Fan WM, Zhao CJ. Effect of electroacupuncture on JAK2/STAT3 pathway in synovial tissues of rats with rheumatoid arthritis. J Acupunct Tuina Sci, 2019, 17(4): 223-230
DOI: https://doi.org/10.1007/s11726-019-1121-z

Basic Study

Effect of electroacupuncture on JAK2/STAT3 pathway in synovial tissues of rats with rheumatoid arthritis

电针对类风湿关节炎大鼠膝关节滑膜组织JAK2/STAT3通路的影响

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Abstract
Objective: To observe the effect of electroacupuncture (EA) on Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway in knee joint synovial tissues of rats with rheumatoid arthritis (RA) and to explore the action mechanism of EA on RA.
Methods: Twelve of the 48 SPF male Sprague-Dawley (SD) rats were assigned to a normal group by the random number table method. The remaining 36 rats were subjected to RA model preparation by intradermal injection of the Freund's complete adjuvant into the right hind foot pad of each rat under sterile conditions. After the model was successfully prepared, rats were then divided into a model group, a drug group and an EA group according to a random number table method (n=12). Rats in the drug group were treated with 2 mL aqueous solution of tripterygium glycosides [8.1 mg/(kg·bw)]; rats in the EA group were treated with EA at bilateral Yanglingquan (GB 34) and Zusanli (ST 36), for 30 min each time; rats in the normal group and the model group were placed in a special rat fixation tank for 30 min each time, and received the same dose of normal saline as those in the drug group. Rats in all groups received intervention once a day for 4 weeks. Diameter of rat ankle joint and rat arthritis index were measured before and after the intervention. At the end of the experiment, the expressions of phospho-JAK2 and phospho-STAT3 were determined by immunohistochemistry. Quantitative real-time polymerase chain reaction (RT-qPCR) was used to detect JAK2 and STAT3 mRNAs expressions.
Results: After the model was produced, the arthritis index >2 was considered successful in model preparation. Compared with the model group, the ankle joint diameters and arthritis indexes of rats in the drug group and the EA group were significantly lower (all P<0.01); immunohistochemical staining cells with phospho-JAK2 and phospho-STAT3 were significantly decreased (all P<0.01); the expression levels of JAK2 and STAT3 mRNAs were decreased with statistical differences (all P<0.01). There were no significant differences between the EA group and the drug group (all P>0.05).
Conclusion: EA can alleviate the inflammatory response of RA rats, improve their pathological conditions, reduce the expressions of phospho-JAK2 and phospho-STAT3 in the synovial tissue of knee joint, and decrease the expressions of JAK2 and STAT3 mRNAs. The therapeutic effect of EA is comparable to that of the tripterygium glycosides. The mechanism of EA treatment may be related to the inactivation of the JAK2/STAT3 pathway.
Keywords: Acupuncture Therapy; Electroacupuncture; Point, Yanglingquan (GB 34); Point, Zusanli (ST 36); Arthritis, Rheumatoid; Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT 3) Pathway; Rats

【摘要】目的：观察电针对类风湿关节炎(RA)大鼠膝关节滑膜组织非受体型酪氨酸激酶2/信号转导和转录激活因子3(JAK2/STAT3)通路的影响，探讨电针治疗RA的作用机制。**方法：**采用随机数字表法从48只SPF级雄性SD大鼠中取12只为正常组，其余36只大鼠无菌条件下右后足跖皮内注射弗氏完全佐剂复制RA模型，造模成功后再按照数字表随机分为模型组、药物组和电针组，每组12只。药物组大鼠接受2 mL雷公藤多苷片水溶液灌胃[8.1 mg/(kg·bw)]；电针组大鼠接受电针双侧阳陵泉和足三里治疗，电针刺激每次持续30 min。正常组和模型组大鼠被置于特制大鼠固定筒中，每次持续30 min，并接受与药物组同等剂量的生理盐水灌胃。每组大鼠每天干预1次，连续干预4周。在干预前后检测大鼠踝关节直径数值和大鼠关节炎指数。实验结束后免疫组化法观察磷酸化JAK2(phospho-JAK2)和磷酸化STAT3(phospho-STAT3)的表达，实时荧光定量聚合酶链反应(RT-qPCR)法检测JAK2和STAT3 mRNA表达。**结果：**模型复制后，关节炎指数大于2视为模型制备成功。与模型组比较，药物组和电针组大鼠踝关节直径数值和关节炎指数明显降低，组间具有统计学差异(均P＜0.01)；phospho-JAK2及phospho-STAT3免疫组化阳性染色细胞减少，组间具有统计学差异(均P＜0.01)；JAK2和STAT3 mRNA表达量下降，组间具有统计学差异

