

Acupuncture Research

Electroacupuncture Delays Cartilage Degeneration by Modulating Nuclear Factor- κ B Signaling Pathway*

WU Guang-wen¹, CHEN Jun¹, HUANG Yun-mei¹, PAN Cai-bin¹, CHEN Wen-lie¹, ZHANG Shi-mao², LIN Wei³, LIU Xian-xiang¹, and WU Ming-xia⁴

ABSTRACT **Objective:** To illustrate the molecular mechanisms underlying the therapeutic effects of electroacupuncture (EA) on knee osteoarthritis (OA). **Methods:** Twenty-seven six-month-old New Zealand white rabbits were allocated into three groups in accordance with a random number table: normal group (no surgery-induced OA; without treatment), model group (surgery-induced OA; without treatment) and EA group [surgery-induced OA; received treatment with EA at acupoints Dubi (ST 35) and Neixiyan (EX-LE 5), 30 min twice a day]. After eight consecutive weeks of treatment, the histopathological alterations in cartilage were observed using optical microscopy and transmission electron microscopy, cartilage degeneration was evaluated by modified Mankin's score principles, the synovial fluid concentration of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and matrix metalloproteinase-3 (MMP-3) were evaluated by enzyme-linked immunosorbent assay, and the protein expression levels of IL-1 β , IL-6, TNF- α , MMP-3, I κ B kinase- β (IKK- β), nuclear factor of α light polypeptide gene enhancer in B-cells inhibitor α (I κ B- α) and nuclear factor- κ B (NF- κ B) p65 were quantified by Western blot analysis. **Results:** EA treatment significantly improved cartilage structure arrangement and reduced cellular degeneration. The IL-1 β , IL-6, TNF- α and MMP-3 of synovial fluid in the EA-treated group were significantly decreased compared with the model group (all $P < 0.01$). Compared with the model group, the IL-1 β , IL-6, TNF- α , MMP-3, IKK- β and NF- κ B p65 protein expressions in cartilage of EA-treated group were significantly decreased (all $P < 0.01$), whereas I κ B- α expression was significantly up-regulated ($P < 0.01$). **Conclusion:** EA treatment may delay cartilage degeneration by down-regulating inflammatory factors through NF- κ B signaling pathway, which may, in part, explain its clinical efficacy in the treatment of knee OA.

KEYWORDS osteoarthritis, electroacupuncture, synovial fluid, cartilage, cytokines, nuclear factor- κ B signaling pathway

The most common joint disease, osteoarthritis (OA), also known as degenerative arthritis, bone and joint disease, and is a chronic disease directly associated with cartilage degeneration and joint inflammation.⁽¹⁾ Local increasing of inflammatory stimuli and mediators in the articular tissues leads to secondary synovial proliferation, articular effusion and cartilage malnutrition, resulting in articular cartilage degeneration.⁽²⁾ It is widely accepted that inflammatory cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), etc. serve a crucial function in the pathogenesis of OA, as they induce extracellular matrix degradation by increasing the synthesis of catabolic factors.⁽³⁾ Previous studies demonstrated that nuclear factor- κ B (NF- κ B) transcription factor family is crucially involved in immunity, inflammation, anti-apoptosis, cellular proliferation, and the negative feedback of NF- κ B, which is an important regulator

of IL-1 β , TNF- α and IL-6.⁽⁴⁾ NF- κ B activity may be induced by multiple cellular stimuli, including inflammatory cytokines, extracellular matrix degradation products and mechanical overload.⁽⁵⁾

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1. Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou (350122), China; 2. Fujian Xiangxing Electronic Technology Co., Ltd., Ningde, Fujian Province (352300), China; 3. Fujian Provincial Key Laboratory of Integrative Medicine on Geriatrics, Fuzhou (350122), China; 4. Department of Acupuncture, Second Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou (350003), China

Correspondence to: Prof. WU Ming-xia, Tel: 86-591-22861586, E-mail: wumingxiafz@163.com

