Effects of electro-acupuncture on oestrogen levels, body weight, articular cartilage histology and MMP-13 expression in ovariectomised rabbits

Yuxi Qin, Jing He, Lu Xia, Hua Guo, Chengqi He

ABSTRACT

Background Electro-acupuncture (EA) treatment has been shown to decrease pain and improve the function of ovariectomised (OVX) rats with osteoarthritis (OA); however, the underlying mechanisms remain unclear.

Object We used OVX rabbits to replicate natural human menopausal processes and to evaluate whether EA could be used to prevent and treat postmenopausal OA.

Methods The rabbits were randomly divided into four groups of eight: a normal control group (NC), an OVX group, an ERT group (oestrogen replacement therapy after OVX) and an EA group (EA therapy after OVX). After the interventions, all of the animals were killed. Serum oestrogen levels and body weight were measured. The transcription of matrix metalloproteinase-13 (MMP-13) mRNA was detected using reverse transcriptase-PCR. Modified Mankin scores were used for histological assessment. Expression of MMP-13 in cartilage was determined by immunohistochemistry.

Results Both the EA group and the ERT group had increased serum oestrogen levels (p=0.028, p=0.037 respectively), as well as decreased expression of MMP-13 (p=0.000, p=0.000, respectively), relative to the OVX group. The body weight of the EA group was lower than that of the OVX group and the NC group (p=0.007), as well as the ERT group (p=0.010).

Conclusions EA could be a new method for preventing and treating postmenopausal OA by producing endogenous sex hormones that inhibit the expression of MMP-13 and cause weight loss with no side effects and a relatively low cost.

INTRODUCTION

Menopause is a natural consequence of aging that occurs in older women. The reduction of female hormone production by the ovaries leads to the transition, which is accompanied by menopausal symptoms and other processes, such as acceleration of bone loss and atherosclerosis. Clinical and epidemiological studies have revealed that postmenopausal women have an increased prevalence, incidence and severity of osteoarthritis (OA) due to a sharp decrease in oestrogen levels. OA is a common condition that causes progressive damage to the joints and irreversible disability in adults, with the knee almost always being affected. Various studies have suggested that, among the many physiopathological mechanisms, sex difference and oestrogen level may correlate with the maintenance of normal joint function and prevention of tissue breakdown in the joint. As a result of these studies, there is increased interest in determining the effects of oestrogen on joint tissues and cartilage. Clinical reports and animal studies have demonstrated that oestrogen replacement therapy (ERT) can effectively suppress cartilage degradation. These studies suggest that oestrogens may maintain the structural integrity of articular cartilage and play an important role in the development of OA. Although many studies have suggested that ERT reduces the incidence of OA, the majority of the oestrogen present in women receiving ERT is not generated by the body, which can result in aggravation of serious conditions. This important issue has not been well addressed, and efforts are being made to identify potential therapeutic interventions with comparable effectiveness to ERT.
Acupuncture typically incorporates traditional Chinese medicine as an integral part of its practice and theory, and it has been shown to be an effective and economical therapy for menopausal and peri-menopausal syndromes, which can cause endogenous serum oestrogen levels to increase significantly.\textsuperscript{13–17} There is considerable clinical evidence that manual acupuncture and electro-acupuncture (EA) can produce significant improvement in symptoms of OA of the knee.\textsuperscript{18–20} However, the mechanisms by which manual acupuncture and EA exert chondroprotective effects remain unclear.

The characteristic pathological changes in OA are articular cartilage degeneration, a process that is irreversible. Articular cartilage is a relatively simple structure consisting of extracellular matrix (ECM) and chondrocytes; it contains no nerves or blood vessels, and cartilage ECM is primarily composed of proteoglycans and collagens. The excessive degradation of proteoglycans and collagens is key to the pathological process of OA, and is caused by deleterious proteinases produced by articular chondrocytes.\textsuperscript{21} The matrix metalloproteinase family (MMPs) includes many of these deleterious proteinases. They are expressed in articular cartilage and degrade the ECM. MMP-13 is thought to be the most important collagenase in the degradation of articular cartilage because of its preferential digestion of type II collagen. MMP-13 expression is limited in normal articular cartilage, but its increased expression in degenerative cartilage suggests that MMP-13 is involved in the destruction of articular cartilage.\textsuperscript{22} Animal studies have confirmed the close connection between MMP-13 expression and OA development, suggesting that MMP-13 may play a key role in the destruction of articular cartilage.\textsuperscript{23, 24} Therefore, in our study, we have confirmed the close connection between MMP-13 expression and OA development, suggesting that MMP-13 may play a key role in the destruction of articular cartilage. Therefore, in our study, we used prepared ovariectomised (OVX) rabbits to duplicate the natural processes of human menopause and investigated the effects of EA on oestrogen levels, articular cartilage histology and MMP-13 expression to determine whether EA could become a new method for the prevention and treatment of OA.