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(均 $P<0.01$)。电针组与药物组比较，均无统计学差异(均 $P>0.05$)。**结论：**电针可以减轻 RA 大鼠的炎性反应，改善其病理状况，减少膝关节滑膜组织 phospho-JAK2 和 phospho-STAT3 的表达，降低 JAK2 及 STAT3 mRNA 表达量，其治疗作用与雷公藤多苷片相当，电针治疗作用机制可能与抑制 JAK2/STAT3 通路激活有关。

【关键词】针刺疗法；电针；穴，阳陵泉；穴，足三里；关节炎，类风湿；Janus 激酶 2/STAT3 通路；大鼠

【中图分类号】R2-03　　**【文献标志码】**A

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disorder that primarily affects the synovial membrane of the joints. It has a high incidence and disability rate and can greatly affect the patients' quality of life (QOL)[1]. At present, immunosuppressive agents and anti-rheumatic drugs are the main treatments for RA in Western medicine; but long-term use will cause adverse reactions and complications. Chinese medicine has a unique advantage in RA treatment. Studies have confirmed the therapeutic effects of acupuncture, moxibustion, electroacupuncture (EA), bee-needle, and other special therapies on RA[2-4]. However, there are few studies on the underlying mechanisms. In this study, adjuvant arthritis was used as an animal model, and tripterygium glycosides was used as a positive control to observe the efficacy of EA for the experimental RA in rats, and its effect on the non-receptor tyrosine kinase Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, thus to explore the mechanism of EA and provide an experimental basis for promoting RA treatment with EA.

1 Materials and Methods

1.1 Laboratory animals and grouping

Forty-eight SPF male Sprague-Dawley (SD) rats, weighing (190±10) g, were provided by Shanghai Xipuer-Bikai Laboratory Animal Co., Ltd. [license number: SCXK (Shanghai) 2013-0016; certificate number: 2008001680636]. Animals were kept in the animal room of the Nanjing General Hospital of the People's Liberation Army [animal room license number: SYXK (Army) 2012-0047]. Animal experiments strictly adhered to the *Guiding Opinions on the Treatment of Experimental Animals* issued in 2006 by the Ministry of Science and Technology, and were approved by the ethics committee (approval number: 2017DWLS-0705).

After one-week adaptive feeding, 12 rats were selected as a normal group by the random number table, and the other 36 rats were randomly divided into a model group, a drug group and an EA group after successful modeling (n=12).

1.2 Main drugs, reagents and instruments

Tripterygium glycosides tablets (10 mg/tablet, Cat. No. 160903, Jiangsu Meitong Pharmaceutical Co., Ltd., China); normal saline (Cat. No. 170312F18, Zhejiang Jimin Pharmaceutical Co., Ltd., China); Freund's complete adjuvant (Sigma, USA); phospho-JAK2 antibody (Cat. No. ab108596) and phospho-STAT3 antibody (Cat. No. ab76315), (Abcam, UK); hematoxylin (Cat. No. H9627, Sigma, USA); embedding paraffin (Cat. No. 69019361) and neutral gum (Cat. No. 10004160), (Sinopharm Chemical Reagent Co., Ltd., China); trizol (Cat. No. 252250AX, Beijing Aidelai Biotechnology Co., Ltd., China); HiScript reverse transcriptase (RNase H) (Cat. No. R101-01/02, VAZYME, USA); ribonuclease inhibitor (Cat. No. LOT#J11202, Beijing TransGen Biotech Co., Ltd., China); Taq plus DNA polymerase (Cat. No. ET105-01) and DL2000 DNA Marker (Cat. No. MD114-02), (TIANGEN, Germany); primer synthesis (Tianyi Huiyuan Biotechnology Co., Ltd., China). Acupuncture needle (Cat. No. 160320) and SDZ-II electronic needle therapy instrument (Suzhou Medical Products Factory Co., Ltd., China); RM 2016 pathology slicer (Leica, Germany); JK-6 tissue water bath-slide drier (Wuhan Junjie Electronics Co., Ltd., China); DHG 9203A electric heating constant temperature air drying oven (Shanghai Jinghong Experimental Equipment Co., Ltd., China); QuantStudio 6 real-time fluorescence-quantitative polymerase chain reaction (RT-qPCR) instrument (ABI, USA); Nano-100 micro spectrophotometer (Hangzhou Aosheng Instrument Co., Ltd., China); HW-SY11-KP2 electric thermostatic water bath (Beijing Changfeng Instrument Co., Ltd., China); JY300 horizontal electrophoresis instrument and JY02S UV analyzer (Beijing Junyi Oriental Electrophoresis Equipment Co., Ltd., China).