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OA has a major economic and social impact on populations and health-care systems worldwide.⁽⁶⁾ Although widely prescribed non-steroidal anti-inflammatory drugs is able to reduce joint pain and stiffness, the inflammatory component reduction of OA is usually minimal.⁽⁷⁾ Currently, advanced OA is only managed by surgical joint replacement, however, difficulties regarding to the degree of invasion, cost and long-term prognosis still remain.⁽⁸⁾ The disadvantages call for an evaluation of the risks and benefits of the therapies for OA and require less toxic options. Therefore, an increasing number patients suffering from OA turn to treatments from complementary and alternative medicine.⁽⁹⁾

Acupuncture, which has long been used for the treatment of various types of diseases in Eastern medicine, is currently gaining acceptance as an alternative medicine in Western countries.⁽¹⁰⁾ Electroacupuncture (EA) is a modified acupuncture technique that utilizes electrical stimulation instead of manual manipulation. Previous studies have demonstrated that EA possesses therapeutic effects on chondral defects including knee OA and produces cytokines with multiple biological activities in various types of diseases.⁽¹¹⁻¹⁴⁾ Our previous study showed that EA can be employed as a novel non-drug-inducing measure for the differentiation of bone marrow mesenchymal stem cells (BMSCs) into chondrocytes and promote chondrocyte proliferation via promoting G₁/S check point transition in cell cycle.^(15,16) However, it remains unclear that whether EA is able to attenuate the inflammatory response on knee OA and the underlying mechanism is remained to be explored. Therefore, we try to answer the questions by investigating cartilage morphology, cytokine production in synovial fluid, and NF- κ B signaling in cartilage upon EA treatment.

METHODS

Experimental Animals

A total of 27 six-month-old male New Zealand White rabbits (clean grade), weighing 2.0–2.2 kg, were purchased from the Shanghai City Songjiang District Songlian Experimental Animal Farm [laboratory animal use certificate No. SCXK (SU)2012-0011]. The rabbits were raised in the Fujian University of Traditional Chinese Medicine Laboratory Animal Center (Fuzhou, China) under controlled light (12-h light: 12-h dark cycle) and temperature (18–22 °C) conditions, with *ad libitum* access to water and chow. All experiments involving

the animals complied with Guidance Suggestions for the Care and Use of Laboratory Animals developed by the Ministry of Science and Technology of China.⁽¹⁷⁾ It was approved by Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine.

Reagents

Pentobarbital sodium was purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 β , IL-6, TNF- α and matrix metalloproteinase-3 (MMP-3) were obtained from Cloud-Clone Co. (Wuhan, China). Rabbit polyclonal to human I κ B kinase- β (IKK- β) and rabbit polyclonal (IgG) to human nuclear factor of α light polypeptide gene enhancer in B-cells inhibitor α (I κ B- α) antibodies were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). Anti-NF- κ B p65 antibody, anti-IL-1 β antibody, anti-IL-6 antibody, anti-TNF α antibody, anti-MMP3 antibody and anti- β actin antibody were obtained from Abcam (Cambridge, MA, USA). Horseradish peroxidase-labeled goat anti-rabbit immunoglobulin IgG and horseradish peroxidase-labeled goat anti-mouse IgG were from Merck Millipore (Darmstadt, Germany). Clarity Western electrochemiluminescence substrate was from Bio-Rad (Hercules, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China).

Knee OA Model and Grouping

Twenty-seven animals were divided into two groups: normal group ($n=9$) and model group ($n=18$) by a random number table method. The experiments were repeated 3 times and 3 animals in the normal group and 6 in the model group were used in batches in each of the following experiment. The rabbits in the normal group received no surgery-induced OA, whereas the rabbits in the model group, both knees underwent the surgical procedure to induce OA according to the modified Hulth's modeling procedure.⁽¹⁸⁾ One week after surgical procedure, all animals in the model group were subjected to passive movement of the knee for 0.5 h daily for 4 weeks. And previous studies have shown that this method produces a reliable and reproducible degradation of articular cartilage.⁽¹⁹⁾ Those surgery-induced OA rabbits were subsequently randomly divided equally into two groups: model group and EA treatment group.