**MATERIALS AND METHODS**

**Laboratory animals**

All animal care and experimental procedures were conducted with approval of the animal centre (Experimental Animal Center of West China Hospital, Sichuan University; certificate number SYXK (Sichuan) 2009-045) and the Moral Committee on Research Animals of the People’s Republic. According to the animal centre guidelines for the care and use of laboratory animals, 18.5-month-old (2 kg body weight), skeletally mature female New Zealand White rabbits were obtained from the animal centre. All were housed individually in stainless-steel cages in a standard animal facility, where the room temperature was maintained at 21–26°C with 40–60% relative humidity and a 12 h light/dark cycle. The light cycle coincided with daylight hours. Standard chow and tap water were available ad libitum. At the end of quarantine and an acclimatisation period, all of the rabbits were randomly divided into four groups of eight: a normal control group (NC), an OVX group, an ERT group (ERT after OVX) and an EA group (EA therapy after OVX). All of the animals underwent bilateral OVX except for those in the NC group.

**Bilateral ovariectomy**

The OVX group, ERT group and EA group underwent bilateral OVX through a ventral incision under general anaesthesia with an intramuscular injection of 5% chloral hydrate (3 ml/kg), and antibiotic prophylaxis with gentamicin (40 000 U) was administered once a day for the 3 days after surgery to minimise complications.

**Treatment**

After 8 weeks, different interventions were applied to the four groups. All rabbits had both hind legs shaved and fixed to a board in the prone position. In the EA group, acupuncture was applied to ‘ganshu (BL18)’, ‘pishu (BL20)’ and ‘shenshu (BL23)’ points on the back and ‘zusanli (ST 36)’ and ‘sanyinjiao (SP6)’ points on the legs. EA (10 Hz, 1–5 mA) was applied to two points—‘shenshu (BL23)’ and ‘zusanli (ST 36)’—for 30 min once a day. The treatment lasted 14 days. The acupuncture treatment points were selected according to the animal-standardised version of the acupuncture formula\textsuperscript{25} traditionally used for treatment of OA of the knee as a type of Chinese medicine. The acupuncture needles used were solid, disposable filiform stainless-steel needles that were 25 mm long with a diameter of 0.3 mm. The depth of needle insertion varied with the thickness of the skin and subcutaneous fatty tissues at the sites of the acupuncture points; the depth was usually 10–15 mm. In the ERT group, all of the rabbits received ERT with orally conjugated oestrogen tablets (Premarin; Wyeth-Ayerst Laboratories, Philadelphia, Pennsylvania, USA) at a dosage equivalent to 0.625 mg/day in humans. The treatments continued for 2 weeks at five times a week. The NC group and OVX group received no treatment.

**Sample preparation**

Blood samples were obtained from all of the experimental animals; blood was drawn during the initial phase and at 8 and 10 weeks (after treatment) for the assessment of serum 17β-oestradiol. All of the samples were refrigerated for up to 1 h and then centrifuged to extract the serum (2 ml), which was then frozen at −20°C until assay. Ten weeks after OVX, all of the experimental animals were killed by air embolism to obtain tissue. The distal femur was removed from the rabbits’ left knee, cleaned and fixed in 10% buffered formalin. Paraffin-embedded blocks were longitudinally cut into
sections 5 μm thick and mounted on precoated slides for histological classification and immunohistochemical studies. Reverse transcriptase (RT)-PCR was used to semiquantify mRNA expression of MMP-13 in cartilage collected from the right femoral condyle and the tibial plateau of the rabbits.

Oestrogen assay

ELISA (R&D, USA) was performed to estimate the levels of serum 17β-oestradiol. Portions (25 μl each) of the NC, OVX and treated serum samples were added to 200 μl of enzyme conjugate for 2 h at 36°C. Subsequently, 100 μl substrate was added and incubated for 15 min at room temperature. Stop solution (50 ml) was used to terminate the reactions, and absorbance was measured at 450 nm. Each sample was run twice.†

Histological examination

Cartilage samples were stored in alcohol and embedded in paraffin; 5 μm sections were cut and stained with haematoxylin and eosin and toluidine blue for microscopic analysis (digital images at 400× magnification). Sections of articular cartilage were classified for OA using the modified Mankin score.‡ Scoring was performed by two experienced histopathologists, who were blinded to the treatment assignments and other data.