1.3 Modeling methods

Except the normal group, rats in the other three groups were injected with Freund's complete adjuvant (0.15 mL/rat) in the right hind foot pad under sterile conditions to produce the adjuvant arthritis rat model[5-6].

On the 15th day of the experiment (the 1st day of the experiment was defined as the day preparing the models), the arthritis index was observed by the 5-level scale method. The index >2 indicated the success.

1.4 Interventions

On the 16th day of the experiment, rats of each group received the specified intervention for 4 consecutive weeks.

1.4.1 Normal group

Rats in the normal group were placed in a special rat fixation tank for 30 min with intragastric administration of normal saline (2 mL/rat), once a day.

1.4.2 Model group

Rats in the model group were placed in a special rat fixation tank for 30 min with intragastric administration of normal saline (2 mL/rat), once a day.

1.4.3 Drug group

Rats in the drug group received intragastric administration of 2 mL tripterygium glycosides aqueous solution [8.1 mg/(kg·bw)], once a day.

1.4.4 EA group

Points: Bilateral Zusanli (ST 36) and Yanglingquan (GB 34).

Methods: Rats were placed in a special rat fixation tank; localization of point was performed according to the standard acupuncture point map of *Experimental Acupuncture Science*[7].

The acupuncture needle of 0.30 mm in diameter and 40 mm in length was inserted into the point for 3.5 mm, and then connected to the SDZ-Ⅱ type electronic acupuncture instrument. The positive and negative electrodes in each group were respectively connected to the 2 homolateral points and subjected to the sparse-dense wave with a frequency of 2 Hz/10 Hz and current intensity of 1 mA for 30 min. The intervention was performed once a day (Figure 1).

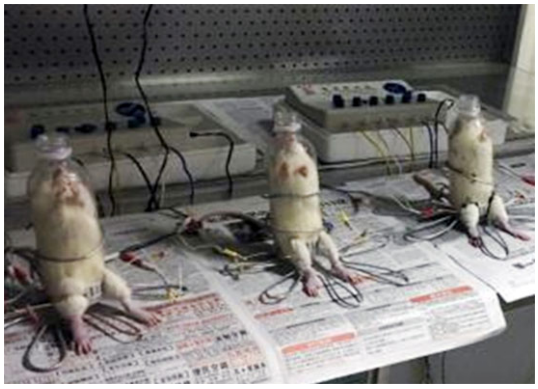


Figure 1. Rats were receiving EA treatment

1.5 Observation items and detection methods

1.5.1 Ankle diameter

The diameter of the right hind ankle joint was measured using a digital vernier caliper before and after the intervention. Specific method: the right hind limb of the rat was dorsiflexed to 90°, the vernier caliper was placed at the point of the right angle, and the diameter of the rat ankle joint was measured.

1.5.2 Arthritis index

Rat arthritis scores were evaluated in each group before and after the intervention using a 5-point scale. The sum of the arthritis indexes of the extremities represented the arthritis index of each rat with a maximum score of 16 points. The higher the score, the severer the joint symptoms.

1.5.3 Ankle joint pathology

After the experiment, the right ankle joints of the rats were separated, and the pathological images of the ankle joints were observed under a microscope following the conventional procedures, such as decalcification, fixation, embedding, sectioning and hematoxylin-eosin (HE) staining.

1.5.4 Phospho-JAK2 and phospho-STAT3 expressions

The expressions of phospho-JAK2 and phospho-STAT3 were determined by immunohistochemistry. At the end of the experiment, the rats were sacrificed and the synovial tissue of the knee joint was removed for 4% paraformaldehyde fixation, paraffin embedding and sectioning. After antigen retrieval, blocking endogenous peroxidase, incubating with primary and secondary antibodies and chromogenic agentchromogenic agent, counterstaining, dehydration and sealing, 5 fields were randomly selected under a 400× microscope. Brown-yellow particles in the cytoplasm/nucleus were positive markers. The expression of positive substances was semi-quantitatively determined by Image pro-Plus 6.0 image analysis software.

