanism of phlegm-resolving therapy.

**MATERIALS AND METHODS**

**Animals**

Specific pathogen-free Wistar male rats weighting (200±20) g were purchased from the Animal Experimental Center of Xiamen University [Animal license number: SCXK (Shanghai) 2012-0002]. The rats were acclimatized in the animal facility for 1 week before the experiments. The study was approved by the experimental animal ethics committee of Xiamen University.

**Drugs and reagents**

The ingredients of HTTL were purchased from Kang-mei Pharmaceutical Co., Ltd. (Guangdong, China) in the same batch. The following drugs and reagents were used: tripterygium polyglycoside (TP, positive control) tablets (10 mg/tablet; Fujian Huitian Bio-pharma Co., Ltd., Fujian, China; SFDA approval number: Z35020431); immunization grade bovine type II collagen (Chondrex, Redmond, WA, USA; batch No. 20021; stored at 4 °C); complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA; F-5881); anti-Vascular endothelial growth factor receptor 2 (VEGFR2) and anti-CD34 antibodies (Boster Biological Technology, Wuhan, China); Trizol (cat #: 15596026; Invitrogen, Carlsbad, CA, USA); cDNA synthesis kit (K1622, Thermo Fisher Scientific, Waltham, MA, USA); TransStart Top Green qPCR SuperMix (K10225, TransGen Biotech, Beijing, China); diaminobenzidine (DAB) staining kit (Maixin Biotechnologies, Fuzhou, China); chloral hydrate and paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA); glacial acetic acid (Xilong Scientific, Guangdong, China).

**Devices**

The following devices were used: biosafety cabinet (BSL2 ACS-4S1; Esco, Singapore); PCR system (S1000; Bio-Rad, Hercules, CA, USA); inverted microscope (BX53F; Olympus, Tokyo, Japan); Microphotograph system (U-TV05XC-3; Olympus); Precision balance (AL204; Mettler, Columbus, OH, USA); Tissue grinder (TissueLyser-24; Jingxin Industrial Development Co., Ltd., Shanghai, China); Rotary evaporator (Precision HL G3; Heidelberg, Germany); Paraffin embedder (EG1160), paraffin slicer (RM2235), slide...
warmer (HI1220) and slide spreader (HI1210) (Leica, Germany); Constant temperature water bath (DK-8D; Jinghong Laboratory Instrument Co., Ltd., Shanghai, China); Magnetic stirrer (C-MAG HS4; IKA, Guangdong, China).

**Induction of collagen-induced arthritis (CIA)**

Bovine type II collagen was dissolved under sterile conditions in 0.01 mmol/L glacial acetic acid to a concentration of 2 mg/mL, and emulsified with an equal volume of complete Freund’s adjuvant. To induce CIA, rats were intradermally injected at three sites (front, middle and rear) on the back and at the tail base with 0.2 mg of the emulsified bovine type II collagen. After 7 d, another intradermal injection of 0.1 mg of the emulsified bovine type II collagen was given at the back and tail base for booster immunization.

**Clinical scoring regime**

The individual clinical score was assessed every 6 d for both hind limbs as follows: 0 = no swelling; 1 = mild swelling or redness; 2 = swelling and redness of one hind foot that can move normally; 3 = moderate swelling and redness of both hind feet whose moving ability is limited; 4 = the joints are severely swollen, redness, and/or ankylosis, and the hind limbs movement is restricted. Unilateral or bilateral ankle swelling was first observed in some rats 12 d after the first immunization. On day 14, bilateral ankle swelling was observed in all rats. Arthritis index (AI) was the sum of joint swelling scores of two hind limbs, ranging from 0 to 8. At AI ≥ 4, CIA was regarded as successfully established, and these animals were randomly divided into model group, positive control group and HTTL group (10 rats in each group).

**Preparation of the decoction**

As this formula consists of a few medicinal components in small quantities, 10 doses of the formula were soaked in 3000 mL of tap water for 30 min before decoction. The mixture was decocted on high heat to 100 ºC, and then decocted with mild heat for 25 min. The decoction was moved into a container, and the medicinal components were decocted again for 20 min after adding 2000 mL of tap water. The decoction was mixed with the previous decoction to a volume of 1000 mL for later use.

**Concentrating the decoction**

The 1000-mL liquid mixture was placed in a rotary evaporator and concentrated to 90 mL in a water bath at 60 ºC and 7-8 r/min.