Treatment

EA treatment was applied to acupoints for knee

OA in Dubi (ST 35) and Neixiyan (EX-LE5). ST 35, also called Waixiyan, is located in a depression lateral to the patellar ligament. EX-LE5 is located in the center of the depression of the patellar ligament of the knee and is opposite ST 35, according to the National Standard of the People's Republic of China, the name and location of acupoints, issued in 2006 (GB/T 12346-2006). The acupuncture needle were inserted into the acupoint and connected to an electrical stimulator (Type SDZ-II, Suzhou Medical Appliance Factory Co., Ltd., China) and stimulated electrically with low-frequency EA of 2 Hz with square-wave burst pulses (duration 1.1 s) and a burst frequency of 100 Hz (duration 2.2 s) with alternating polarity. The intensity of the output voltage was 17.3 V and the pulse width was shorter than 1 ms. The acupuncture stimulation was applied for 30 min per day for a period of 8 weeks. The model and normal groups receive no treatment.

Tissue Collection

Following 8 weeks treatment with or without EA, all animals were anesthetized with 3% pentobarbital sodium (30 mg/kg weight via ear vein injection), 1.5 mL of saline was injected into knee joints at the upper part of the patellar ligament about 0.5 cm. Synovial fluid was collected with a needle after activating the knee joints 30 times, and the joints were then opened and the femur and tibia were collected for further investigation. The right medial femoral condyle was prepared for hematoxylin and eosin (HE) and Safranin O staining and the right tibial plateau cartilage for transmission electron microscopy. The synovial fluid was collected for ELISA, and the left femoral condyle and tibial plateau cartilage were collected for immunoblotting.

Optical Microscopy Analysis and Histological Evaluation

The right femoral specimens were harvested and fixed in 4% paraformaldehyde for 2 days, decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 8 weeks. The medial femoral condyle was longitudinally cut into 1.2 cm × 1.2 cm × 0.5 cm sections and embedded in paraffin. Then, 4- μ m paraffin sections were cut using a microtome, dewaxed in xylene and stained with HE and Safranin O. The cartilage morphological changes were observed under a microscope (Olympus Corporation, Tokyo, Japan) and images were captured at a magnification of ×100. Following that a modified Mankin's score principle was used to evaluate the degeneration of each femoral condyle.⁽²⁰⁾ All the samples

were scored blindly. An average of the individual observer scores was calculated for each knee.

Transmission Electron Microscopy Analysis

The right tibial plateau cartilage specimens were sectioned into 1 mm × 1 mm × 2 mm sections, pre-fixed in 4% glutaraldehyde [Alfa Aesar (China) Chemicals Co., Ltd., Shanghai, China] and 1.5% paraformaldehyde solution (pH 7.3) at 4 °C for 3 days, post-fixed with 1% osmium tetroxide (Ted Pella, Inc, Redding, CA, USA) at 4 °C for 2 h following decalcification in 5.5% EDTA for 12 weeks at 4 °C. The tissue specimens were dehydrated with graded alcohol-acetone, and embedded in Epoxy resin 618 (E-51, Ganxi Chemical Co. Ltd., Jiangxi, China). The 1- μ m resin semi-thin sections were subsequently cut using a microtome and stained with azur-methylene blue. The structure of the cartilage was observed and ultrathin sections were observed under an optic microscope. The 70-nm ultrathin sections were cut using an ultramicrotome (EM UC6; Leica Microsystems GmbH, Wetzlar, Germany), stained with 2% aqueous uranyl acetate and counter-stained with 0.3% lead citrate. The ultrastructure of the articular cartilage was observed using a transmission electronic microscope (H7650; Hitachi, Ltd., Tokyo, Japan) at 80 kV.

ELISA Analysis

The synovial fluid was collected, and the levels of IL-1 β , IL-6, TNF- α and MMP-3 were measured using an ELISA kit according to the manufacturer's protocol. The experiment was repeated 3 times.