1.5.5 JAK2 and STAT3 mRNAs expressions

JAK2 and STAT3 mRNAs expressions were detected by RT-qPCR. After the experiment, the rats were sacrificed, and the synovial tissues of the knee joints were removed for total RNA extraction and cDNA reverse transcription. Primers were designed for RT-qPCR assay (Table 1).

Table 1. Primer sequences in the RT-qPCR reaction

Primer name	Primer sequence	Amplification length (bp)
JAK2	Forward: 5'-TACTTCCTGACCTTTGCCGT-3' Reverse: 5'-TGATACTGTCTGAGCGCACA-3'	172
STAT3	Forward: 5'-GGAAGGAGGGGT CACTTTCA-3' Reverse: 5'-TCTCGGGGCGACAATACTTT-3'	227
GAPDH	Forward: 5'-ACAGCAACAGGGTGGTGGAC-3' Reverse: 5'-TTTGAGGGTGCAGCGAACTT-3'	253

The Ct difference (△Ct) between the test gene JAK2 or STAT3 and the reference gene GAPDH in the sample was calculated. Subtracted the △Ct of the normal group from the △Ct of each experimental group to calculate $2^{-\triangle\triangle Ct}$, which represented the fold change of JAK2 and STAT3 expressions in the experimental group versus the normal group.

1.6 Statistical methods

Data analysis was performed using IBM SPSS Statistics 22.0 statistical software. Measurement data presented as mean ± standard deviation ($\bar{x}$ ±*s*). Multiple group comparison was performed by one-way analysis of variance. The least significant difference (LSD) method was used for comparison between groups. Repeated measure analysis of variance was used for the same group before and after the intervention. *P*<0.05 indicated statistically significant difference.

2 Results

2.1 Comparing ankle joint diameters of rats

Before the intervention, the diameters of the ankle joints in the model, the drug and the EA groups were all statistically significantly increased than the diameter of the normal group (*P*<0.01); there were no significant differences in the ankle joint diameters among the model, the drug and the EA groups (all *P*>0.05), suggesting that rats in the model, the drug and the EA groups had obvious arthritis reaction and the model was successful. After the intervention, the ankle joint diameters of the drug group and the EA group were significantly lower compared with the model group (both *P*<0.01), indicating that the ankle joint diameters of the drug and EA groups were decreased, and the inflammatory response was alleviated. There was no significant difference between the EA group and the drug group (*P*>0.05), (Table 2).

Table 2. Comparison of ankle joint diameter among groups ($\bar{x}$ ±*s*, mm)

Group	*n*	Before intervention	After intervention
Normal	12	5.85±0.85	6.57±0.60
Model	12	9.35±1.14[1)]	10.05±0.77
Drug	12	9.05±1.23[1)]	8.18±1.07[2)]
EA	12	9.22±1.09[1)]	7.98±1.09[2)]

Note: Compared with the normal group, 1) *P*<0.01; compared with the model group, 2) *P*<0.01

2.2 Comparison of rat arthritis index

Before the intervention, there were no significant differences in the arthritis index among the model, the drug and the EA groups (all *P*>0.05). After the intervention, compared with the model group, the arthritis indexes of the drug and EA groups were significantly lower (both *P*<0.01), suggesting that the arthritis indexes of the rats in the drug and the EA groups were decreased, and the inflammatory response of rats was alleviated. There was no significant difference between the EA and the drug groups (*P*>0.05), (Table 3).

Table 3. Comparison of arthritis index among groups ($\bar{x}$ ±*s*)

Group	*n*	Before intervention	After intervention
Model	12	8.41±1.05	8.39±1.25
Drug	12	8.11±0.93	6.50±1.14[1)]
EA	12	8.67±0.90	6.54±1.04[1)]