**Drug administration**

After grouping, intragastric administration was performed once daily for 30 d. HTTL and TP were given at 7.5 and 7.81 mg/kg, respectively. The control and model groups received an equal volume of saline by gavage.

**Observation of pannus formation and synovial thickness in the left ankle by color Doppler ultrasound**

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g body weight) as described previously. Fur was shaved from the left lower limbs, including the left abdomen area, when necessary. Next, rats were fixed in a supine position on a plate by an assistant as instructed by a color Doppler ultrasound doctor. The surface of the left ankle was coated with a coupling agent. Both longitudinal and cross-sectional views were obtained using an 18 MHz probe. Sections that clearly showed a complete articular cavity and synovial changes were used for assessment of pannus formation and synovial thickness. The images were interpreted by a color Doppler ultrasound doctor at the end of the ultrasound procedure.

**Sample preparation**

Rats were weighed, anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g body weight), sacrificed and fixed in the supine position. The synovial and fibrous layers of the joint capsule were bluntly dissected, and the synovial tissue was gently clamped and stored in liquid nitrogen until later use. Next, a longitudinal skin incision was made in the middle of the right hind limb to expose the knee joint, and after the muscle was stripped off, the knee joint was cut with scissors approximately 1 cm from its upper and lower edges, and then fixed in 10% neutral formalin (using 20 times the volume of the joint) for 10 d. Then, the joint was decalcified in 10% ethylene-diamine tetraacetic acid (EDTA) (using 10 times the volume of the joint) for 30 d. EDTA was replaced every 2 d. Finally, the joint was dehydrated and embedded in paraffin.

**Hematoxylin and eosin (HE) staining and immunohistochemical detection of VEGF, VEGFR2 and CD34 in synovial tissues**

The embedded joint specimens mentioned above were deparaffinized, hydrated and placed in an automatic HE staining machine, mounted under coverslips with neutral balsam for microscope observation. For immunohistochemistry, the specimens were sliced, spread, dried, deparaffinized and hydrated. After antigen retrieval, the slices were incubated overnight at 4 ºC with primary antibodies for VEGF, VEGFR2 and CD34 at dilutions of 1: 100, 1: 50 and 1: 50, respectively. After washing, the slices were incubated with 100 mL of highly sensitive enzyme-labeled mouse/rabbit IgG antibodies for 20 min at room temperature, and color was developed with freshly prepared DAB solution for 3-10 min. Slices were then mounted under coverslips with neutral balsam for microscope observation.
Measurement of VEGF and VEGFR2 mRNA expression in synovial tissues by real-time quantitative reverse transcription (RT-PCR)

The mRNA was extracted from 15 mg of knee synovial tissue from each rat by grinding the tissue with a tissue grinder after addition of Trizol. Total RNA was extracted by precipitation with chloroform, phenol and isopropanol; the precipitate was dissolved in 12 μL of diethyl pyrocarbonate (DEPC) water and stored at −80°C. cDNA was synthesized using a cDNA synthesis kit (ThermoFisher Scientific, Waltham, USA) following the manufacturer’s instructions. PCR was performed using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) following the manufacturer’s instructions. The primers used are shown in Table 1. The amplification program consisted of initial denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s and extension at 60°C for 30 s, with a final extension at 65°C for 30 s. The reaction was terminated at 4°C.

Statistical analysis

Statistical analysis was performed by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Experimental data are expressed as mean ± standard deviation (x ± s). Data were analyzed using one-way analysis of variance (ANOVA) and Student’s t-test. P < 0.05 indicates a statistically significant difference.

RESULTS

General observations

Compared with the control group, the body weight increased less in the model, positive control and HTTL groups 12 d post modeling. While after 30 d of treatment, the body weight remained constant in both the HTTL and positive control groups compared with the model group, which the body weight lost significantly (Table 2). CIA was symmetrical in rats and the movement of ankle and toe joints were affected. The incidence rate was 100% in the 30 rats. Ecchymosis and mild swelling were observed in some rats 12 d after immunization. The swelling gradually increased in the bilateral ankle and toe joints, and some joints became shiny and congested 14 d after immunization. After that, the joint symptoms continued to progress in the model group, resulting in limited mobility. However, the swelling did not increase in the positive control and HTTL groups. After 30 d of treatment, the joint swelling gradually decreased in both these groups, while it gradually increased in the model group.