Immunohistochemical assessment

After histological examination, immunohistochemistry was performed according to the following steps. The sections were dewaxed and rehydrated by sequential immersion in xylene and a descending ethanol series after blocking for endogenous peroxidase activity with 3% hydrogen peroxide in methanol at room temperature for 10 min. After a wash in phosphate-buffered saline, the slides were exposed to bovine serum albumin for 30 min at 37°C to reduce non-specific staining. Incubation with primary monoclonal antibodies against MMP-13 (Boster Biological Technology (Wuhan, Hubei, China); 50 μl; 1:40 dilution) was performed overnight at 4°C. The sections were washed and counterstained with haematoxylin and eosin and toluidine blue for microscopic analysis (digital images at 400× magnification).

After an incubation step at 72°C for 5 min and incubation at 16°C for 20 min, each cycle was repeated at least three times. After normalisation against GAPDH, the expression levels of the products were determined by agarose gel electrophoresis and then calculated relative to the mean values for specimens without treatment and the mean values for the normal groups.

In addition to the measurement indicators described above, the body weights of the rabbits at 0, 8 and 10 weeks were recorded for statistical analysis.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package, V.17.0. The data are expressed as mean±SD. The significance of differences in oestrogen levels between pre-OVX and post-OVX was analysed using Student’s t test. One-way analysis of variance and the χ² test were performed to compare differences among all the groups. p<0.05 was considered significant.

RESULTS

Comparisons of levels of serum oestradiol

Before the experiments, differences in the level of serum 17β-oestradiol among the four groups were not statistically significant. After OVX but before treatment, the levels of serum 17β-oestradiol in the rabbits in the OVX group, ERT group and EA group were significantly lower than before OVX, and the differences among the groups were significant (p<0.05) (table 1). After the intervention, levels of serum 17β-oestradiol in the rabbits in the EA group and ERT group were higher than the levels after OVX but before treatment, and the between-group differences were significant (p<0.05). The difference between the EA group and NC group was not significant (p>0.05).

Modified Mankin score of cartilage samples

We evaluated the Mankin score to assess the histology of articular cartilage damage in rabbits receiving OVX, EA treatment and ERT (table 2). The score of sections 5 μm thick and mounted on precoated slides for histological classification and immunohistochemical studies. Reverse transcriptase (RT)-PCR was used to semiquantify mRNA expression of MMP-13 in cartilage collected from the right femoral condyle and the tibial plateau of the rabbits.

RNA extraction and RT-PCR testing

RNA was extracted using Trizol reagent (Takara Holdings, Japan) and processed as follows using an RT-PCR Kit from Takara Holdings. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The reaction mixtures were incubated at 50°C for 30 min, followed by 94°C for 2 min, and then 30 PCR cycles were performed with the following temperature profile: 94°C, 30 s; 54°C (temperature (Tm) of MMP-13) or 49°C (Tm of GAPDH), 30 s; 72°C, 30 s. The PCR cycles were followed by an extension step at 72°C for 5 min and incubation at 16°C for 20 min. Each cycle was repeated at least three times. After normalisation against GAPDH, the expression levels of the products were determined by agarose gel electrophoresis and then calculated relative to the mean values for specimens without treatment and the mean values for the normal groups.

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Modified Mankin score of cartilage samples

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the OVX group was significantly higher than that of the NC group (p<0.01). After EA and ERT, relative to the OVX group, the scores of the EA and ERT groups were decreased (p<0.01), but they were higher than the scores of the NC group (p<0.01). Furthermore, the scores of the EA group and ERT group were significantly different (p<0.05).

### Immunohistochemical assessment of MMP-13
MMP-13 expression was determined in all of the rabbits by tissue immunohistochemistry as well as by determining the grey value (optical density (OD) score) on electron microscopy (table 3). After OVX, the OD score of rabbits in the OVX group was higher than that in the NC group, ERT group and EA group, and the differences between the groups were significant (p<0.01). The differences between the EA group and NC group and between the ERT group and NC group were significant (p<0.01). There was no significant difference between the EA group and ERT group.

### Body weights of rabbits at different time points
There were no significant differences among the four groups at 0 and 8 weeks. After 2 weeks of treatment, the weight of the EA group was lower than that of the OVX group and NC group (p<0.01) as well as the ERT group (p<0.05; table 4).

