

Electroacupuncture prevents ovariectomy-induced osteoporosis in rats: a randomised controlled trial

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ABSTRACT

Background Electroacupuncture (EA) treatment has been shown to increase bone mineral density (BMD) in ovariectomised (OVX) rats; however, the underlying mechanisms remain unclear.

Objective To systematically evaluate the effects of EA on OVX rats and the Wnt/ β -catenin signalling pathway.

Methods Three-month-old female Sprague–Dawley rats were randomly divided into three different groups ($n=10$ each): sham operated control (sham operated), ovariectomy (OVX) and ovariectomy with EA treatment (OVX+EA). Rats in the OVX+EA group received 12-week EA treatments.

Results Serum bone-specific alkaline phosphatase level ($p<0.01$), BMD of the proximal femoral metaphysis and the fifth lumbar (L5) vertebral body (both, $p<0.05$) and maximum load and energy to failure of L5 vertebral body (both $p<0.01$) were significantly higher in the OVX+EA group than in the OVX group. Trabecular area, trabecular width and trabecular number were significantly higher in the OVX+EA group by 66.9%, 29.2% and 30.3%, respectively, than in the OVX group (all, $p<0.01$). Trabecular separation was 31.9% lower in the OVX+EA group than in the OVX group ($p<0.01$). Quantitative real-time reverse transcription polymerised chain reaction indicated that the expressions of mRNAs for low-density lipoprotein receptor-related protein 5 and β -catenin were significantly increased in the OVX+EA group, as compared with the OVX group ($p<0.01$ and $p<0.05$, respectively).

Conclusion This study demonstrates that EA can prevent OVX-induced bone loss and deterioration of bone architecture and strength by stimulating the Wnt/ β -catenin signalling pathway. These findings suggest that EA may be a promising adjunct method for inhibiting OVX-induced osteoporosis in clinical settings.

INTRODUCTION

Osteoporosis is a chronic disease of the skeleton characterised by progressive bone loss and deterioration of bone microarchitecture and manifests as diminished physical strength of the bone and increased susceptibility to fractures.¹ Several pharmacological agents have been developed for osteoporosis treatment

and are in widespread clinical use, including bisphosphonates, oestrogen, selective oestrogen-receptor modulators, calcitonin, denosumab and human recombinant parathyroid hormone. Administration of these drugs has been shown to effectively prevent and treat osteoporosis, but most are associated with certain undesirable side effects.^{2,3} Thus, identification and development of safer and more effective bone protective treatments remain a focus of experimental and clinical research.

Acupuncture is a well-established component of traditional Chinese medicine and has been suggested as a promising alternative to drug-based treatments. Since its first documented use over 2000 years ago in China, acupuncture has been applied to treat a multitude of different human diseases. Previous studies of patients with osteoporosis have demonstrated the positive effects of acupuncture on maintaining bone mass,⁴ regulating bone metabolism,⁴ relieving pain⁵ and improving life quality.⁶ Animal studies, using the ovariectomised (OVX) rat model of osteoporosis, have also demonstrated that acupuncture can increase serum 17 β -oestradiol (E2) level, bone mineral density (BMD)⁷ and bone strength.⁸

Electroacupuncture (EA) is a modern modification of the traditional acupuncture method that stimulates acupuncture points with electrical current instead of manual manipulations. Previous studies have demonstrated that EA can increase BMD and prevent bone loss in OVX rats,^{9,10} and promote bone healing and callus formation in a rat model of tibia fracture.¹¹ Although studies have indicated that EA has a positive effect on preserving bone mass, the underlying mechanisms by which EA prevents and treats osteoporosis remain unclear.

Recently, the Wnt/ β -catenin signalling pathway was shown to be involved in the regulation of osteoblastogenesis and osteoblast activity,^{12,13} presumably playing a critical role in maintenance of bone mass.¹⁴ Low-density lipoprotein receptor-related protein 5 (LRP5), a key co-receptor for Wnt signals, can induce a cascade of intracellular events and is essential for skeletal development and maintenance. Moreover, different LRP5 mutations have been correlated with high or low bone