Note: Compared with the model group, 1) *P*<0.01

2.3 Pathological comparison of rat ankle joints

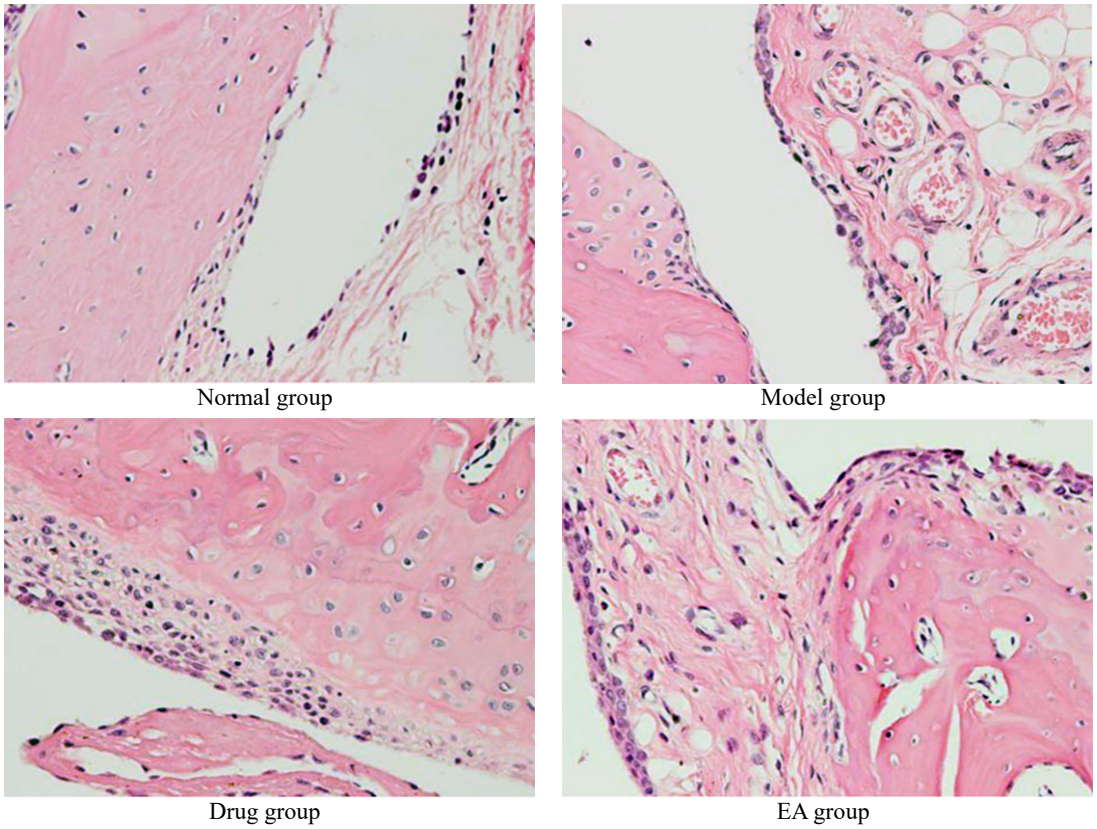
In the normal group, rats showed no obvious inflammatory infiltration, and the synovial cells were arranged neatly with newly formed individual blood vessels. In the model group, rats showed a large number of vascular hyperplasia tissues and pannus. Rats in the drug and the EA groups showed a small amount of inflammatory cell infiltration and angiogenic tissues (Figure 2).

2.4 Comparison of phospho-JAK2 and phospho-STAT3 expressions

A small amount of phospho-JAK2 and phospho-STAT3 positive staining cells were showed in the normal group; compared with the normal group, the phospho-JAK2 and phospho-STAT3 positive staining cells in the model group were significantly increased (*P*<0.01); compared with the model group, the phospho-JAK2 and phospho-STAT3 positive staining cells were decreased in the drug and the EA groups, and the between-group differences were statistically significant (*P*<0.01). There was no significant difference between the EA and the drug groups (both *P*>0.05), (Figure 3-Figure 6).

