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Journal of the Chinese Medical Association 79 (2016) 264–271

www.jcma-online.com

Original Article

Effects of total saponins from Rhizoma Dioscoreae Nipponicae on expression of vascular endothelial growth factor and angiopoietin-2 and Tie-2 receptors in the synovium of rats with rheumatoid arthritis

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Received June 16, 2015; accepted October 20, 2015

Abstract

Background: This study aimed to determine the effects of total saponins from Rhizoma Dioscoreae Nipponicae (TS-RDN) on the expression of vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-2 and Tie-2 (endothelial tyrosine kinase receptor) receptors in the synovium of rats with rheumatoid arthritis (RA) (collagen-induced arthritis; CIA), and to examine the mechanisms of TS-RDN in alleviating RA.
Methods: The CIA rat model was established and the animals were randomly divided into control, CIA model, TS-RDN, diosgenin, and tripterygium groups. Fluorescent polymerase chain reaction was performed to detect VEGF expression in the rat knee joint synovium. Additionally, immunohistochemical assay was used to detect protein expression of Ang-2 and Tie-2 in the rat knee joint synovium.
Results: Expression of VEGF, Ang-2, and Tie-2 in the model group was significantly higher than in the control group ($p < 0.01$). After TS-RDN, tripterygium and diosgenin treatment, VEGF and Ang-2 expression was lower than in the model group ($p < 0.01$). However, Tie-2 expression showed no significant difference. The effects of TS-RDN on VEGF expression were more marked than those of tripterygium and diosgenin ($p < 0.01$).
Conclusion: TS-RDN might reduce the expression of VEGF, Ang-2, and Tie-2 in the synovium, thus inhibiting synovial angiogenesis and playing a therapeutic role in RA.

Keywords: angiogenesis; angiopoietin 2; Rhizoma Dioscoreae Nipponicae; Tie-2; total saponins; vascular endothelial growth factor

1. Introduction

Rheumatoid arthritis (RA) is a systemic, chronic, autoimmune disease characterized by joint inflammation. RA has a high prevalence worldwide, with the global incidence rate being 0.5–1.0% and that in Chinese population being 0.4%. RA severely affects patients' quality of life, since it is difficult to treat and is associated with high morbidity.[1] The basic pathological feature of RA is multiple, chronic, progressive synovitis of the joints;[2–4] however, other body organs and tissues might also be involved.[5,6] Neoangiogenesis has been observed to be the main underlying cause of synovitis, pannus growth, bone and cartilage destruction, and osteophyte formation.[7–10] A variety of angiogenic factors could lead to angiogenesis,[11] among which vascular endothelial growth factor (VEGF) plays a crucial role in promoting angiogenesis. VEGF was found to be closely associated with synovial angiogenesis in RA,[12] promoting angiogenesis during the

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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http://dx.doi.org/10.1016/j.jcma.2015.10.012

early stages of RA and playing an important role in neovascularization.[13] In addition, angiopoietin (Ang) was correlated with angiogenesis. Tie-2, a co-receptor of Ang, is a tyrosine kinase receptor that is specifically expressed in endothelial cells and certain hematopoietic progenitor cells, and plays an important role in vascular development.[14] Among the Ang family members, Ang-2 can promote angiogenesis, vascular remodeling, and vascular regression.[15] Ang-2 has VEGF-dependent actions: in the presence of VEGF, it promotes vascular sprouting, whereas in VEGF-deficient conditions, Ang-2 promotes vascular regression.[16] Therefore, the VEGF/VEGF receptor pathway[17] and the Ang/Tie-2 (endothelial tyrosine kinase receptor) signaling pathway have become the focus of RA angiogenesis research.

Our previous research suggested that total saponins from Rhizoma Dioscoreae Nipponicae (TS-RDN) inhibit neoangiogenesis and synovial VEGF expression.[18]

This study examined the expression levels of synovial VEGF, Ang-2, and Tie-2, aiming to explore the roles of VEGF and Ang/Tie-2 signaling pathways in synovial angiogenesis in a collagen-induced arthritis (CIA) model to ascertain whether they had synergistic effects. We further aimed to investigate the mechanisms of TS-RDN in inhibiting synovial angiogenesis in a CIA model, to provide experimental evidence for the use of TS-RDN in RA treatment.