Comparison of AI before and after treatment

Compared with the control group, significantly higher AI was noted in the model (P < 0.01), TP (P < 0.01) and HTTL groups (P < 0.01) 12 d after modeling. There was no significant difference in AI among the model, TP and HTTL groups before treatment. However, compared with the model group, a significant decrease in AI was observed in the TP group (P < 0.01) and HTTL group (P < 0.01) after 30 d of treatment (Table 3).

Comparison of pannus formation

Pannus formation and synovial thickness in the left ankle of rats in each group were observed by color Doppler ultrasound. Compared with the control group, the

<table>
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<tr>
<td>GAPDH</td>
<td>Forward: 5'-CCCTGTTGCTGTAGCCATATT-3'</td>
<td>131</td>
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Notes: RT-PCR: real-time quantitative polymerase chain reaction; VEGFR-2: vascular endothelial growth factor receptor 2; VEGF: vascular endothelial growth factor; GAPDH: glyceraldehyde phosphate dehydrogenase.

<table>
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<th>Table 2</th>
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Notes: control group: normal rat treated with physiological saline; model group: CIA rat treated with physiological saline; positive group: CIA rat treated with tripterygium polyglycoside at doses of 7.81 mg/kg; HTTL group: CIA rat treated with Huatan Tongluo decoction at doses of 7.5 g/kg. All treatment was Intragastric administration. ⁃P < 0.01, compared with control group at Day 12; ⁃P < 0.05, compared with control group at Day 12; P < 0.01, compared with control group at Day 42; P < 0.05, compared with control group at Day 42.
model group showed significant pannus formation and thickened synovium. Conversely, pannus formation and synovial thickness decreased in the Positive control and HTTL groups compared with the model group (Figure 1).

**Histopathological changes in joint tissues**

In the control group, orderly arranged cells were observed in the soft tissue around the joints and synovial tissue. In contrast, proliferation, inflammatory infiltration and disordered arrangement of synovial cells were observed in the model group, which well simulated the pathological features of human RA. However, synovial cell proliferation and inflammatory cell infiltration were reduced in rats treated with positive control or HTTL (Figure 2).

**Immunohistochemical detection of CD34 and VEGFR2 in synovial and soft tissues**

The level of CD34, VEGFR2 and VEGF expression in the joints of rats in each group were measured by immunohistochemistry. Compared with the control group, the model group demonstrated vascular proliferation and positive CD34, VEGFR2 and VEGF staining in synovial and soft tissues. However, compared with the model group, vascular proliferation in synovial and soft tissues was reduced, and CD34, VEGFR2 and VEGF staining was decreased or negative in the positive control and HTTL groups (Figure 3).

**VEGF and VEGFR-2 mRNA expression in the synovial tissue**

The mRNA expression of VEGF and VEGFR2 in the synovial tissue of rats in each group was measured by real-time quantitative RT-PCR with glyceraldehyde phosphate dehydrogenase (GAPDH) as the internal control. The mRNA expression of VEGF (Figure 4A) and VEGFR2 (Figure 4B) was significantly increased in the model group compared with the control group ($P < 0.01$). However, compared with the model group, the mRNA expression of these genes was significantly decreased in the synovial tissue of rats treated with positive control or HTTL ($P < 0.01$, Figure 4A, B).

**DISCUSSION**

RA is an autoimmune disease, with synovitis as the primary pathological process. The main pathological changes are infiltration by inflammatory cells and pannus formation, which lead to abnormal vascular proliferation in RA. The angiogenesis equilibrium is regulated by angiogenesis-stimulating and -inhibiting factors, which maintain the balance of angiogenesis in normal conditions. When this balance is disrupted because of an abnormal increase in angiogenesis-stimulating factors, pathological vascular proliferation occurs. The most important factor that promotes angiogenesis in pathological vascular proliferation in RA is VEGF.
It has been shown that CIA could be inhibited by neutralizing VEGF in mice, indicating that VEGF-induced angiogenesis plays an important role in the pathogenesis of RA. Based on reviews, clinical observations and practice, we believe that phlegm and blood stasis are the key factors in the development of RA. Moreover, beneficial effects on RA were observed after removing sputum to smoothen collateral therapy in animal experiments and clinical practice. Therefore, we propose that normalizing the functional activities of Qi, eliminating phlegm and dredging collaterals should be applied throughout the treatment of RA.