2.5 Comparison of JAK2 and STAT3 mRNA expressions

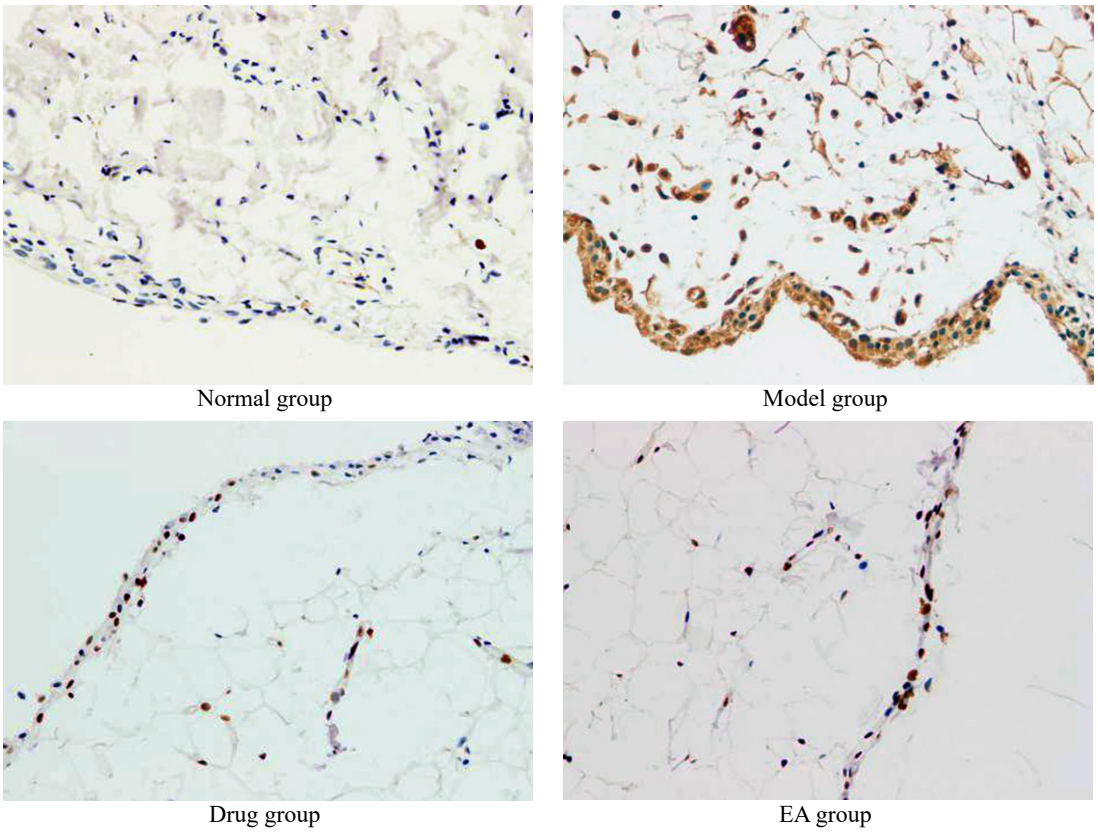
The RT-qPCR results showed that the mRNA expressions of JAK2 and STAT3 in the model group were significantly higher than the expression in the normal group with 1.5 times and 1 time increase, respectively (*P*<0.01). Compared with the model group, the mRNA expressions of JAK2 and STAT3 in the drug and the EA groups were significantly decreased (all *P*<0.01). No statistically significant differences were found between the EA and drug groups (all *P*>0.05), (Figure 7 and Figure 8).



Normal group　　Model group

Drug group　　EA group

Figure 2. Ankle joint pathology of rats in each group (HE, ×400)



Normal group　　Model group

Drug group　　EA group

Figure 3. Phospho-JAK2 expression of rats in each group (SP method, ×400)