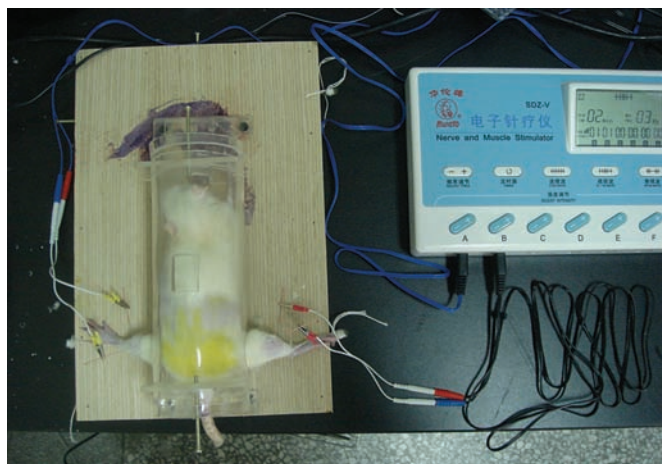


Figure 1 Procedure for electroacupuncture at ST36 and SP6 in a conscious rat.

mass.^{15–17} Another key component of canonical Wnt signalling, β -catenin, activates transcription of genes necessary for osteoblast differentiation.^{18,19} However, there is a paucity of research on the effects of EA on the Wnt signalling pathway in vivo.

The aim of this study was to determine whether EA has positive effects on prevention of bone loss in OVX rats and to investigate the molecular signals underlying these effects. To this end, we examined the effects of EA on serum biochemical markers, BMD, bone microarchitecture and bone biomechanical properties. In addition, we assessed the mRNA expressions of LRP5 and β -catenin in bone marrow cells of EA-treated OVX rats.

METHODS

A randomised controlled trial was used in this study. The study was single blind because it was not possible to blind the acupuncturist.

Animals

Thirty 3-month-old female Sprague–Dawley rats, weighing 261.3 ± 11.3 g, were purchased from the Experimental Animal Center of West China Hospital, Sichuan University. All rats were housed in cages at room temperature (20 – 26°C) in an atmosphere of 60 – 70% humidity and under a $12/12$ h light/dark cycle. Access to water and food were unrestricted. Thirty rats were randomly divided into the following three groups ($n=10$ each), according to random digits tables: the sham operated control group (sham operated), ovariectomy group (OVX) and ovariectomy with EA treatment group (OVX+EA). After 1 week of acclimatisation, all rats underwent either a sham surgery or bilateral ovariectomy using the standard protocol.²⁰ To prevent infection after surgery, a single daily intramuscular injection of penicillin was administered for 3 days. The experimental procedures were in compliance with the Animal Protection Law of the People's Republic of China (2001). This study was approved by the ethics committee at Sichuan University.

Table 1 Primer sequences and PCR product lengths

Target	5'–3'	Sequence	Size, bp	Gene ID
LRP5	Forward	GACATTTACTGGCCCAATGG	131	293649
	Reverse	CTGCCCTCCACCACCTTCT		
β -Catenin	Forward	GGAAAGCAAGCTCATCTTCT	171	84353
	Reverse	AGTGCCTGCATCCACCA		
β -Actin	Forward	GCCAACACAGTGTGTCT	114	81822
	Reverse	AGGAGCAATGATCTTGATCTT		

LRP, low-density lipoprotein receptor-related protein.

Experimental groups

Sham operated group ($n=10$)

No EA treatment, or any other type of therapeutic intervention, was given.

OVX group ($n=10$)

No EA treatment, or any other type of therapeutic intervention, was given.

OVX+EA group ($n=10$)

At 1 week after surgery, rats in the OVX+EA group were treated with EA using the bilateral *Zusanli* (ST36) and *Sanyinjiao* (SP6) acupuncture points. Disposable sterile acupuncture needles (Huatuo, Suzhou Medical Instruments Factory, Suzhou, China), with a length of 25 mm and diameter of 0.25 mm, were inserted perpendicularly into the acupuncture points at a depth of about 3–5 mm for ST36 and 2–3 mm for SP6 and electric stimulation was generated by the SDZ-V nerve and muscle stimulator (Huatuo, Suzhou Medical Instruments Factory) with a frequency of 3 Hz and an intensity of 1 mA. The intensity of the stimulation was sufficient to produce local muscle contractions, but not strong enough to exhibit an escape response or squeaking. Since the rats remained relaxed during stimulation, anaesthesia was not performed. The 12-week EA regimen included 30 min/day for 5 days/week. The EA procedure is shown in figure 1.

