JTCM Journal of Traditional Chinese Medicine

Online Submissions: http://www.journaltcm.com
ISSN 0255-2922
info@journaltcm.com

Original Article

Efficacy of electroacupuncture stimulating Zusanli (ST36) and Xuanzhong (GB39) on synovial angiogenesis in rats with adjuvant arthritis

JIANG Jianzhen, ZHANG Xin, LUO Zhenguo, SU Chengguo, ZHOU Haiyan, JIANG Yuqing, XIAO Xianjun, CHEN Yunfei, ZHU Jun

JIANG Jianzhen, ZHANG Xin, SU Chengguo, ZHOU Haiyan, JIANG Yuqing, XIAO Xianjun, ZHU Jun, School of Acupuncture-Moxibustion and Tuina, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China
LUO Zhenguo, Vocational Training Department, Acupuncture and Moxibustion School Affiliated to Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China
CHEN Yunfei, Department of Acupuncture, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China
Supported by Natural Science Foundation-funded Project: Study of Electroacupuncture on the Angiogenesis in Rheumatoid Arthritis Rats by HIF-1α / MDM2 / p53 Signaling Pathway (No. 81704152); Study on Immune Mechanisms of Macrophage M1/M2 Polarization in the Treatment of Rheumatoid Arthritis by Moxibustion "trengthening Body Resistance and Eliminating Evil" (No. 81973959); Foundation of Science and Technology Department of Sichuan Province: Study of Electroacupuncture on the Punnus in Rheumatoid Arthritis Rats by HIF-1 α， STAT3, Noth1 Signaling Pathway (No. 2019YJ0491)
Correspondence to: ZHU Jun, School of Acupuncture-Moxibustion and Tuina, the Third Affiliated Hospital, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China. zhujuntcm@163.com; **CHEN Yunfei,** Department of Acupuncture, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China. icyf1968@163.com
Telephone: + 86-28-87689918
DOI: 10.19852/j.cnki.jtcm.20221111.002
Received: April 12, 2022
Accepted: July 21, 2022
Available online: November 11, 2022

Abstract

OBJECTIVE: To investigate the efficacy of electroacupuncture (EA) stimulating Zusanli (ST36) and Xuanzhong (GB39) on synovial angiogenesis in rats with adjuvant arthritis (AA).

METHODS: AA models were established by bilateral injection of Freund's complete adjuvant (FCA) in male Sprague-Dawley rats. Three days after injection, rats were given EA at Zusanli (ST36) and Xuanzhong (GB39) acupoints, once every other day, for 16 d. The arthritis index score, paw volume, and hematoxylin-eosin (HE) staining was performed for each animal. Angiogenesis marker cluster of differentiation 34 (CD34) expression and synovial cell apoptosis in synovial tissue were observed. The levels of Notch1, hairy and enhancer of split homolog-1 (Hes1), transforming growth factor-beta (TGF-β) and basic fibroblast growth factor (bFGF) were subsequently detected.

RESULTS: We found that EA significantly decreased arthritis index scores, paw volume, and HE staining scores. EA could significantly inhibit the expression of CD34, promoting apoptosis of synovial cells in the joint synovial tissue of AA rats. The expression of Notch1 signaling pathway proteins and mRNAs (Notch1, Hes1, TGF-β, and bFGF) were markedly downregulated by EA treatment.

CONCLUSIONS: These results prove that EA attenuates synovial angiogenesis by inhibiting the Notch1 signaling pathway in AA rat models. Based on our findings, we propose that EA is a promising complementary and alternative therapy in rheumatoid arthritis.

Keywords: electroacupuncture; arthritis, experimental; synovial angiogenesis; receptor, Notch1; signal transduction

1. INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, progressive, autoimmune disease with a very high disability rate, occurring in about 5 per 1000 people.[1] Synovial angiogenesis is a crucial contributing factor of RA, facilitating an influx of macrophages and lymphocytes that transform synovial cells into an invasive tumor-like pannus, inducing cartilage erosion and ultimately causing disability.[2,3] Indeed, there are large numbers and densities of synovial angiogenesis in RA, which provides the expansion of synovial tissue and develops the invasive nature of the RA synovial membrane and increases local pain receptors that cause structural damage and pain. Synovial cells also seem an active target for cytokines, chemokines, and permeability factors. There is a positive feedback loop between

synovial inflammation and angiogenesis.[4,5] Therefore, synovial angiogenesis plays an indispensable role in the development of RA.

Notch signaling molecules are highly expressed in synovium from RA patients, regulating cell proliferation, differentiation, and death.[6,7] The Notch1 signaling pathway is activated by the binding of its ligands to its receptors, leading to cleavage of the Notch receptor and release of the Notch intracellular domain, which translocates into the nucleus and activates transcription of the Notch target gene, hairy and enhancer of split homolog-1 (Hes1), which induces the production of pro-inflammatory cytokines and pro-angiogenic factors, transforming growth factor-beta (TGF-β) and basic fibroblast growth factor (bFGF), and participates in the development of RA.[8] Notch1 is overexpressed in RA-activated macrophages and synovial tissues. Inhibition of Notch1 signaling with Notch1 targeting siRNAs has been shown to reduce the severity of inflammatory arthritis and downregulate pro-inflammatory cytokines in mouse models of arthritis.[9] Notch1 signaling plays an important role in the process of angiogenesis, activates angiogenic regulators, and mediates hypoxia-induced angiogenesis in RA patients.[10,11] These researches reveal that Notch1 signaling is a potential therapeutic target in RA therapy.