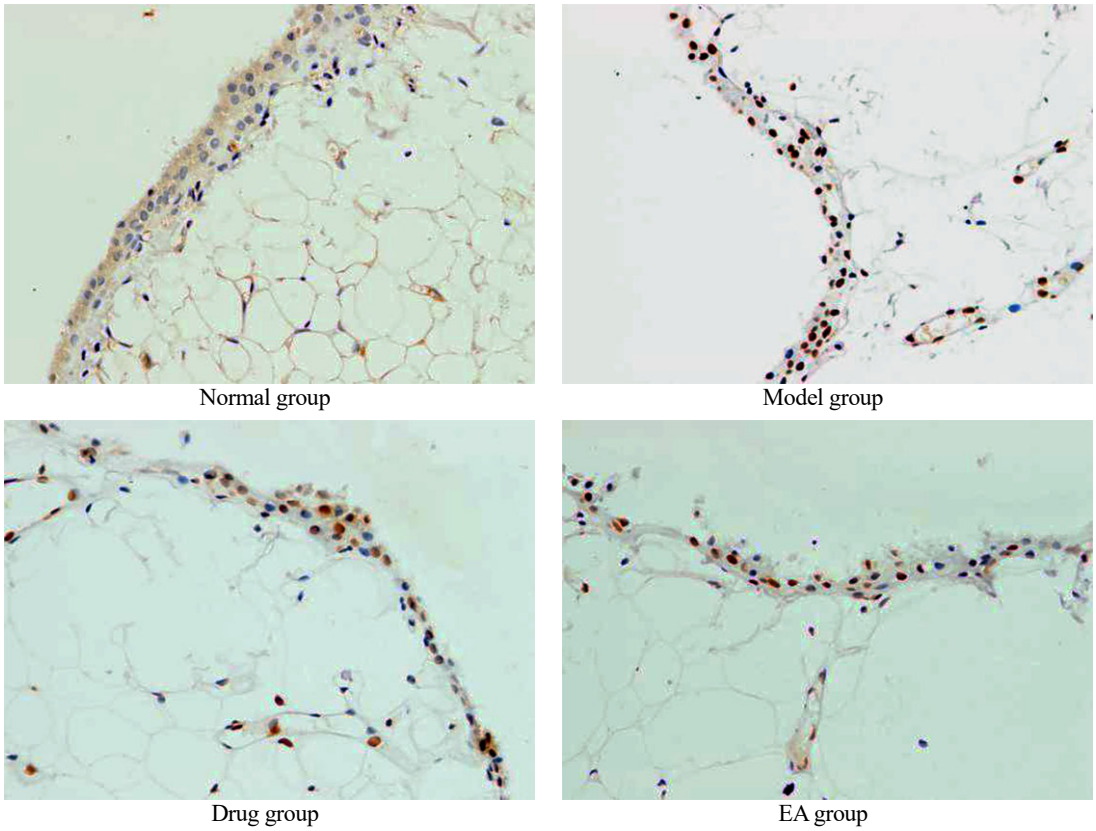


Figure 4. Phospho-STAT3 expression of rats in each group (SP method, ×400)