Thus, we believe, by composing Dannanxing (Rhizoma Arisaematis Cum Bile), Taoren (Semen Persicae), Jiangcan (Bombyx Batryticatus), Baijiezi (Semen Sinapis) and Shancigu (Pseudobulbus Cremastrae) at a ratio of 1:1:1:0.5 to formulate HTTL which should have therapeutic effect for RA. As proved by the results, the therapeutic effects of HTTL on RA in CIA rats were observed, with TP as the positive control, HTTL significantly decreased AI, intra-articular pannus formation, synovial thickening and inflammatory cell infiltration. Moreover, they significantly decreased VEGF and VEGFR\textsubscript{2} mRNA and protein levels in the synovial tissue. These results indicated that HTTL reduced pannus formation and synovial thickening by inhibiting the expression of angiogenesis factors and their receptors in the synovial tissue, thereby resulting in decreased AI in rats with RA.

Because Dannanxing (Rhizoma Arisaematis Cum Bile) is Tiannanxing (Rhizoma Arisaematis Erubescentis) treated with cow bile to process and make a cooling agent to treat phlegm heat. It is more appropriate for treating phlegm and heat retention. Taoren (Semen Persicae) activates blood circulation and relieves blood stasis. It treats many blood stasis conditions including menstrual disorders, abdominal pain, trauma, flank pain, lung...
In summary, based on our observations and pathogen TL, which ultimately leads to decreased AI in rats and angiogenesis of RA, also may be affected by HTL and FGF/FGFR, however, because of the complex pathophysiology of ing wind and dredging collaterals.

By herbs, assist the Dannanxing (Rhizoma Arisaematis Cum Swelling through anti-inflammation and inhibition of can clear away heat, dispel /phlegm and eliminate Pseudobulbus Cremastrae seu Pleiones) eliminates phlegm, suppresses wind and stops trem.

Jiangcan (Semen Persicae Dannanxing) is used as a adjuvant component to assistant therapeutic activity of Semen Sinapis and clearing meridians, and eliminating swelling and Qi, opens the meridians and collaterals, spicy and warm. It warms the lungs, expels phlegm

Sanye (Baijiezi (Semen Sinapis) lieving blood stasis, and dredging collaterals to relieve pain. The properties of Baijiezi (Semen Sinapis) are

Bile) and Taoren (Semen Persicae) are used as key com

in the body. Dannanxing (Rhizoma Arisaematis Cum and abscesses. It can sweep away the old, starting afresh abdominal mass, postpartum abdominal pain, and general sores and abscesses. It can sweep away the old, starting afresh in the body. Dannanxing (Rhizoma Arisaematis Cum Bile) and Taoren (Semen Persicae) are used as key components, working as monarch herbs for reducing phlegm and dispelling wind, eliminating swelling and dissipating knots, activating blood circulation and relieving blood stasis, and dredging collaterals to relieve pain. The properties of Baijiezi (Semen Sinapis) are spicy and warm. It warms the lungs, expels phlegm and regulates Qi, opens the meridians and collaterals, dissipates nodules and alleviates pain. For warming and clearing meridians, and eliminating swelling and nodding. Baijiezi (Semen Sinapis) is used as a adjuvant component to assistant therapeutic activity of Dannanxing (Rhizoma Arisaematis Cum Bile) and Taoren (Semen Persicae). Jiangcan (Bombys Batryticatus) eliminates phlegm, suppresses wind and stops tremors. Sancigu (Pseudobulbus Cremasae seu Pleiones) can clear away heat, dispel phlegm and eliminate swelling through anti-inflammation and inhibition of angiogenesis. And therefore, these five ingredients, working as key component, as an adjuvants or as guide herbs, assist the Dannanxing (Rhizoma Arisaematis Cum Bile)’s effect of eliminating phlegm, removing wind and dredging collaterals. However, because of the complex pathophysiology of RA, and other signaling pathways, like TGF/TGFα and FGF/FGFR, also contributed to inflammation and angiogenesis of RA, also may be affected by HTL, which ultimately leads to decreased AI in rats with RA. Hence, further investigation is required. In summary, based on our observations and pathogenesis analysis in terms of modern medicine and traditional Chinese medicine, we believe that HTTL has anti-inflammatory effects on arthritis of CIA rats. However, its mechanism for treating RA should be further investigated from the perspective of VEGF-mediated microvascular proliferation.

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