2. Methods

2.1. Model preparation

Sixty female Wistar rats, clean grade, weighing 140 ± 20 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Beijing. China (Certificate Number: 0158513), and the CIA model was established as described previously.[19] This study was carried out in strict accordance with the recommendations noted in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Chengde Medical College, Hebei, China. Bovine type II collagen dissolved in acetic acid (2 mg/mL; Chondrex, Redmond, WA, USA, Lot Number: 200202) and Freund's complete adjuvant (Sigma-Aldrich Corp., St. Louis, MO, USA, Lot Number: 9007-81-2) were mixed in equal volume and emulsified to form type II collagen emulsion. Under sterile conditions, the above emulsion was injected into multiple points on the back and tail root of rats (0.2 mL at each point); the same dose was re-injected on Day 7 for potentiation of excitation. The arthritis index (AI) was used as the standard to evaluate whether the model succeeded or failed.[20] The limb joint swelling was scored on a scale of 0–IV; 0: no swelling; I: slight swelling in toe joint; II: swelling in toe joint and foot toe joint; III: swelling in paws under ankle joint; and IV: swelling in all paws, including the ankle joint. AI value was the sum of swelling scores of four limbs joints [0, 1, 2, 3, and 4 points for, Grade 0, I II, III, and IV, respectively (the total score was 16 points]. An AI value $\geq$ 4 points indicated successful establishment of the model.

2.2. Animal grouping and treatment

Of the 60 Wistar rats, 12 were randomly assigned to the normal control group. Saline (0.2 mL) was injected into multiple points on the back and tail root of rats in the normal control group. A CIA model was successfully established using the remaining 48 rats, and these rats were randomly divided into the CIA model group (CIA group), RDN group (extracted by the Traditional Chinese Medicine Institute of Chengde Medical College), the tripterygium (TP) group (Huangshi Feiyun Pharmaceutical Co. Ltd., Huangshi, China), and diosgenin (DS) group (Nanjing Zelang Medical Technology Co. Ltd., Nanjing, China), with 12 rats in each group. Fourteen days post-modeling, according to the dosage we confirmed in the preliminary experiment,[21] the RDN, TP, and DS groups were intragastrically administered tripterygium glycoside tablet (12 mg/kg/d), TS-RDN (25 mg/kg/d), and diosgenin (50 mg/kg/d), respectively, whereas the control and the CIA model group were intragastrically administered double distilled water at 1 mL/100 g body weight. The period during which medication was administered for each group was 21 days.

The preparation and quality control of TS-RDN were carried out as follows: diosgenin (China Institute of Drug and Biological Products, Beijing, China; Number: 111539) was dissolved in methyl alcohol to a final concentration of 0.5 mg/mL and was used as control.

RDN (1 g) was dissolved in methyl alcohol (50 mL) with ultrasonic processing (250 W, 33 kHz, 30 minutes), filtered, and the residue was collected, washed with methyl alcohol (20 mL), evaporated to remove methyl alcohol, and the resulting precipitate was dissolved in hydrochloric acid (3 mol/L), 20 mL, 10 mL, 5 mL, and 5 mL. The solution was transferred to a conical flask, heated in water bath (30 minutes), and the sample was refluxed twice with trichloromethane in a water bath, with another washing of the container. The resulting solute was dissolved with methyl alcohol to 25 mL.

TS-RDN was extracted, refluxed 10 times and eight times with ethyl alcohol (50%) at 85°C for 1 hour, and the filter liquor was collected for spray drying to obtain TS-RDN.

Reversed-phase high-performance liquid chromatography was used to test the TS-RDN (Fig. 1).

2.3. Sampling treatment

The rats in each group were killed after peritoneal venous blood sampling, and the joint synovium of six rats in each group was sampled (in the DS group, sampling was performed in rats administered 50 mg/kg/d DS), followed by fixation in 4% paraformaldehyde solution, gradual alcohol dehydration, xylene hyalinization, paraffin embedding (automatic dryer and embedding machine; Thermo Fisher Scientific Inc., Waltham, MA, USA), slicing (Leica paraffin slicing machine; Leica Camera AG, Solms, Germany), and immunohistochemical staining. The joint synovium of the remaining six rats in each group was