Electroacupuncture (EA), as an adjuvant therapy, effectively reduces the suffering and improves the quality of life of RA patients.[12,13] EA is a prospective adjuvant therapy for RA clinical therapeutics, as it could inhibit the expression of inflammatory markers, interleukin-17 (IL-17), C-reactive protein, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF).[14] However, its molecular mechanism remains to be revealed. Animal experiments also proved that EA reduced inflammation pain intensity and inhibited synovitis and osteoclastogenesis in the RA model.[15,16] Our previous research indicated that EA attenuated the synovial angiogenesis by upregulating the p53 signaling pathway and downregulating hypoxia-inducible factor-1α (HIF-1α)/VEGF expression in a rat model of adjuvant arthritis (AA).[17,18] Furthermore, HIF-1α directly regulates the expression of Notch1in RA synovial fibroblasts.[7] Nevertheless, it is still not clear how EA inhibits synovial angiogenesis in RA rats. Therefore, the present study aimed to verify the hypothesis that EA could inhibit synovial angiogenesis via modulating the Notch1 signaling pathway to suppress the inflammatory responses in RA rats.

2. MATERIALS AND METHODS

2.1. Animals

Thirty-two male Sprague-Dawley (*SD*) rats (6 weeks of age, 180 g), provided by the Animal Experiment Center of Chengdu University of Traditional Chinese Medicine, were dwelled in a pathogen-free environment. A 12 h light-dark cycle, temperature of (23 ± 3) ℃, and humidity of 60% ± 10% were maintained, and rats had free access to standard rodent chow and water. All animal experiments were designed according to the principles of the 3Rs (Replacement, Reduction, and Refinement) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the Scientific Investigation Board of the Chengdu University of Traditional Chinese Medicine, Chengdu, China (approval No. CUTCM-2018-05).

2.2. Experimental design and induction of AA

SD rats were randomly divided into four groups (i.e., Control, AA, AA + EA, and AA + sham EA), with eight rats in each group. The rat models of AA were established as described previously.[19] All rats were anesthetized with an intraperitoneal injection of pentobarbital (38 mg/kg). AA rats were injected with 0.1 mL Freund's complete adjuvant (FCA, Product Number: F5881, Sigma, St. Louis, MO, USA) in the bilateral hind footpads, while rats in the Control group were injected with 0.1 mL saline. Three days after FCA injection, the degree of swelling of the joints was observed and scored (maximum score 5 points) according to previous research. A score > 3 points indicated that the AA model was established.[20]

Additionally, rats in the AA + EA group were given EA treatment, and rats in the AA + sham EA group were subjected to electrical stimulation at a nonacupoint. Rats were sacrificed on the 18th day, and the synovial tissues in bilateral ankles were sampled for further detection.

2.3. EA treatment

Three days after FCA injection, EA treatment was performed as described previously.[17] In brief, two stainless acupuncture needles (0.25 mm × 25 mm) were inserted plumb at Zusanli (GB39, 3 mm depth) and Xuanzhong (ST36, 6 mm depth). The handle of the needle was further connected to an EA treatment instrument (Hwato SDZ-Ⅱ, Suzhou Medical Products Factory Co., Ltd., Suzhou, China). Finally, a constant electrical stimulus (2 Hz, 0.2 ms pulse width, intensity: 6-7 mA) was applied continuously for 15 min. Rats in the AA + sham EA group received the same electrical stimulation procedures at 10 mm lateral to the side of Zusanli (GB39) and Xuanzhong (ST36) (2 mm depth). The EA and AA + sham EA groups received the intervention once every other day, for 16 d.

2.4. Arthritis index scoring and paw volume

The arthritis index score was evaluated 3 d after the FCA injection. According to the arthritis index scoring system, the rats were scored on the 3rd, 8th, 13th, and 18th days after the injection of FCA reagent, respectively, by two independent experimenters. The arthritis index score was assessed using a scoring system with five grades: 0, no swelling; 1, swelling of finger joints; 2, mild swelling of the ankle or wrist joints; 3, severe inflammation of the entire paws; and 4, paws with deformity or ankylosis. The mean values of arthritis index scores at each time

point were analyzed and plotted. The higher the score is, the more severe the disease is. The paw volume of the left hind paw of the rats was measured by volume drainage method using a plethysmograph apparatus (YLS-7A, Yiyan Sci Ltd., Jinan, China) every 5 d for 18 d.

2.5. Hematoxylin-eosin (HE) staining

The isolated ankle joint was washed in phosphate-buffered saline, fixed with 4% paraformaldehyde, and embedded in paraffin wax. After sectioning, 4-μm thick sections were deparaffinized in xylene followed by rehydration through an ethanol gradient. HE staining was performed before histopathological examination by a blinded assessor (microscope magnification: ×200). Three fields of each sample were quantified by 2 investigators who were blinded to the treatments according to previous literature report.[18]

2.6. Cluster of differentiation 34 (CD34) immunohistochemistry

Briefly, paraffin sections (4-μm thick) were deparaffinized with xylene, rehydrated, and subjected to microwave antigen retrieval. Sections were incubated with the primary antibody of CD34 (1∶200, Abcam, Cambridge, UK) at 4 ℃ for 12 h. The sections were subsequently incubated with secondary antibodies for 50 min, followed by 3, 3 diaminobenzidine (DAB) staining. CD34 expression was recorded under a 200-fold light microscope. Three fields of each sample were quantified by 2 investigators who were blinded to the treatments.