Western Blot Analysis

The left femoral condyle and the tibial plateau cartilage tissue specimens were obtained from each group. Total proteins from the tissue specimens were isolated, and protein concentrations were determined using a bicinchoninic acid method according to the manufacturer's protocol (Pierce Biotechnology Inc., IL, USA). Equal quantities of protein (50 μ g) were separated by 12% SDS-PAGE, transferred onto polyvinylidene fluoride membranes (Pierce Biotechnology Inc., IL, USA), and blocked with 5% W/V non-fat dry milk. Membranes were exposed to primary antibodies for IL-1 β (1:10000), IL-6 (1:1000), TNF- α (1:1000), MMP-3 (1:1000), IKK- β (1:600), I κ B- α (1:600), NF- κ B p65 (1:500) and β -actin (1:2000) overnight at 4 °C, and then to secondary horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG antibodies (1:10000) at room temperature for 2 h. Finally, the antibody-bound protein bands were detected

with enhanced chemiluminescence, and images were captured using ChemiDoc XRS+ (Bio-Rad Laboratories Inc., CA, USA). The grayscale value ratio of the target protein to the internal control was used to measure the relative expression levels of IL-1 β , IL-6, TNF- α , MMP-3, IKK- β , I κ B- α and NF- κ B p65.

Statistical Analysis

Data were analyzed using the SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Data presented are expressed as mean \pm standard deviation ($\bar{x} \pm s$). The Shapiro-Wilk test was used to determine the normality of all groups of data. If the data exhibited a normal distribution, they were analyzed with one-way analysis of variance (ANOVA) followed by least significant difference or Games Howell post hoc tests; if not, the Kruskal-Wallis test was used and the Mann-Whitney *U* with Bonferroni's correction was applied as a post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

EA Impacts on Cartilage Morphology

Cartilage sections were stained with HE and Safranin O, and observed under an optical microscope. The articular cartilage surfaces of the normal group were smooth, the four structural layers of the articular cartilage were visible, the chondrocytes were lined up in neat rows, and the caryotin was uniform and clear (Figure 1A). However, in the model group, the cartilage surface was partially damaged, disrupting the four-layer structure. Disordered chondrocyte clusters appeared, the tide mark was partially replicated, cartilage matrix staining intensity was decreased to some extent and chondrocyte apoptosis was observed (Figure 1B). Following treatment with EA, the four-layer tissue structure remained clear while the cartilage surface was rough. The number of chondrocytes was increased and arranged regularly. The tide mark was partially replicated, and the cartilage matrix was homogeneous stain (Figure 1C). Safranin O staining was used to further evaluate the degeneration of cartilage morphology. The cartilage matrix was stained pink by Safranin O, the tidal line between the radiation layer and the calcification was complete in the normal group (Figure 1D). However, the Safranin O staining was greatly reduced in the model group. The tide mark was partially replicated or defected (Figure 1E). While, after EA treatment, the Safranin O stained is looked similar to the normal group and the tide mark was partially replicated (Figure 1F).

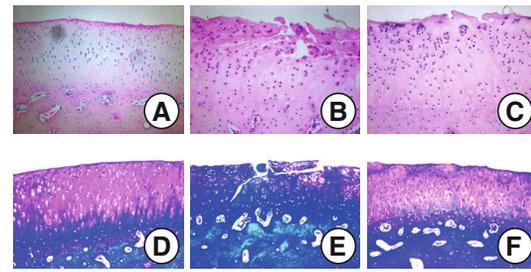


Figure 1. Effect of EA Treatment on the Microstructure of Cartilage Tissue in Rabbits (HE and Safranin staining, $\times 100$)

Notes: A, D: normal group; B, E: model group; C, F: EA group.

The mean of modified Mankin's scores in the model group (10.13 ± 0.65 scores) were significantly higher compared to normal group (0 score, $P < 0.01$). However, the scores of EA group were significantly lower than the model group (6.50 ± 0.76 scores, $P < 0.05$).