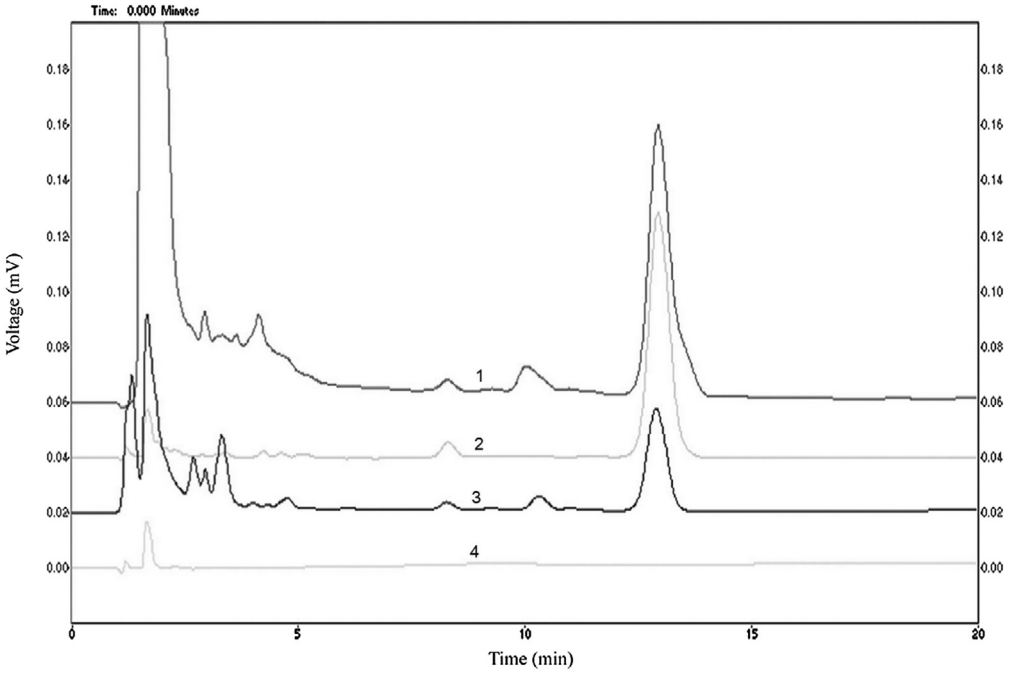

Fig. 1. Reversed-phase high-performance liquid chromatography. 1: RDN; 2: control; 3: TS-RDN; 4: solution. TS-RDN = total saponins from Rhizoma Dioscreae Nipponicae.

quickly sampled (in the DS group, sampling was performed in rats treated with 25 mg/kg/d DS), placed in Eppendorf tubes, and immediately stored in liquid nitrogen.

2.4. Immunohistochemical staining and image analysis

Staining was performed according to the instructions of the streptavidin-peroxidase immunohistochemical staining kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Rabbit anti-Ang-2 polyclonal primary antibody (Abcam, Cambridge, MA, USA, Lot: ab65835) was diluted to 1:250 and the rabbit anti-Tie-2 polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Lot: sc-9026) was diluted to 1:50. Phosphate-buffered saline was used instead of the primary antibody as the negative control. The MiVnt image analysis system (Shandong Echung Electronics Co., Ltd., Jinan, Shandong, China) was used to quantitatively analyze immunohistochemical results at 400-fold magnification, and the ratio of immunohistochemical-positive products to the vision field was recorded as the relative expression level of the target protein. Six slices were selected for each rat and each slice was observed across three visual fields for the final average value. Two investigators blinded to group allocation performed image analysis, and the average value was used.

2.5. Real-time polymerase chain reaction

Total RNA was extracted from the synovial tissues stored in liquid nitrogen using the Trizol method (Invitrogen, Carlsbad, CA, USA), and UV-visible spectrophotometer (DU800) (Beckman Coulter Inc., Brea, CA, USA) was used to detect the RNA purity and concentration. The sample with good purity was then used for reverse transcription reaction (RT master Mix, TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning, China, Lot number: DRR036s). The polymerase chain reaction (PCR) mixture (10 μL) consisted of 0.5 μg total RNA template and 2 μL 5 × PrimeScript RT Master Mix, diluted up to 10 μL with diethypyrocarbonate water. The reaction conditions were 37°C for 15 minutes and

Table 1
Primer and probe sequence of VEGF and GADPH.

Gene		Sequence	Product size
GADPH	Forward	5′-TGGTCTACATGTTCCAGTATGACT-3′	134 bp
	Reverse	5′-CCATTTGATGTTAGCGGGATCTC-3′	
	Probe	5′-(FAM)CCACGGCAAGTTCAACGGCACAGT(Eclipse)-3′	
VEGF	Forward	5′-TCACTCCCTCAAATTACTTCGG-3′	87 bp
	Reverse	5′-TCTCTCTCTTCCTTGACTTCTCTC-3′	
	Probe	5′-(FAM)TCCTGCTACCTCTTTCCTCTGCTGA(Eclipse)-3′	

GADPH = glyceraldehyde-3-phosphate-dehydrogenase; VEGF = vascular endothelial growth factor.

85°C for 5 seconds. The cDNA was then stored at −20°C for future use.