2.7. Terminal deoxynucleotidyl transferase (Tdt) dUTP nick-end labeling (TUNEL)

According to the manufacturer's protocol, synovial cell apoptosis was determined using a TUNEL assay (Roche Diagnostics, Mannheim, Germany). In brief, slices were washed with phosphate buffer saline and fixed with 4% paraformaldehyde. Sections were incubated for 30 min in the dark and then incubated in a TUNEL reaction mixture for 60 min at 37 ℃. Finally, the slides were counterstained with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) and mounted in fluorescence mounting medium. Four images were randomly captured by a fluorescence microscope (×200, Olympus BX53; Olympus Corporation, Tokyo, Japan) to quantify the number of apoptotic cells. TUNEL + nuclei were quantified by automatic counting using Image-pro plus 6.0 (Media Cybernetics, Rockville, MD, USA) software.

2.8. RNA isolation and real-time quantitative polymerase chain reaction (PCR)

Total RNA from synovial tissue was extracted using an RNA extraction kit (cat. No. G3013; Servicebio, Wuhan, China) according to the manufacturer's instructions. cDNA was subsequently synthesized from total RNA using the cDNA Synthesis Kit (cat. no. #K1622; Thermo, Mannheim, Germany). Real-time quantitative PCR was performed with an SYBR-green detection kit (cat. No. 04913914001; Roche, Basel, Switzerland) on an ABI Stepone plus real-time PCR System (Applied Biosystems, Waltham, MA, USA). β-actin was used as a reference gene for the normalization of different transcript values. The PCR cycle was as follows: 95 ℃/10 min, 40 cycles of 94 ℃/15 s, 60 ℃/60 s, 72 ℃/30 s, and 72 ℃/5 min. The primer sequences for Notch1, Hes1, TGF-β, bFGF, and β-actin are listed in Table 1. All PCR assays were performed in triplicate. The relative mRNA level was calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.9. Western blot

A protein concentration assay kit determined the protein concentrations in the synovial tissue (Servicebio, Wuhan, China). Samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Applygen, Beijing, China) and electrically transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% bull serum albumin for 2 h and incubated overnight at 4 ℃ with primary antibodies directed against Notch1 (cat. No. ab52301; abcam, Cambridge, UK; in dilution 1∶1000), Hes1 (cat. No. GB11374; Servicebio, Wuhan, China; in dilution 1∶1000), TGF-β (cat. No. GB11179; Servicebio, Wuhan, China; in dilution 1∶1000), bFGF (cat. No. bs-0217R; Bioss, Wuhan, China; in dilution 1∶1000), and GAPDH (cat. No. Mab5465-100; Multi sci, Hangzhou, China; in dilution 1∶1000). The membranes were incubated with secondary antibodies (cat. No. GB23303; Servicebio, Wuhan, China; in dilution 1∶1000) for 2 h

Table 1 Primers used for real-time quantitative PCR in this study

Gene		Primers sequence (5'-3')
β-Actin	Forward	TGTCACCAACTGGGACGATA
	Reverse	GGGGTGTTGAAGGTCTCAAA
Notch1	Forward	TGCCGAGTGTGAGTGGGATGG
	Reverse	AAGTGGAAGGAGTTGTTGCGTAGC
Hes1	Forward	TCCTGACGGCCAATTTGCTTTCC
	Reverse	CTGGAAGGCGACACTGCGTTAG
TGF-β	Forward	GACCGCAACAACGCAATCTATGAC
	Reverse	CTGGCACTGCTTCCCGAATGTC
bFGF	Forward	GAGCGACCCACACGTCAAACTAC
	Reverse	CAGCCGTCCATCTTCCTTCATAGC

Notes: PCR: polymerase chain reaction; Hes1: hairy and enhancer of split homolog-1; TGF-β: transforming growth factor-beta; bFGF: basic fibroblast growth factor.

at 25 ℃ and visualized using the chemidoc™ XRS Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.10. Statistical analysis

Statistical analysis was performed by SPSS v.20.0 software (IBM Corp., Armonk, NY, USA). Multi-group comparison was performed using a one-way Analysis of Variance (ANOVA) test followed by Tukey or Dunnett's T3 multiple comparison tests. ANOVA of repeated measurement data was used to measure arthritis index scores and paw volume. All data were expressed as mean ± standard deviation ($\bar{x} \pm s$). P values < 0.05 were considered statistically significant.

3. RESULTS

3.1. EA reduces arthritis index scores and paw volume in AA rats

Compared with the Control group, the scores of the AA and AA + sham EA groups increased significantly ($P > 0.05$) and did not decrease over time. However, compared with the AA group and the AA + sham EA group, EA treatment can effectively reduce the arthritis index score of AA rats ($P < 0.05$). Compared with the AA group, there was no significant difference in the scores of AA + sham EA group ($P > 0.05$) (Table 2).

The AA group and the AA + sham EA group scores were significantly higher than those of the control group ($P > 0.05$). However, compared with the AA and AA + sham EA groups, EA treatment can effectively reduce the paw volume of AA rats ($P < 0.01$). Compared with the AA group, there was no significant difference in the paw volume of AA + sham EA group ($P > 0.05$) (Table 2).

3.2. EA reduces HE staining scores of synovial tissue in AA rats

HE staining analysis revealed no signs of inflammatory infiltration and synovial cell proliferation in the ankle joint of Control group (Figure 1A). By comparison, animals in the AA group displayed a massive accumulation of inflammatory cells in the synovial tissue and synovial cell proliferation (Figure 1B). The FCA-induced joint inflammation was significantly inhibited by EA treatment but not sham EA treatment (Figure 1C, 1D).