### RT-PCR testing
RT-PCR testing was used to determine whether the EA or ERT treatment was effective in downregulating the transcription of MMP-13 mRNA in the cartilage of OVX rabbits. The four groups showed the same levels of expression of GAPDH mRNA, and the expression level of MMP-13 mRNA in the EA and ERT groups was lower than that in the OVX group but higher than that in the NC group (figure 1).

### DISCUSSION
Our findings show that OVX-induced cessation of endogenous oestrogen production influenced the integrity and morphology of articular cartilage, and EA and ERT prevented the further incidence of cartilage surface erosion in OVX rabbits. Furthermore, EA increased the OVX-induced reduction in serum oestrogens levels while decreasing MMP-13 mRNA and protein levels in OVX rabbits. In addition, the body weight of the rabbits decreased after acupuncture. All of these findings suggest that EA could exert chondroprotective effects by increasing oestrogen levels, inhibiting the production of MMP-13 and reducing body weight.

Studies have shown that OVX cynomolgus monkeys and OVX rats can be used to model postmenopausal OA, which presents OA-like pathological changes. The results are consistent with the results of our study. Several experimental studies have clearly shown that oestrogen increases cartilage turnover and surface erosion through complex molecular mechanisms on multiple levels. In studies in vitro, Maneix et al²⁹ observed that 17β-oestradiol upregulated the uridine diphosphate glucose dehydrogenase gene and influenced the production of glycosaminoglycan, which promotes the synthesis of joint chondrocytes in rabbits. Furthermore, Morisset et al³⁰ and Claassen et al³¹ observed that 17β-oestradiol protected bovine articular chondrocytes from reactive oxygen species-induced damage by suppressing cyclo-oxygenase-2 mRNA expression. It is generally acknowledged that there are oestrogen receptors (ERs) present in articular cartilage. Roman-Blas et al³² summarised the four signalling pathways used to regulate the activity of oestrogen through ERs in articular tissues: the canonical oestrogen signalling pathway; the non-oestrogen response element oestrogen signalling pathway; the non-genomic oestrogen signalling pathway; and the
ligand-independent pathway. The expression of ERs and affinity of ER for its ligand is significantly decreased in postmenopausal women relative to early pubertal women. The decreased levels of oestrogen and ERs and decreased ER affinity increase articular cartilage turnover, which results in menopausal OA.³³

Oestrogen deficiency may influence the development of OA through MMP family expression. The excessive destruction of articular cartilage ECM components by proteinases is considered the major mechanism of OA. The ECM is primarily composed of proteoglycans and collagens. In the early stages, proteoglycans are initially depleted by proteinases, followed by collagen fibril degradation, which leads to collagen fibril fibrillation and articular cartilage laceration, and finally the arcade structures of the collagen fibrils are destroyed in the articular cartilage.³⁴ The role of MMPs in OA is mainly mediated through aggrecan and type II collagen.³⁵ Aggrecan contains three globular domains, G1, G2 and G3. MMPs are capable of cleaving the G1 domain, which binds to hyaluronan chains through a linking protein and disrupts the aggrecan–hyaluronan network.³⁴ MMPs also degrade fibrillar collagen, which leads to degradation of the cartilage ECM and results in the development of OA. MMP-13 is considered to be the most important of the MMP family proteins in OA pathophysiology because of its preferential cleavage of type II collagen, which is the major fibrillar collagen in articular cartilage and is highly resistant to most proteinases triple-helical structure.³⁴ Many studies have shown that MMP-13 plays an important role in the development of OA. Kamekura et al²³ showed, using experimental OA models, that MMP-13 expression is associated with OA development. They showed that, in the early stages of development, chondrocytes undergo hypertrophic differentiation, and that type X collagen is expressed, including the existence of hypertrophic chondrocytes. In contrast, Jiménez et al²⁴ found increased expression of Runx2, which is a transcriptional activator upregulated by MMP-13 and chondrocyte hypertrophy in the articular cartilage of patients with OA. In experimental OA models, mRNA and protein expression of MMP-13 is increased in OA cartilage; these changes are consistent with the development of OA.²³ The present study confirms that MMP-13 in cartilage significantly degrades type II collagen.³⁶ Claassen et al³⁷ determined that the suppression of 17β-oestradiol may reduce the expression of insulin receptor proteins and influence the formation of type II collagen. Høegh-Andersen et al²⁸ indicated that oestrogen deficiency produced by ovariectomy

[Figure 1] RT-PCR: the expression levels of GAPDH mRNA (top) and the expression levels of MMP-13 mRNA (bottom).
increases levels of C-Telopeptide of Type II Collagen, which is a characteristic metabolic product of type II collagen, and increases cartilage surface erosion. Lee et al reported that oestrogen decreased the expression of MMP-1, MMP-3 and MMP-13 in osteoarthritic chondrocytes. Our study confirmed that after O VX, with the development of oestrogen deficiency, the expression of MMP-13 was significantly higher than in the NC group.