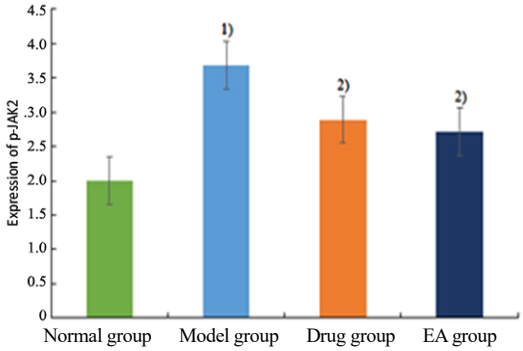


Figure 5. Comparison of phospho-JAK2 expression

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$

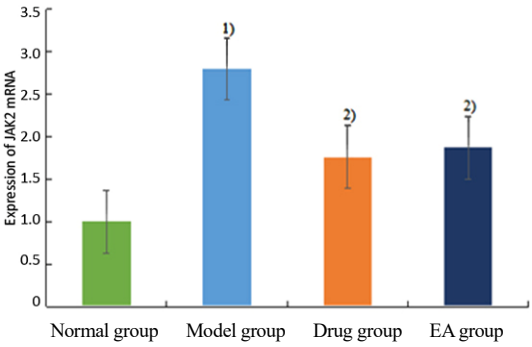


Figure 7. Comparison of JAK2 mRNA expression

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$

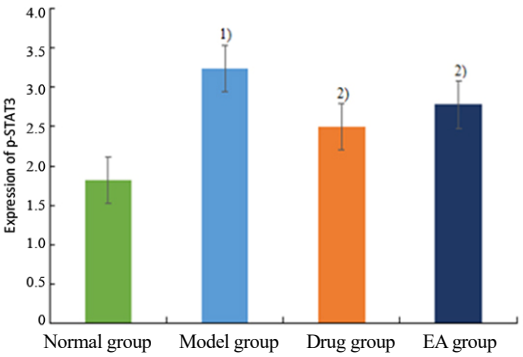


Figure 6. Comparison of phospho-STAT3 expression

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$

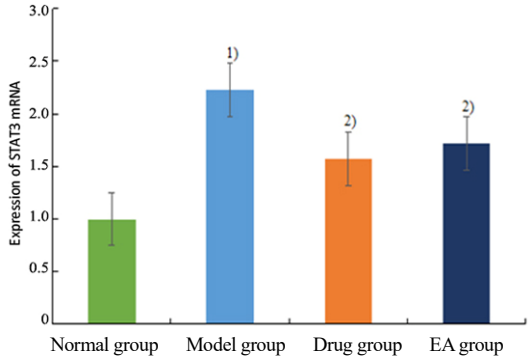


Figure 8. Comparison of STAT3 mRNA expression

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$

3 Discussion

RA belongs to the Bi-impediment syndrome, joint wind or severe joint pain like being bitten by a white tiger in traditional Chinese medicine (TCM). Contributing factors mainly include wind, cold, summer heat and dampness. These factors may affect the sinews, muscles or joints and cause pain, numbness, soreness, distension, or in severe cases, deformity. Chinese herbal medicine or acupuncture is commonly used to reinforce healthy qi, unblock meridians and remove pathogenic factors. Zusanli (ST 36) is the He-Sea point of the Stomach Meridian, and the lower He-Sea point of the stomach. It can replenish the foundation of acquired constitution, nourish qi and blood, and strengthen the vitality. Zusanli (ST 36) is a local point for RA-induced knee joint pain and deformation. Zusanli (ST 36) also has anti-inflammatory and immune-regulating effects[7-9]. Yanglingquan (GB 34) is the He-Sea point of the Gall Bladder Meridian and the lower He-Sea point of the gall bladder. It is the main point for Bi-impediment and bone pain[10]. Fan W, *et al*[11] also found that acupuncture at Yanglingquan (GB 34) had an anti-inflammatory effect. Our previous clinical practice also confirmed that EA at Zusanli (ST 36) and Yanglingquan (GB 34) significantly relieved joint pain and reduced inflammatory indexes in RA patients. In this study, we found that compared with the model group, the ankle joint diameters were significantly decreased, the arthritis indexes were decreased, and the pathological conditions of the ankle joints were improved in the EA group. There were statistical differences between the groups. There was no significant difference in each observation item between the EA and the drug groups, suggesting that the therapeutic effect on RA of EA is equivalent to that of tripterygium glycosides.

RA affects the joint synovium and extra-articular organs. Synovium angiogenesis is an important feature of RA[12-13]. The main pathological changes of RA are synovitis, vascular hyperplasia and pannus of synovium[14-15]. Angiogenesis is widespread and involved in the development and progression of autoimmune diseases such as RA[16]. There are 4 members in JAK family. JAK3 is expressed in the bone marrow and lymphatic system. JAK1, JAK2 and tyrosine kinase (TYK2) are widely distributed in cells and tissues. The signal transducer and activator of transcription (STAT) is located downstream of JAK, and it is a signalling and transcriptional activator. The activated STAT dimer is transferred into the nucleus and combined with the corresponding target gene promoter to activate the signal transduction[17].

Studies have shown that the JAK2/STAT3 signalling pathway plays an important role in the synovium angiogenesis of RA[18-20]. Inflammatory factors activate JAK2 kinase. The activated JAK2 kinase promotes STAT3 phosphorylation to form homo/dimers and bind to the promoter region of the corresponding target gene after entering into the nucleus, which further activates the corresponding gene transcription[21], causing synovial cell damage and apoptosis, the occurrence or aggravation of RA. Therefore, it is important to study the prevention and treatment of RA from inactivation of the JAK2/STAT3 pathway[22]. In this study, the pathological images of the ankle joints in the model group showed the vascular hyperplasia and pannus. Immunohistochemistry and RT-qPCR detection found that EA inhibited the expressions of phospho-JAK2 and phospho-STAT3, mRNAs of JAK2 and STAT3, and the activation of JAK2/STAT3 pathway, thus improve the joint synovium angiogenesis, thereby exerting the anti-inflammatory and immunosuppressive effects.

In conclusion, we found that the JAK2/STAT3 pathway in the rats with adjuvant arthritis was activated, which resulted in the development of arthritic lesions. EA significantly reduced the diameters of rat ankle joints and arthritis indexes, and improved the pathological conditions of the ankle joints. The mechanism may be related to the inactivation of JAK2/STAT3 pathway, improving the joint synovium angiogenesis, and regulating the immunological function. Therefore, as a non-drug therapy, EA is effective in the treatment of RA. In future study, cell research should be involved, and the mechanism of EA treatment of RA should be elucidated on multiple levels and in multiple aspects.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

Acknowledgments

This work was supported by Research Fund Project for Introduced Talents of Yijishan Hospital (弋矶山医院引进人才科研基金项目, No. YR201809).

Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria.

Received: 28 September 2018/Accepted: 11 November 2018

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