To identify the effect of EA on FCA-induced inflammatory response in AA rats, H&E staining scores were quantified (Figure 1E). HE staining scores were increased in the AA and AA + sham EA groups ($P < 0.01$), while the scores were reduced by EA treatment ($P < 0.01$). Compared with the AA group, there was no significant difference in the scores of AA + sham EA group ($P > 0.05$).

3.3. EA decreases CD34 expression in synovial tissue of AA rats

CD34 is a characteristic marker of synovial angiogenesis.[21] Immunohistochemistry was used to detect the effect of EA on CD34 protein expression of synovial tissue. Only a relatively small amount of CD34 was expressed in the synovial tissue of the Control group rats (Figure 2A). By contrast, CD34 was highly expressed in rat synovial tissue of the AA group (Figure 2B), indicating that angiogenesis was significantly increased ($P < 0.01$, Figure 2E). EA treatment significantly reduced the expression of CD34 in rat synovial tissue of the AA group ($P < 0.01$, Figure 2C, 2E), while sham EA intervention did not show the same influence ($P > 0.05$, Figure 2D, 2E). These results indicated that EA treatment significantly inhibited synovial angiogenesis.

3.4. EA improves the apoptosis rate of synovial cells in AA rats

The proliferation of synovial cells is an indispensable basis of angiogenesis.[22] The TUNEL assay was used to detect whether EA promoted the apoptosis rate of synovial cells. Our analysis displayed that the number of cells expressing green signals increased significantly in AA rats. Moreover, EA intervention further increased the number of cells expressing green signals (Figure 3A).

To clarify the effect of EA on the proliferation of synovial cells in AA rats, the apoptosis rate of synovial cells was calculated (Figure 3B). Compared with the

Table 2 Effects of EA on arthritis index scores and paw volume in synovial tissue of ankle joint in AA rats ($n = 8$, $\bar{x} \pm s$)

Index	Group	Days after FCA injection				
		0	3rd	8rd	13rd	18rd
Arthritis index scores	Control	-	0	0	0	0
	AA	-	6.75±0.70	7.50±0.53	7.50±0.53	7.62±0.51[a]
	AA+EA	-	7.75±0.46	7.37±0.51	6.25±0.46	5.50±0.53[ab]
	AA+sham EA	-	7.37±0.51	7.25±0.46	6.87±0.35	6.75±0.70[a]
Paw volume (mL)	Control	1.20±0.11	1.23±0.12	1.29±0.09	1.33±0.10	1.38±0.14[c]
	AA	1.17±0.09	1.83±0.10	1.98±0.15	2.09±0.17	2.24±0.19[a]
	AA+EA	1.20±0.13	1.83±0.09	2.02±0.13	1.97±0.15	1.89±0.13[ac]
	AA+sham EA	1.21±0.12	1.87±0.15	2.09±0.11	2.13±0.14	2.16±0.16[a]

Notes: Three days after injection of FCA to make AA rats model. Control group and AA group: only fixed on self-made fixator; AA + EA group: treated with EA; AA + sham EA Group: treated with sham EA; Treatment course: once every other day, 15 min each time, lasting for 16 d. The toe volume was measured at different times for comparison. EA: electroacupuncture; FCA: freund's complete adjuvant; AA: adjuvant arthritis. Compared with the control group, [a]$P < 0.01$; compared with the AA group, [b]$P < 0.05$, [c]$P < 0.01$.

Control group, the apoptosis rate of synovial cells increased markedly in the AA group ($P < 0.01$). However, compared with the AA group, EA stimulation led to a greater increase in the apoptosis rate of synovial cells ($P < 0.01$). Additionally, there was no significant difference in the apoptotic cell ratio between the AA + sham EA and AA groups ($P > 0.05$).

3.5. EA reduces the expression of the Notch1 singling pathway proteins and mRNAs in synovial tissue

The expressions of Notch1, Hes1, TGF-β, and bFGF were detected by real-time quantitative PCR and Western blotting. The results showed that the expression of Notch1, Hes1, TGF-β, and bFGFmRNA in the AA group was significantly higher than that in the Control group ($P < 0.01$, Table 3). However, Notch1, Hes1, TGF-β, and bFGF mRNA expression levels were markedly reduced in the AA + EA group compared with the AA group ($P < 0.01$). Compared with the AA + sham EA group, EA intervention also can significantly reduce the mRNA expression of Notch1singling pathway. Nevertheless, compared with the AA group, there was no significant difference in the mRNA expression of AA + sham EA group ($P > 0.05$).

The protein expression levels of Notch1, Hes1, TGF-β, and bFGF in the AA group were significantly higher than those in the Control group ($P < 0.01$, Figure 4). However, the protein expression of Notch1, Hes1, TGF-β, and bFGF in the AA + EA group was markedly reduced than that in the AA group ($P < 0.01$). Compared with the AA + sham EA group, EA intervention also can significantly reduce the protein expression of Notch1 singling pathway ($P < 0.01$). Compared with the AA group, there was no significant difference in the protein expression of AA + sham EA group ($P > 0.05$). Therefore, the above results demonstrated that the EA intervention significantly reduced the expression of the Notch1 singling pathway in synovial tissue.

4. DISCUSSION

The present study suggests that EA can effectively inhibit arthritis in AA rats, attenuate CD34 expression of synovial angiogenesis, promote synovial cell apoptosis rate, and decrease the expression levels of Notch1 and its downstream factors, Hes1, TGF-β, and bFGF.