The primers and probes of VEGF and of GAPDH were synthesized by Takara (primer sequences are shown in Table 1). The PCR mixture (25 μL; Taq DNA polymerase; TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning, China; DRR039s) consisted of 12.5 μL Taq enzyme, 0.5 μL upstream and downstream primers (both 10μM), 1 μL 3μM fluorescent probe, and 2 μL template (cDNA), diluted up to 25 μL with diethypyrocarbonate water. The reaction conditions were 40 cycles of denaturation at 95°C for 60 seconds, 95°C for 10 seconds, and 6°C for 30 seconds. SLAN fluorescence quantitative PCR detection system (Shanghai Hongshi Medical Technology Co. Ltd., Shanghai, China) was used to read the fluorescence at 60°C. The fluorescence intensity of each sample was read three times and averaged. Based on the Ct value of obtained target gene to that of the internal reference gene (the Ct value corresponded to the cycle number at which the fluorescence signal of each reaction well reached the preset threshold), the internal reference was used for the normalization process. Relative quantitative analysis of gene expression was performed in accordance with the $2^{-\Delta\Delta Ct}$ method where $2^{-\Delta\Delta Ct}$ represented the relative expression of the target gene.[4] $^{\Delta\Delta}$Ct was calculated according to the following formula:

$^{\Delta\Delta}$Ct = (average Ct value of target gene in the test group − average Ct value of internal reference gene in the test group) − (average Ct value of target gene in the control group − average Ct value of internal reference gene in the control group).

The experimental data were then analyzed.

2.6. Statistical analysis

SPSS version 11.5 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as mean ± standard deviation. The intergroup comparison was performed with analysis of variance and pairwise comparison was performed using the q test, with $\alpha = 0.01$ set as the significance.

Table 2
Thickness of paw pad, volume of paw and AI score.

Groups	Thickness of paw pad (mm)	Volume of paw (mL)	AI score
Control	3.25 ± 0.30	1.24 ± 0.86	–
Model	6.69 ± 0.29*	2.61 ± 0.15*	7.87 ± 0.52*
Tripterygium	5.43 ± 0.26*,**	2.78 ± 0.12*,**	6.42 ± 0.24*,**
TS-RDN	5.37 ± 0.19*,**	2.69 ± 0.11*,**	6.43 ± 0.23*,**
Diosgenin	5.50 ± 0.19*,**	3.20 ± 0.08*,**	6.40 ± 0.13*,**

Results are presented as mean ± standard deviation.
* $p < 0.01$ compared with control group.
** $p < 0.01$ compared with model group.
AI = Arthritis Index; TS-RDN = total saponins from Rhizoma Dioscreae Nipponicae.

3. Results

3.1. Clinical manifestations in paws of rats

After the first immunization, multiple small lesions were observed on the back and tail of rats, which had lustrous coats. On Day 12, the paws were swollen with redness over the limbs, including the knee joint. The swelling was severe in the CIA model group and caused limb deformities, subsequently impairing locomotion. By contrast, in the other groups (except for the control group), swelling and redness were alleviated; no significant changes were observed in the control group. The thickness of the paw pad, hind paw volume, and AI score increased significantly in the model group compared to that in the control group ($p < 0.01$, Table 2).

3.2. Protein expression of Ang-2 in synovial tissues

As shown in Fig. 2A, Ang-2-postive staining was observed as yellow or brown granules, which was mainly observed in the cytoplasm, with slight expression in the nucleus. As shown in Fig. 2B, compared with the control group, the CIA model group was characterized by thickened synovial tissues, infiltration of inflammatory cells, and a 45-fold increase in protein expression of Ang-2 ($p < 0.01$). Compared with that in the CIA model group, the protein expression of Ang-2 in the RDN, TP, and DS groups significantly decreased by 76.7%, 71.7%, and 59%, respectively ($p < 0.01$); however, the difference was not significant among the treatment groups ($p > 0.05$).

3.3. Protein expression of Tie-2 in synovial tissues

As shown in Fig. 3A, Tie-2-positive staining was observed as yellow or brown granules, which were mainly located in the cytoplasm or on the membrane. As shown in Fig. 3B, compared with the control group, the CIA model group was characterized by thickened synovial tissues and infiltration of inflammatory cells. The protein expression of Tie-2 increased by 29.75-fold ($p < 0.01$) compared to that in the control group. Compared with the CIA model group, protein expression of Tie-2 in the RDN, TP, and DS groups decreased; however, the difference was not significant among the treatment groups ($p > 0.05$).