Ultrastructural of the chondrocytes was observed by transmission electron microscopy. In the normal group, chondrocytes were nearly oval, with intact cell membrane, abundant rough endoplasmic reticulum, dictyosomes, mitochondria and glycogen in cytoplasm (Figure 2A). In the model group, the chondrocytes exhibited marked atrophy. Degeneration and necrosis of chondrocytes were presented with irregular shape, the areolae surrounding the cells were missing, organelles in the cytoplasm were hardly distinguish (Figure 2B). The degradation of the chondrocytes in the EA group was less severe compared with the model group (Figure 2C).

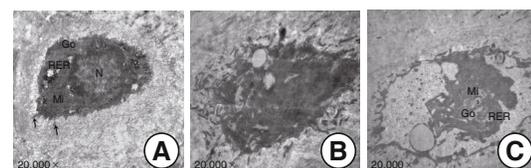


Figure 2. Effect of EA Treatment on Ultrastructure of Chondrocytes in Rabbits ($\times 20,000$)

Notes: A: normal group; B: model group; C: EA group. Black arrows indicate microvilli-like protrusions; N: nuclei; RER: rough endoplasmic reticula; Go: Golgi apparatus; Mi: mitochondria.

EA Regulated IL-1 β , IL-6, TNF- α and MMP-3 of Synovial Fluid

IL-1 β , IL-6, TNF- α and MMP-3 in the model group were significantly higher compared with those in the normal group (all $P < 0.01$). However, the IL-1 β , IL-6, TNF- α and MMP-3 expressions in the EA group were significantly lower compared with the model group, respectively (all $P < 0.01$; Figure 3).

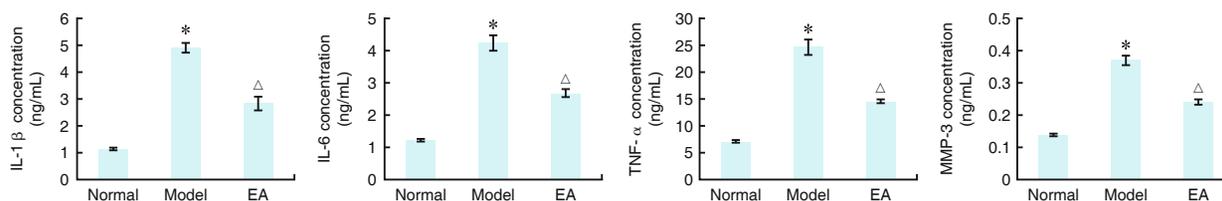


Figure 3. Effect of EA Treatment on IL-1 β , IL-6, TNF- α and MMP-3 of Synovial Fluid ($\bar{x} \pm s$)

Notes: * $P < 0.01$ vs. the normal group; $\Delta P < 0.01$ vs. the model group

EA Regulated Protein Expressions of IL-1 β , IL-6, TNF- α and MMP-3 in Cartilage

The protein expressions of IL-1 β , IL-6, TNF- α and MMP-3 were significantly higher in the model group, as compared with the normal group (all $P < 0.01$). However, EA treatment decreased IL-1 β , IL-6, TNF- α and MMP-3 expressions significantly in the treatment group, as compared with the model group (all $P < 0.01$, Figure 4).

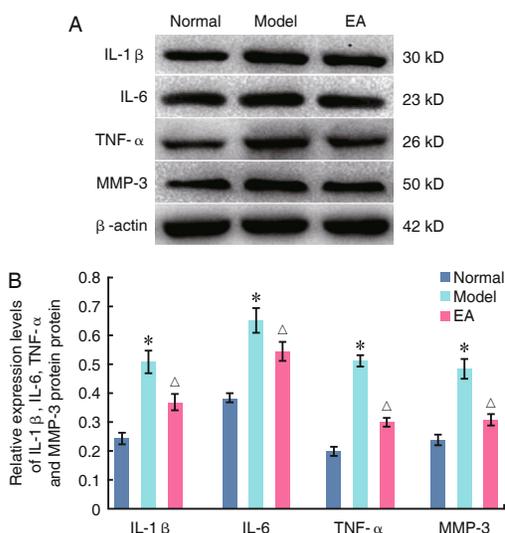


Figure 4. Effect of EA Treatment on Protein Expression of IL-1 β , IL-6, TNF- α and MMP-3 in Cartilage