EA stimulation has a good clinical effect on RA patients, and the related molecular mechanism remains to be explored.[13,14] Clinical studies have also confirmed that the clinical efficacy of EA stimulation on corresponding

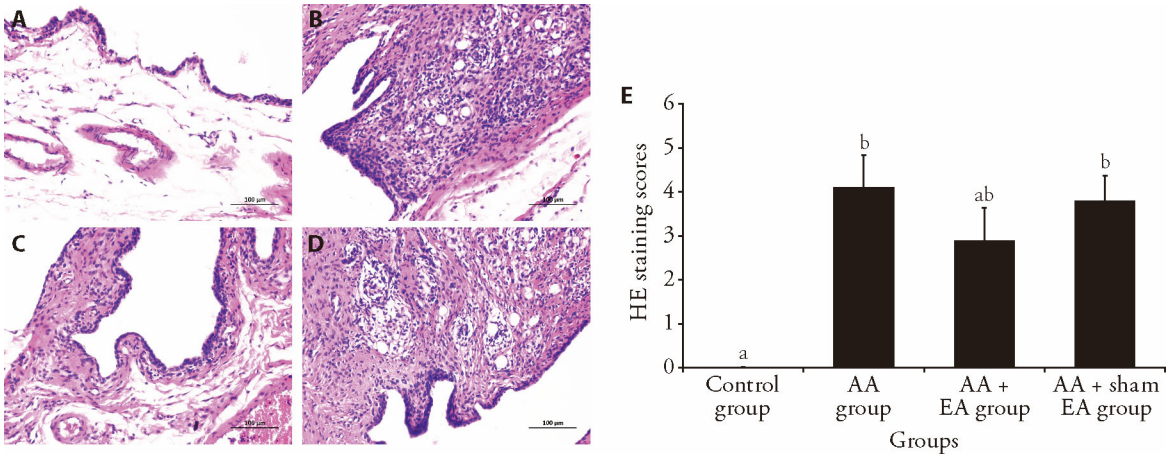

Figure 1 Effects of EA on HE staining scores in synovial tissue of the ankle joint in AA rats

A-D: HE staining pictures of groups Control, AA, AA+ EA and AA+ sham EA group (×200); E: HE staining scores. Representative HE-stained sections from the Control group, demonstrating no signs of inflammatory infiltration and synovial cells proliferation (A), the AA group and the AA + sham EA group illustrating massive accumulation of inflammatory cells, synovial proliferation and angiogenesis (B, D); and the AA + EA group, demonstrating less inflammatory cell infiltration, synovial proliferation and angiogenesis in the ankle joint (C). EA: electroacupuncture; HE: hematoxylin-eosin; AA: adjuvant arthritis. Data are shown as mean ± standard deviation ($n = 8$). Compared with the AA group, $^{a}P < 0.01$; compared with the Control group, $^{b}P < 0.01$.

Table 3 Effects of EA on Notch1 signaling mRNA expression in synovial tissue ($\bar{x} \pm s$)

Group	*n*	Notch1	Hes1	bFGF	TGF-β
Control	8	1.1±0.4[a]	1.2±0.4[a]	1.0±0.4[a]	1.0±0.4[a]
AA	8	52.4±17.1[b]	58.8±21.7[b]	73.2±18.9[b]	43.7±15.8[b]
AA+EA	8	4.1±1.9[ab]	5.6±1.7[ab]	5.4±1.9[ab]	3.0±1.7[ab]
AA+sham EA	8	30.8±11.9[b]	38.0±16.1[b]	49.5±17.1[b]	25.0±9.0[b]

Notes: three days after injection of FCA to make AA rats model. Control group and AA group: only fixed on self-made fixator; AA + EA group: treated with EA; AA + sham EA Group: treated with sham EA; Treatment course: once every other day, 15 min each time, lasting for 16 d. EA: electroacupuncture; FCA: freund's complete adjuvant; AA: adjuvant arthritis; Hes1: hairy and enhancer of split homolog-1; bFGF: basic fibroblast growth factor; TGF-β: transforming growth factor-beta. Compared with the Control group, $^{b}P < 0.01$; compared with the AA group, $^{a}P < 0.01$.