3.4. mRNA expression of VEGF in synovial tissues

Fluorescence intensity curves were generated (Fig. 4A). The real-time PCR products were analyzed using 2% agarose gel electrophoresis (Fig. 4B) and two gene bands were observed at 134 bp and 87 bp. Expression of VEGF target gene in the CIA model group increased by 43.48% compared to that in the control group (Fig. 5; $p < 0.01$). Compared with the CIA model group, expression of VEGF target gene in the RDN, TP, and DS groups decreased significantly ($p < 0.01$) by 59%, 32.67%, and 29%, respectively. Expression of VEGF target gene in the RDN group decreased by 39% and 42.27%,

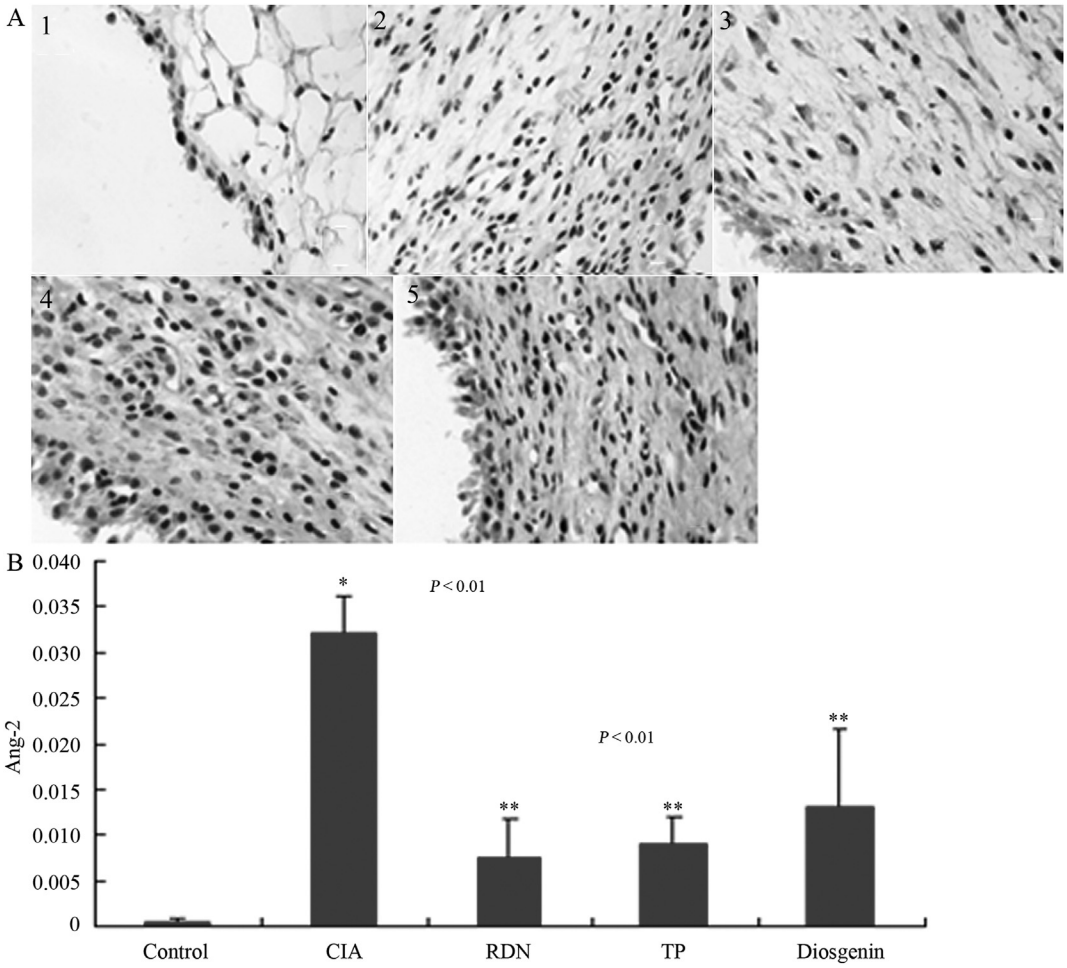

Fig. 2. (A) Expression of Ang-2 in synovial membrane of rats in each group (400×). (1) Normal control group; (2) CIA model group; (3) TS-RDN group (25 mg/kg/d); (4) tripterygium group (12 mg/kg/d); (5) diosgenin group (50 mg/kg/d). (B) Expression of Ang-2 protein is significantly higher in the synovium of rats in the CIA model group, compared with the normal control group. Values are expressed as mean ± SD. * $p < 0.01$; expression of Ang-2 protein is significantly reduced in synovium of rats in the TS-RDN, tripterygium and diosgenin groups compared with the CIA model group. ** $p < 0.01$; expression of Ang-2 protein is similar in the TS-RDN, tripterygium and diosgenin groups. Ang-2 = angiopoietin-2; CIA = collagen-induced arthritis; SD = standard deviation; TS-RDN = total saponins from Rhizoma Dioscreae Nipponicae.

respectively, compared to that in the TP and DS groups ($p < 0.05$).