Assessment

Biochemical analysis of serum

After the 12-week EA regimen, blood samples were collected from all of the experimental animals (including the sham operated and OVX groups) and centrifuged at $2000g$ for 20 min at 4°C . The supernatants were stored at -20°C until analysis. Serum bone-specific alkaline phosphatase (BALP) and tartrate-resistant acid phosphatase 5b (TRACP5b) levels were estimated using an ELISA kit (Shanghai QaYee Biological Technology, Shanghai, China), according to the manufacturer's instructions.

Bone densitometry

After rats were killed, BMD of the left femur and the fifth lumbar (L5) vertebral body was measured using dual energy X-ray absorptiometry (DEXA) (Lunar-DPX-IQ; Lunar, Madison, WI, USA). Briefly, samples were submerged in

2.5 cm of tap water (to simulate the presence of soft tissue around the bones) and then scanned using Lunar's small animal analysis protocol. Scans were recorded for two regions of interest in each femur (proximal femoral metaphysis and femur mid-shaft) and for the L5 vertebral body. After measuring BMD, the samples were stored at -20°C until subsequent biomechanical testing.

Biomechanical examination

Quantitative assessment of the biomechanical integrity in the left femur and L5 vertebral body was performed using an AG-IS biomechanical testing system (Shimadzu, Kyoto, Japan). Bones were removed from frozen storage and allowed to thaw gradually by warming to room temperature overnight. For the left femur, the three-point bending test was performed. The samples were placed with their natural curvature upwards and immobilised on two fixing supports with a span of 20 mm. For the L5 vertebral body specimens, the compression examination was performed. In the two tests mentioned above, load was applied at a constant displacement rate of 2 mm/min until a fracture occurred. The load–displacement curves were analysed to determine maximum load (the maximum tensile load that the bones sustained before failure) and energy to failure (the area under the load–displacement curve).

Histomorphometric analysis

The rats were killed and their left tibias were resected and fixed by immersion in buffered formalin for 72 h, then

decalcified in 10% EDTA for 4 weeks and embedded in paraffin. Several 5 μm thick longitudinally oriented sections were obtained. Sections were stained with haematoxylin and eosin for histomorphometric analysis. Bone histomorphometric parameters were quantified using the ImagePro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Static parameters, including the percentage of trabecular area (%Tb.Ar), trabecular width (Tb.Wi), trabecular number (Tb.N) and trabecular separation (Tb.Sp), were calculated and expressed according to previous studies.^{20 21}

Quantitative real-time reverse transcription PCR (RT-PCR) measurements of gene expression

Total RNA was extracted from bone marrow cells obtained from the right femur using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instructions. cDNA was reverse transcribed from 5 μg RNA using the RevertAid First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany), according to the manufacturer's instructions. The resultant cDNA was amplified in a FTC-2000 real-time PCR machine (Funglyn, California, USA) using reagents from a Taq DNA real-time PCR reaction kit (Takara, Dalian, China) and gene-specific primers (table 1). The mRNA expressions of target genes, LRP5 and β -catenin, were assessed using the comparative cycle threshold method and normalised against the detected expression of the house-keeping gene, β -actin.

Statistical analysis

Data were expressed as the mean $\pm$ SD for each group. The statistical significance of the differences between means was assessed using one-way analysis of variance followed by Tukey's post hoc test. All statistical comparisons were performed using SPSS version 13.0 statistical software (USA). Differences between groups were considered significant when the p value was <0.05 .

RESULTS

Serum BALP and TRACP5b levels

As shown in figure 2, the serum BALP and TRACP5b levels in the OVX group were significantly higher than those

Table 2 Changes in BMD values of proximal femoral metaphysis, femur mid-shaft and L5 vertebral body

BMD (g/cm^2)	Sham operated	OVX	OVX+EA
Proximal femoral metaphysis	0.327 ± 0.142	$0.271 \pm 0.011^*$	$0.299 \pm 0.030^{* \dagger}$
Femur mid-shaft	0.232 ± 0.024	0.224 ± 0.022	0.229 ± 0.018
L5 vertebral body	0.179 ± 0.023	$0.123 \pm 0.022^*$	$0.155 \pm 0.022^{\dagger}$

Data are expressed as mean $\pm$ SD.

* $p < 0.01$ vs sham operated group; $\dagger p < 0.05$ vs OVX group.