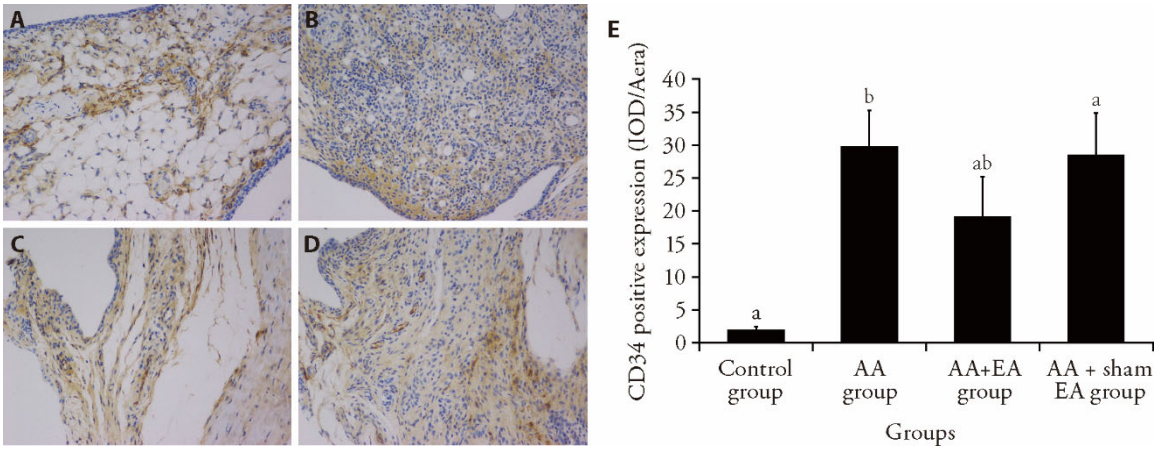

Figure 2 Effects of EA on CD34 expression in synovial tissue of the ankle joint in AA rats
A-D: CD34 immunohistochemistry pictures of groups .A: Control; B: AA; C: AA + EA; D: AA + sham EA group (×200); E: CD34 positive expression (IOD/Aera). HE-stained sections from the Control group, demonstrating CD34 was only expressed in a relatively small amount in the synovial tissue, the AA group and the AA + sham EA group illustrating a large number of CD34 expression in the synovial tissue, and the AA + EA group, demonstrating a less number of CD34 expression in the synovial tissue. Mean inflammation score (+*SD*) on a 3-point scale. EA: electroacupuncture; CD34: cluster of differentiation 34; AA: adjuvant arthritis; HE: hematoxylin-eosin. Data are shown as mean ± standard deviation (*SD*) (n = 8). Compared with the AA group, $^{a}P < 0.01$; compared with the Control group, $^{b}P < 0.01$.

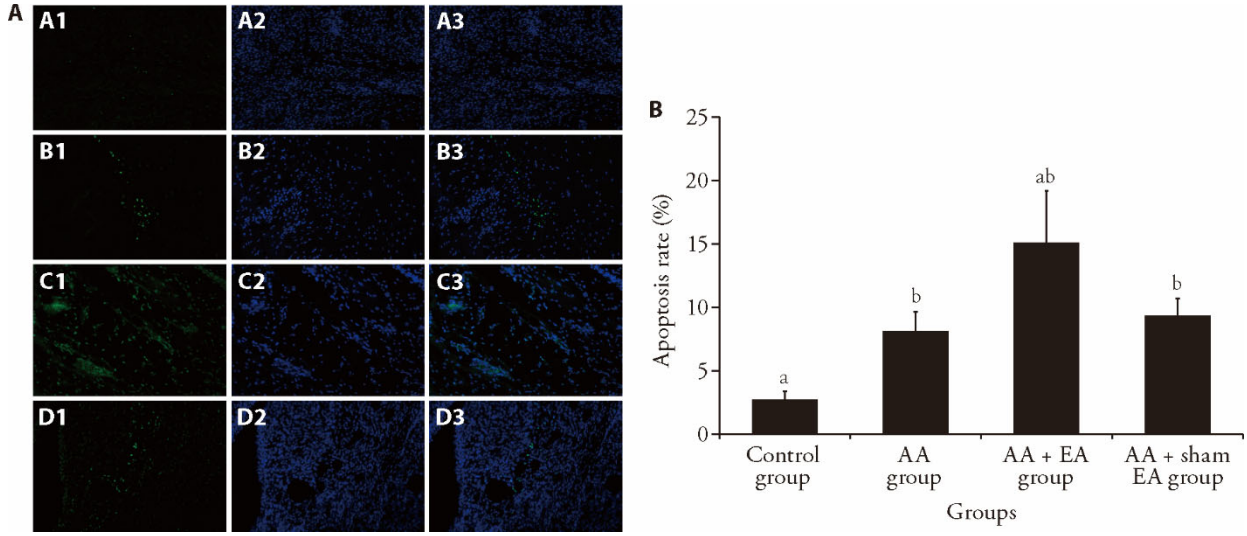

Figure 3 Effects of EA on the apoptosis rate of synovial cells in synovial tissue of the ankle joint in AA rats
A1-D1: TUNEL assay; A2-D2: counterstained with DAPI; A3-D3: merge pictures; A1-A3: pictures belonging to control group (×200); B1-B3: pictures belonging to AA group; C1-C3: pictures belonging to AA + EA group; D1-D3: pictures belonging to AA + EA + sham group. B: apoptosis rate. EA: electroacupuncture; AA: adjuvant arthritis; TUNEL: terminal deoxynucleotidyl transferase (Tdt) dUTP nick-end labeling; DAPI: 4′,6-diamidino-2-phenylindole. Data are shown as mean ± standard deviation (n = 8). Compared with the AA group, $^{a}P < 0.01$; compared with the Control group, $^{b}P < 0.01$.

acupoints is significantly better than that of non-acupoints.[23] Our research also suggests that the EA intervention's inhibition effect at Xuanzhong (ST36) and Zusanli (GB39) is significantly better than that of sham EA intervention in arthritic rats, consistent with previous reports.[17]

Angiogenesis is a characteristic sign of the progression of RA and is also a potential target for therapeutic intervention.[24] Studies have confirmed that inflammatory cytokines cause hypoxia in the synovium, which in turn leads to the proliferation of synovial cells and an abnormal increase in blood vessels in the synovial tissue; inflammatory factors further erode chondrocytes through new blood vessels and destroy the joint cavity.[25] Our previous studies have preliminarily confirmed that EA has a significant effect on synovial inflammation in arthritic rats and has a significant intervention effect on synovial cell proliferation and angiogenesis.[17,18] This study also confirmed that EA could inhibit the expression of CD34, a characteristic marker of synovial angiogenesis, through Notch1 and its downstream molecules.