4. Discussion

RA is a systemic inflammatory disease that involves multiple joints of the body, and the main pathological feature is synovitis. RA is associated with tumor-like growth characteristics such as proliferation of synovial cells, hypertrophy of synovial tissues, infiltration of inflammatory cells, neovascularization, pannus formation, and fibrotic changes. While the etiology and pathogenesis of RA are unknown, angiogenesis has been suggested to play an important role in the early synovial hyperplastic course of RA, and some studies have shown that the numbers of synovial neovessels is positively correlated with the AI integration. Expression of synovial VEGF is positively correlated with the number of neovessels, indicating that VEGF plays an important role in the progression of synovitis.[22] The VEGF family comprises VEGFA, VEGFB, VEGFC and VEGFD, and their receptors Flt-1, KDR, Flt-4, and NRP-1. VEGFs, along with their receptors, promote the migration of endothelial cells and increase the permeability of the endothelial barrier, thereby initiating and maintaining angiogenesis.[23] VEGF induces chemokine production by endothelial cells; chemokines attract the mononuclear cells to invade and release inflammatory cytokines such as tumor necrosis factor-α and interleukin (IL)-6. The interactions among the cytokines further increase the vascular permeability as well as the expression of adhesion factors, enhancing the migration of inflammatory cells towards the inflammation sites, thereby prolonging inflammation.[24] Agents that block expression of VEGF or interfere with the VEGF signal transduction pathway could inhibit angiogenesis and control progression of RA.[25] This study also demonstrated that expression of VEGF in the synovial tissues of the CIA model group was significantly higher than that in the normal group, which confirmed that VEGF had an important role in the pathogenesis of RA.