Although considerable effort has been made to demonstrate the effect of oestrogen on the maintenance of the integrity and homoeostasis of articular cartilage, the effects of ERT on joint cartilage in vivo in animals and in clinical studies have been paradoxical. Compared with postmenopausal women not receiving E RT, the risk of radiographic knee OA apparently decreases in women receiving E RT.7 38 Nevitt et al performed a meta-analysis of four prevalence studies and found that the prevalence of OA in postmenopausal women using ERT was lower than that in women who were not. In vivo animal studies have revealed side effects of ERT in OA. High-dose responsiveness in cartilage cultures produced deleterious effects on joint cartilage,43 and in some oestrogen-treated OVX animals, the incidence of OA increased.44 These conflicting results may arise from the use of different doses and time points to evaluate the effect of oestrogen on articular cartilage. Most research has suggested that low-dose oestrogen inhibits inflammatory factor-induced cartilage degradation, whereas high-dose oestrogen increases cartilage digestion;43; early and long-term oestrogen treatment has beneficial effects on cartilage.44 In clinical applications, the majority of oestrogen used in ERT is not endogenously derived, which can increase the risk of cancer of the uterus, ovaries and breasts and also cause strokes and cardiovascular disorders.12–14 Concerns about individual doses and negative side effects are critical issues that remain to be addressed in a systematic manner.

Acupuncture is a type of physical therapy based on the theory of traditional Chinese medicine, which has existed in China for more than 2500 years and has continuously improved and evolved. Recently, high-quality meta-analyses of pooled data and clinical studies have indicated the clinical benefits of acupuncture treatment for OA.18 26 45–48 In addition, some research has confirmed that acupuncture promotes the production of endogenous oestrogens by modulating neurotransmitter function in the central nervous system and improving natural functional homoeostasis.15–17 Acupuncture transfers signals through nervous pathways to the brain and modulates a series of neurotransmitters that regulate the hypothalamic–pituitary–ovarian axis.15 16 There is also evidence that acupuncture influences the activation of endocrine, hormonal, dielectric and other pathways.49–52 Yu and Chen found that EA may affect neuroendocrine–immune metabolic systems and increase the levels of ERs and ER mRNA in the hypothalamus and peripheral organs in patients and in aging rats. These studies indicate that acupuncture exerts an integrated and complex effect on the hypothalamic–pituitary–ovarian axis through the regulation of multiple systems in the body.

Several reports have indicated that OA is associated with excess weight, especially OA in weight-bearing joints, such as the knees and hips. Obesity and over-weight are recognised as risk factors for developing OA. Felson et al found that weight loss reversed the development and progression of knee OA in women with a normal or high body mass index. Other studies have shown that weight loss slows the progression and reduces the pain of knee OA and is beneficial for quality of life.55 56 In our study, the weight of rabbits after EA decreased relative to those in the OVX and NC groups, which potentially provides an important method for the behavioural treatment of knee OA.

CONCLUSION
Our results show that EA produced endogenous sex hormones without side effects and may thus be a new method for preventing and treating OA safely and relatively inexpensively; however, the mechanism of treatment should be further investigated.

Acknowledgements We are grateful to Lu Xia, MD, and Hua Guo, MD, for their contributions to RT-PCR, histological analysis of articular cartilage and immunohistochemical assessment. Yuxi Qing assisted in the preparation of ELISA. We also thank Xueqing Chen, PhD, for her contributions to the MMP-13 immunohistochemistry.

Contributors LX and HG for their contributions to RT-PCR, histological analysis of articular cartilage and immunohistochemical assessment. YQ assisted in the preparation of ELISA and statistic analysis.

Funding The study was supported by the National Natural Science Foundation of China (Grant No 30672215).

Competing interests None.

Ethics approval Moral Committee on Research Animals of People’s Republic.

Provenance and peer review Not commissioned; internally peer reviewed.

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*Acupunct Med* 2013 31: 214-221 originally published online March 21, 2013
doi: 10.1136/acupmed-2012-010289

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