Notes: (A) Representative images of Western blot. (B) Relative protein expression levels of IL-1 β , IL-6, TNF- α and MMP-3. * $P < 0.01$ vs. the normal group; $\Delta P < 0.01$ vs. the model group

EA Regulated Protein Expressions of IKK- β , I κ B- α and NF- κ B p65 in Chondrocytes

The protein expression of IKK- β and NF- κ B p65 were significantly higher, while those of I κ B- α were significantly lower in the model group, as compared with the normal group (all $P < 0.01$). However, following treatment with EA, the expression of IKK- β , NF- κ B p65 decreased and that of I κ B- α increased significantly in the treatment group (all $P < 0.01$, Figure 5).

DISCUSSION

OA is mainly characterized by articular cartilage degeneration and chondral matrix degradation, in

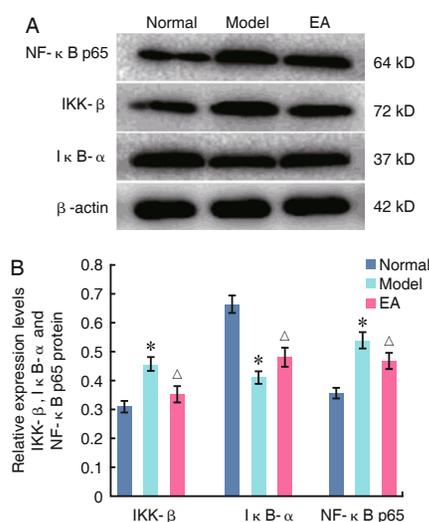


Figure 5. Effect of EA Treatment on Protein Expressions of IKK- β , I κ B- α and NF- κ B p65 in Cartilage ($\bar{x} \pm s$)

Notes: (A) Representative images of Western blots. (B) Relative protein expression levels of IKK- β , I κ B- α and NF- κ B p65. * $P < 0.01$ vs. the normal group; $\Delta P < 0.01$ vs. the model group

which the alteration of chondrocytic function is crucially involved. Such an alteration is primarily associated with the complex network of interactions among inflammatory factors, resulting in synovial inflammation and chondrocytic disturbance.⁽²¹⁾ Articular cartilage is crucial for the function of the knee joint, and can be divided into two parts: chondrocytes and cartilage matrix. Chondrocytes are the only cell type present in mature cartilage, and are responsible for extracellular signals as well as the regulation the maintenance of cartilage homeostasis.⁽²²⁾ In other word, the functional changes of chondrocytes have an important role and contribute to the degradating of articular cartilage, and therefore to the pathogenesis of knee OA.

EA therapy has been reported to have numerous biological effects, both locally and globally.^(11,23) In our study, EA therapy was applied to a rabbit knee OA model, and observed a protective effect on the destabilized articular cartilage surface compared with the non-EA treatment group. HE staining demonstrated that following EA treatment, the four-layer tissue structure

remained clear, although the cartilage surface was rough. The number of chondrocytes was increased and arranged regularly. The tide mark was partially replicated, and the cartilage matrix was homogeneous stain. Besides, the Safranin O stained in EA-treated group is near the normal group. Mankin scores showed that the cartilage morphologic changes in the EA group were significantly lower compared with the model group. Transmission electron microscopy observations further indicated that the mechanisms underlying the therapeutic effects of the treatment of knee OA involved the improvement of chondrocyte function. These results suggested that EA is able to effectively delay the degeneration of articular cartilage.