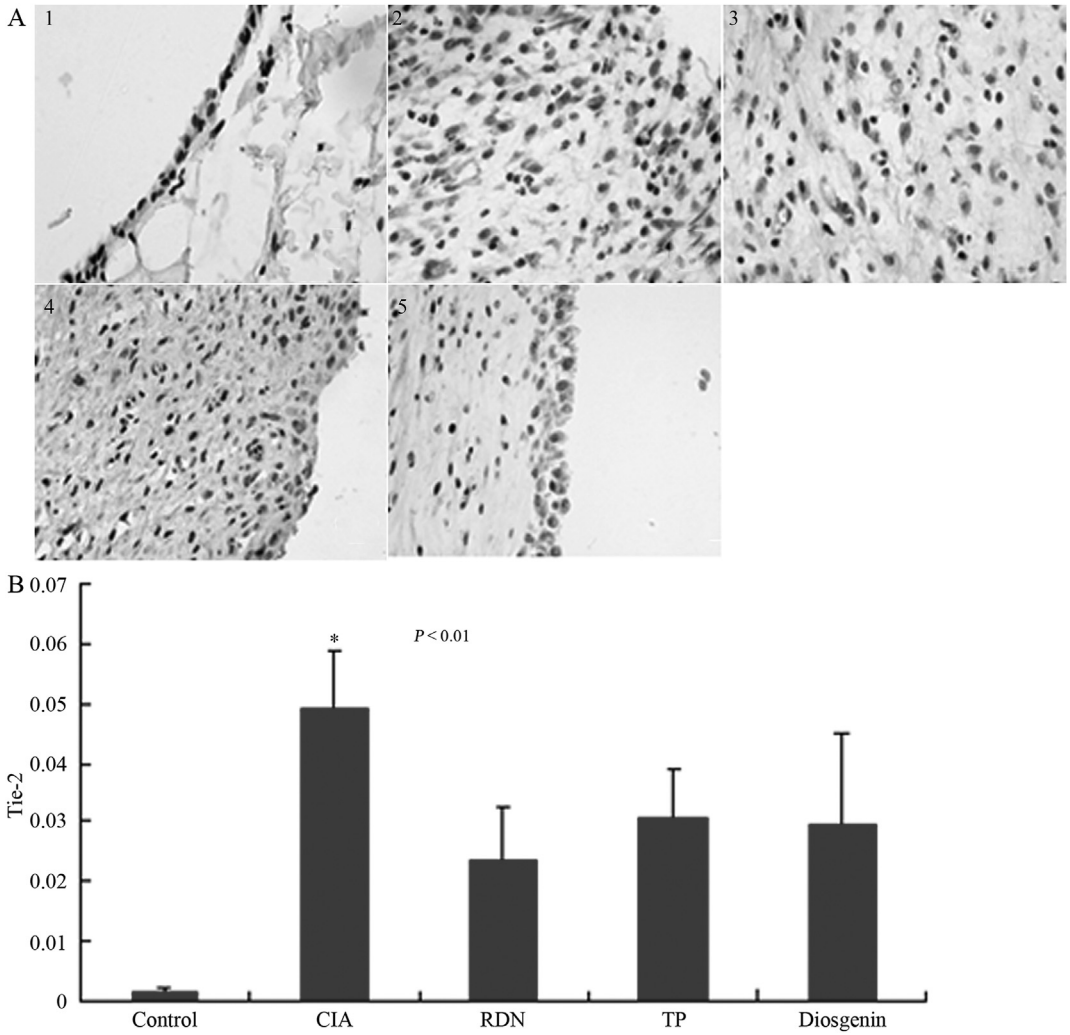

Fig. 3. (A) Expression of Tie-2 in synovial membrane of rats in each group (400×). (1) Normal control group; (2) CIA model group; (3) TS-RDA group (25 mg/kg/d); (4) tripterygium group (12 mg/kg/d); and (5) diosgenin group (50 mg/kg/d). (B) Expression of Tie-2 protein is significantly higher in synovium of rats in the CIA model group, compared with normal control group. Values are expressed as mean ± standard deviation. * $p < 0.01$; expression of Tie-2 protein is similar in the TS-RDA, tripterygium, diosgenin and CIA model groups. CIA = collagen-induced arthritis; Tie-2 = endothelial tyrosine kinase receptor; TS-RDN = total saponins from Rhizoma Dioscreae Nipponicae.

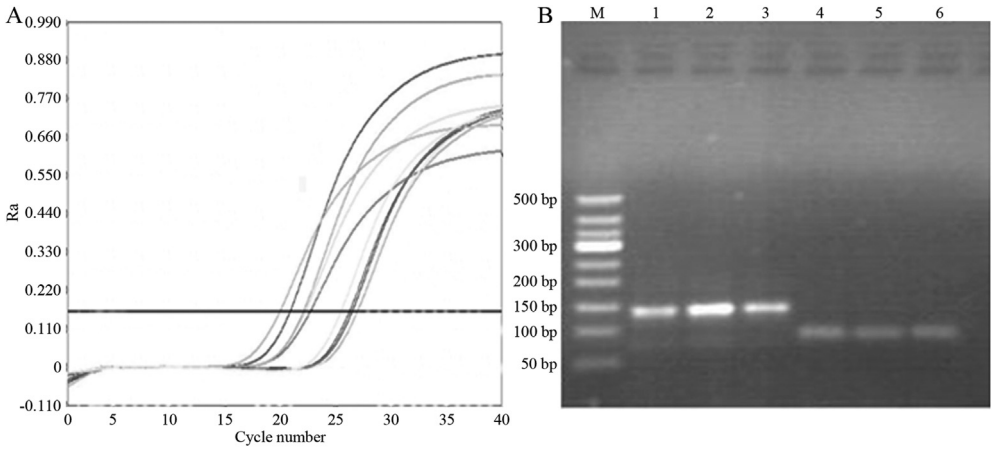

Fig. 4. (A) Amplification curve of VEGF and GAPDH. 1 and 6 normal control group; 2 and 7 CIA model group; 3 and 8 total saponins from Rhizoma Dioscreae Nipponicae group (25 mg/kg/d); 4 and 9 tripterygium group (12 mg/kg/d); and 5 and 10 diosgenin group (50 mg/kg/d). (B) Gel electrophoresis of real-time quantitative reverse transcriptase polymerase chain reaction products. 1−3: GAPDH gene, 4−6: VEGF gene. GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; VEGF = vascular endothelial growth factor.