The occurrence of Notch1 signaling-induced angiogenesis in RA patients suggests that Notch1 is a target of RA treatment.[26,27] Notch1 signaling transmits signals to the nucleus through its downstream signaling molecule, Hes1, prompting synovial cells and vascular endothelial cells, among others, to produce many pro-

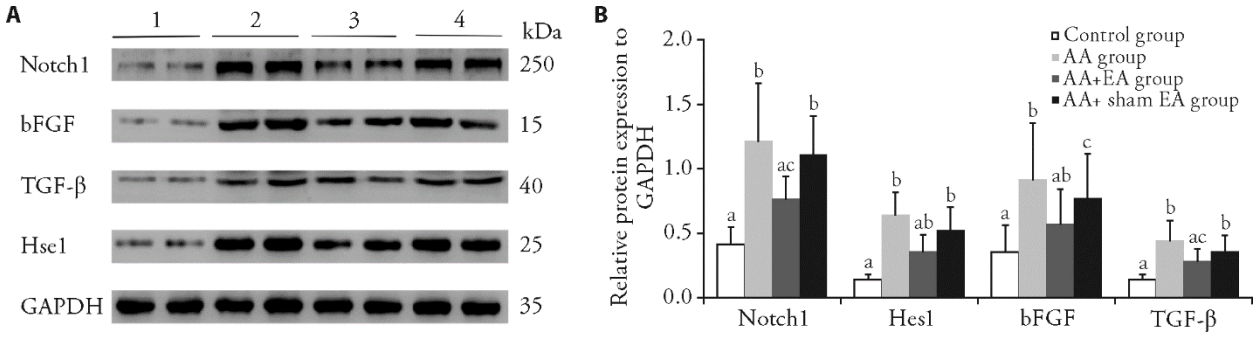

Figure 4 Effects of EA on Notch1 signaling protein expression in synovial tissue.
A: Western blot was used to detect the expression of Notch1, bFGF, TGF-β and Hes1; B: relative protein expression to GAPDH. 1: control group; 2: AA group; 3: AA + EA group; 4: AA + sham EA group. EA: electroacupuncture; FCA: freund's complete adjuvant; AA: adjuvant arthritis; Hes1: hairy and enhancer of split homolog-1; bFGF: basic fibroblast growth factor; TGF-β: transforming growth factor-beta; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Data are shown as mean ± standard deviation (n = 8). Compared with the AA group, $^{a}P < 0.01$; compared with the Control group, $^{b}P < 0.01$ and $^{c}P < 0.05$.

angiogenic factors, such as TGF-β and bFGF.[28] Pro-angiogenic factors cooperate with VEGF to induce excess new blood vessels in the synovium. Pro-angiogenic factors are formed in the medium and transmit foreign inflammatory factors, such as TNF-α, IL-17, and matrix metalloenzymes protein-3 into cartilage and bone, and then destroys cartilage and joints.[29,30] In this animal experiment, Notch1 and its downstream signal factors, Hes1, TGF-β, and bFGF, showed a high expression state, consistent with previous findings.

EA intervention has a significant protective effect on animal models through the Notch1 signal pathway. Research has shown that acupuncture treatment protects cerebral hemorrhage by inhibiting the Notch1 signaling pathway in rats with cerebral hemorrhage.[31] EA treatment improved learning and memory function by downregulating the Notch1 signaling pathway in an Alzheimer's disease rat model.[32] In this animal experiment, EA intervention inhibited the expression of CD34, increased the apoptosis rate of synovial cells, and achieved a protective effect on AA rats by reducing the expression of Notch1 and its downstream signal factors (Hes1, TGF-β, and bFGF).

In conclusion, our findings suggest that EA treatment attenuates synovial angiogenesis in AA rats, probably related to the inhibition of the Notch1 signaling pathway. These results provide a new perspective on the molecular mechanism of EA attenuating synovial angiogenesis, implicating EA as a potential complementary therapy for RA patients.