It is known that proinflammatory cytokines, such as IL-1 β , IL-6, TNF- α , are involved in the pathological process of knee OA.⁽²⁴⁾ Previous study demonstrated that the expressions of IL-1 β , IL-6, TNF- α in the synovial fluid were increased in knee OA.⁽²⁵⁾ IL-1 β is produced locally by OA chondrocytes that has been shown to increase the production of matrix degrading enzymes and inhibit proteoglycan synthesis, resulting to loss of cartilage.⁽²⁶⁾ Furthermore, IL-1 β is crucial for the inflammatory process as well as articular tissue destruction, and release of inflammatory mediators cyclooxygenase-2 which is an important mediator of OA associated inflammation and the anabolic/catabolic process.⁽²⁷⁾ TNF- α is involved in inflammation and induces chondrocytes peroxidation. IL-6 is produced by macrophages, monocytes, and fibroblasts, and stimulates cartilage and synovial cells to produce prostaglandin E, MMPs and collagenase, regulates the inflammatory response by amplifying the biological effects of TNF- α ,^(28,29) thereby enhancing inflammation.⁽³⁰⁾ MMP-3 is an important MMP produced by the synovial joints, which involved in inflammation and the degradation of collagen II that increase knee OA.

Our results showed that IL-1 β , IL-6, TNF- α , and MMP-3 expressions in the synovial fluid were decreased following EA treatment which strongly suggest that EA treatment impaired inflammation response and articular tissue destruction. Previous study proved that the expression of proinflammatory mediators, including IL-1, IL-6 and TNF- α increasing in cartilage, bone, and synovium, contributed to the initiation of OA.⁽³¹⁾ In our study, IL-1 β , IL-6, TNF- α and MMP-3 protein expression in cartilage, quantified

by Western blot, were significantly higher in the model group, as compared with the normal group. However, following EA treatment, IL-1 β , IL-6, TNF- α and MMP-3 expressions decreased significantly in the EA treatment group. These results suggested that EA treatment effectively regulate inflammatory through inflammatory mediator manipulation.

It was known that NF- κ B is one of the most important signaling molecule that regulate the inflammatory response in OA. A positive feedback cycle is formed by the interaction between inflammatory factors and activated NF- κ B to enhance the progression of inflammation.⁽³²⁾ NF- κ B is regulated by a group of inhibitory proteins of the I κ B family, including I κ B- α , I κ B- β , I κ B- γ , I κ B- δ , I κ B- ϵ and B cell lymphoma-3.⁽²⁹⁾ Upon binding to NF- κ B, I κ B blocks its translocation from the cytoplasm to the nucleus and suppresses its gene transcription.⁽³³⁾ The IKK complex is an important factor for regulating the activity of I κ B, including the catalytic subunit IKK- α , IKK- β , and the regulatory subunit IKK- γ .⁽³⁴⁾ IKK- β is primarily involved in inflammatory and innate immune responses, mediating the rapid recruitment of immune cells to inflammatory and injury sites through NF- κ B signaling.⁽³⁵⁾ I κ B is degraded in the presence of activated IKK, releasing NF- κ B to regulate the expression of numerous inflammatory factors. In our experiments, IKK- β , I κ B- α and NF- κ B p65 expressions evaluated by Western blot analysis showed that the protein expressions of IKK- β and NF- κ B p65 in the EA-treated group were significantly decreased compared with those in the model group, whereas I κ B- α expression was significantly up-regulated, suggesting that EA may regulate the inflammatory response in knee OA through NF- κ B signaling pathway.

In summary, EA treatment is able to delay cartilage degeneration by down-regulating the inflammatory response via the NF- κ B signaling pathway, which may partly explain its clinical efficacy in the treatment of knee OA.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Author Contributions

Liu XX and Wu MX were in responsible for designing the project proposal and managing the progress. Wu GW was in

charge of data analysis, and drafted this paper. Lin W revised the manuscript. Chen J was responsible for data collection in each experiment. Huang YM, Pan CB, Chen WL and Zhang SM performed the experiments.

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