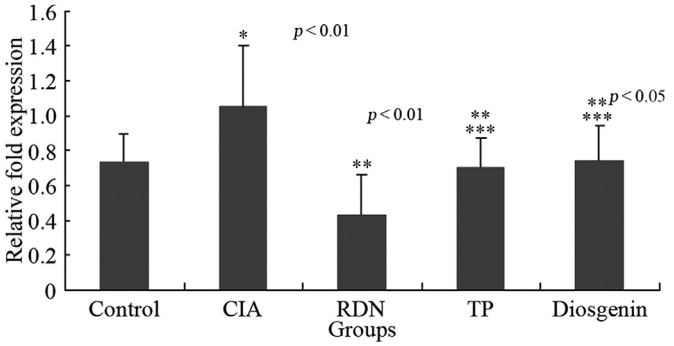

Fig. 5. Expression of VEGF mRNA is significantly higher in synovium in CIA model group, compared with normal control group. Values are expressed as mean ± SD. * $p < 0.01$; expression of VEGF mRNA is significantly reduced in synovium in the TS-RDN (25 mg/kg/d), tripterygium (12 mg/kg/d) and diosgenin (50 mg/kg/d) compared with CIA model groups. ** $p < 0.01$; expression of VEGF mRNA is higher in the tripterygium and diosgenin groups compared with TS-RDN group. *** $p < 0.05$; expression of VEGF mRNA is similar in the tripterygium and diosgenin groups. CIA = collagen-induced arthritis; SD = standard deviation; TS-RDN = total saponins from Rhizoma Dioscreae Nipponicae; VEGF = vascular endothelial growth factor.

Ang is another important factor involved in angiogenesis in RA. The Ang family comprises Ang-1, Ang-2, Ang-3, and Ang-4. Ang-1 and Ang-2 are related to angiogenesis. Ang1 and Tie-2 interact with each other to promote autophosphorylation of the receptor (activation). Endothelial cells expressing activated Tie-2 attract vascular smooth muscle cells, pericytes, and other perivascular cells towards the vascular endothelial cells to form a complete vascular wall, thereby promoting vascular remodeling and maturation, maintaining vascular integrity, and regulating vascular functions. In addition, the effects of Ang-2 on angiogenesis are related to the local microenvironment; in the presence of VEGF and other angiogenic factors, Ang-2 competitively inhibits Ang-1, promoting neoangiogenesis, vascular remodeling, and formation of new capillaries. When the local microenvironment lacks these factors, Ang-2 inhibits Ang-1, contributing to vascular regression.[26] VEGF, Ang-1 and Ang-2 possibly interact synergistically, thus mediating the synovial thickening and pannus formation observed in RA. Therefore, VEGF, Ang-1, and Ang-2 are considered to be important factors in the occurrence and development of synovitis.[16] This study evaluated the expressions of VEGF and Ang-2 in the synovial tissues of CIA model rats and found that they might act synergistically in the pathological process of RA synovitis.

Recently, many studies have reported that tripterygium decreases expression of VEGF in the synovial tissues of CIA rats, thus inhibiting angiogenesis.[27] Therefore, tripterygium glycosides were used as the positive control in this study. TS-RDN is an active ingredient isolated from the dried roots of RDN, which has anti-inflammatory and immunosuppressive effects and therapeutic effects in rat models of adjuvant arthritis. Previous study has shown that TS-RDN inhibits expression of IL-1/IL-6/IL-8/tumor necrosis factor-α in serum and synovial fluid of adjuvant-induced arthritis and CIA.[28] In addition, TS-RDN inhibits T cell lymphopoiesis as well as IL-2 expression *in vitro*.[29] It also inhibits nuclear factor-κB p65/signal transducer and activator of transcription 3/activator protein-1 and VEGF expression.[30,31]

Previous study[32] used conventional reverse transcriptase PCR, which was not a reliable quantitation method owing to large variability. In the present study, real-time fluorescence quantitative PCR was used for analysis of VEGF mRNA expression in rat synovium, and showed that TS-RDN, tripterygium and diosgenin decreased VEGF mRNA expression in the synovial tissues of the CIA rat model. TS-RDN had better effects than tripterygium and diosgenin. TS-RDN contained fat-soluble and water-soluble saponins. Diosgenin is a fat-soluble saponin. The results showed that TS-RDN exhibited better inhibitory effects against VEGF mRNA expression than diosgenin did, suggesting that in addition to the fat-soluble saponins, the water-soluble saponins in TS-RDN were involved in inhibiting the progression of RA. Immunohistochemical staining was performed to detect Ang-2 and Tie-2 in the synovial tissues, and showed that TS-RDN, tripterygium and diosgenin decreased the expression of Ang-2 and Tie-2 in the synovial tissues of the CIA rat model. These results suggest that TS-RDN blocks synovial angiogenesis by inhibiting VEGF and Ang/Tie-2 expression and their synergistic interaction and by reducing the reactivity of vascular endothelial cells toward the angiogenesis factors. This study has provided an experimental evidence for application of TS-RDN to treatment of RA.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (No. 30873420).

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