5. REFERENCES

1. Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis: a review. JAMA 2018; 320: 1360-72.
2. Veale DJ, Orr C, Fearon U. Cellular and molecular perspectives in rheumatoid arthritis. Semin Immunopathol 2017; 39: 343-54.
3. Elshabrawy HA, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The pathogenic role of angiogenesis in rheumatoid arthritis, Angiogenesis 2015; 18: 433-48.
4. Hao F, Wang Q, Liu L, et al. Effect of moxibustion on autophagy and the inflammatory response of synovial cells in rheumatoid arthritis model rat. J Tradit Chin Med 2022; 42: 73-82.
5. MacDonald IJ, Liu SC, Su CM, Wang YH, Tsai CH, Tang CH. Implications of angiogenesis involvement in arthritis. Int J Mol Sci 2018; 19: 2012.
6. Sun W, Zhang H, Wang H, et al. Targeting Notch-activated M1 macrophages attenuates joint tissue damage in a mouse model of inflammatory arthritis. J Bone Miner Res 2017; 32: 1469-80.
7. Jiao Z, Wang W, Guo M, et al. Expression analysis of Notch-related molecules in peripheral blood T helper cells of patients with rheumatoid arthritis. Scand J Rheumatol 2010; 39: 26-32.
8. Keewan E, Naser SA. Notch-1 signaling modulates macrophage polarization and immune defense against mycobacterium avium paratuberculosis infection in inflammatory diseases. Microorganisms 2020; 8: 1006.
9. An L, Li Z, Shi L, et al. Inflammation-targeted celastrol nanodrug attenuates collagen-induced arthritis through NF-κB and Notch1 pathways. Nano Lett 2020; 20: 7728-36.
10. Liu Q, Allen TD, Song W, Wada Y, Lobe CG, Liu J. Notch1 activates angiogenic regulator Netrin4 in endothelial cells. J Cell Mol Med 2019; 23: 3762-66.
11. Gao W, Sweeney C, Walsh C, et al. Notch signalling pathways mediate synovial angiogenesis in response to vascular endothelial growth factor and angiopoietin 2. Ann Rheum Dis 2013; 72: 1080-88.
12. Zeng C, Bai X, Qin H, Wang H, Rong X, Yan J. Effect of adjuvant therapy with electroacupuncture on bone turnover markers and interleukin 17 in patients with rheumatoid arthritis. J Tradit Chin Med 2019; 39: 582-6.
13. Zhou Y, Zhu J, Li LB, et al. Effects of electroacupuncture on joint function in rheumatoid arthritis patients of liver-and kidney-*Yin* deficiency type. Zhen Ci Yan Jiu 2016; 41: 440-6.
14. Ouyang BS, Gao J, Che JL, et al. Effect of electro-acupuncture on tumor necrosis factor-α and vascular endothelial growth factor in peripheral blood and joint synovia of patients with rheumatoid arthritis. Chin J Integr Med 2011; 17: 505-9.
15. Gusmão JNFM, Fonseca KM, Ferreira BSP, et al. Electroacupuncture reduces inflammation but not bone loss on periodontitis in arthritic rats. Inflammation 2021; 44: 116-28.
16. Du ZH, Zhang CW, Xie WX, et al. Adenosine A2A receptor mediates inhibition of synovitis and osteoclastogenesis after electroacupuncture in rats with collagen-induced arthritis. Evid Based Complement Alternat Med 2019; 2019: 4617464.
17. Zhu J, Su CG, Chen YZ, Hao X, Jiang JZ. Electroacupuncture on ST36 and GB39 acupoints inhibits synovial angiogenesis *via* downregulating HIF-1α/VEGF expression in a rat model of adjuvant arthritis. Evid Based Complement Alternat Med 2019; 2019: 5741931.
18. Su CG, Chen YZ, Chen YF, et al. Effect of electroacupuncture at the ST36 and GB39 acupoints on apoptosis by regulating the p53 signaling pathway in adjuvant arthritis rats. Mol Med Rep 2019;

20: 4101-10.
19. Leech M, Metz C, Santos L, et al. Involvement of macrophage migration inhibitory factor in the evolution of rat adjuvant arthritis. Arthritis Rheum 1998; 41: 910-17.
20. Ierna M, Kerr A, Scales H, Berge K, Griinari M. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. BMC Musculoskelet Disord 2010; 11: 136.
21. Majchrzak K, Kaspera W, Szymaś J, Bobek-Billewicz B, Hebda A, Majchrzak H. Markers of angiogenesis (CD31, CD34, rCBV) and their prognostic value in low-grade gliomas. Neurol Neurochir Pol 2013; 47: 325-31.
22. Balogh E, Biniecka M, Fearon U, Veale DJ, Szekanecz Z. Angiogenesis in inflammatory arthritis. Isr Med Assoc J 2019; 5: 345-52.
23. Liu Z, Liu Y, Xu H, et al. Effect of electroacupuncture on urinary leakage among women with stress urinary incontinence: a randomized clinical trial. JAMA 2017; 317: 2493-501.
24. Maracle CX, Kucharzewska P, Helder B, et al. Targeting non-canonical nuclear factor-κB signalling attenuates neo-vascularization in a novel 3D model of rheumatoid arthritis synovial angiogenesis. Rheumatology (Oxford) 2017; 56: 294-302.
25. Nygaard G, Firestein GS. Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. Nat Rev Rheumatol 2020; 16: 316-33.
26. Zhi LQ, Zhong Q, Ma JB, Xiao L, Yao SX, Wang X. LncRNA H19 inhibitor represses synovial cell proliferation and apoptosis in rats with rheumatoid arthritis *via* Notch signaling pathway. Eur Rev Med Pharmacol Sci 2020; 24: 4088-94.
27. Kim MJ, Park JS, Lee SJ, et al. Notch1 targeting siRNA delivery nanoparticles for rheumatoid arthritis therapy. J Control Release 2015; 216: 140-8.
28. Li X, Dong Y, Yin H, Qi Z, Wang D, Ren S. Mesenchymal stem cells induced regulatory dendritic cells from hemopoietic progenitor cells through Notch pathway and TGF-β synergistically. Immunol Lett 2020; 222: 49-57.
29. Ma MJ, Liu HC, Qu XQ, Wang JL. Matrix metalloproteinase-3 gene polymorphism and its mRNA expression in rheumatoid arthritis. Genet Mol Res 2015; 14: 15652-59.
30. Gossage DL, Cieslarová B, Ap S, et al. Phase 1b study of the safety, pharmacokinetics, and disease-related outcomes of the matrix metalloproteinase-9 inhibitor andecaliximab in patients with rheumatoid arthritis. Clin Ther 2018; 40: 156-65.
31. Zou W, Chen QX, Sun XW, et al. Acupuncture inhibits Notch1 and Hes1 protein expression in the basal ganglia of rats with cerebral hemorrhage. Neural Regen Res. 2015; 10: 457-62.
32. Guo HD, Tian JX, Zhu J, et al. Electroacupuncture suppressed neuronal apoptosis and improved cognitive impairment in the AD model rats possibly *via* downregulation of Notch signaling pathway. Evid Based Complement Alternat Med 2015; 2015: 393569.