BMD, bone mineral density; EA, electroacupuncture; OVX, ovariectomised.

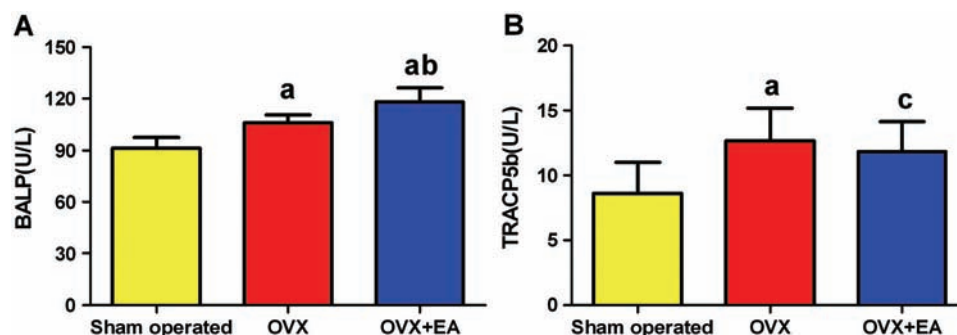


Figure 2 Comparison of changes in biochemical markers of bone turnover. Serum BALP (A) and serum TRACP5b (B) were measured by ELISA. Values are expressed as mean $\pm$ SD. $^a p < 0.01$ vs sham operated group; $^b p < 0.01$ vs OVX group; $^c p < 0.05$ vs sham operated group. EA, electroacupuncture; BALP, bone-specific alkaline phosphatase; OVX, ovariectomised; TRACP5b, tartrate-resistant acid phosphatase 5b.

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in the sham operated group (both, $p<0.01$). After 12 weeks of EA interventions, the level of serum BALP increased significantly in the OVX+EA group, as compared with that detected in the OVX group ($p<0.01$). However, TRACP5b level decreased in the OVX+EA group, but the difference was not statistically significant from that in the OVX group.

BMD measurement

The BMD of the left femur and L5 vertebral body was measured using DEXA scan (table 2). The proximal femoral metaphyseal BMD was significantly lower (-17.1%) in the OVX group than that in the sham operated group ($p<0.01$). As hypothesised, the proximal femoral metaphyseal BMD values were significantly increased (by 10.3% , $p<0.05$) in the OVX+EA group, as compared with the OVX group. There was no significant difference in the femur mid-shaft BMD among the three groups. However, BMD in the L5 vertebral body was significantly lower (-31.3%) in OVX rats, as compared with the sham operated

rats ($p<0.01$). As hypothesised, the BMD values of the L5 vertebral body were significantly increased (by 26.0% , $p<0.05$) in the OVX+EA group, as compared with the OVX group.

Biomechanical property

The results from the biomechanical three-point bending experiment in the left femur are shown in figure 3A,B. Significant decreases in maximum load (-9.5%) and energy to failure (-21.2%) were found in the OVX group, as compared with the sham operated group ($p<0.05$ and $p<0.01$, respectively). The maximum load and energy to failure in the OVX+EA group were higher than those in the OVX group, but the differences were not statistically significant. As shown in figure 3C,D, the results from the L5 vertebral body compression test showed that the maximum load (-22.4%) and energy to failure (-48.8%) in the OVX group were significantly lower than those in the sham operated group (both, $p<0.01$). However, EA treatment significantly increased the maximum load ($+13.6\%$)

Table 3 Changes of static trabecular bone histomorphometric parameters

Group	Tb.Ar (%)	Tb.Wi (μm)	Tb.N (#/mm)	Tb.Sp (μm)
Sham operated	28.28 ± 2.33	62.29 ± 7.78	4.57 ± 0.33	157.84 ± 12.53
OVX	$15.12\pm 1.74^*$	59.88 ± 7.62	$2.54\pm 0.26^*$	$337.27\pm 36.84^*$
OVX+EA	$25.24\pm 2.15^{*\dagger}$	$77.39\pm 11.00^{*\dagger}$	$3.31\pm 0.47^{*\dagger}$	$229.70\pm 31.04^*$

Data are expressed as mean $\pm$ SD.

* $p<0.01$ vs sham operated group; $\dagger p<0.01$ vs OVX group.

EA, electroacupuncture; OVX, ovariectomised; Tb.Ar, trabecular area; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Wi, trabecular width.

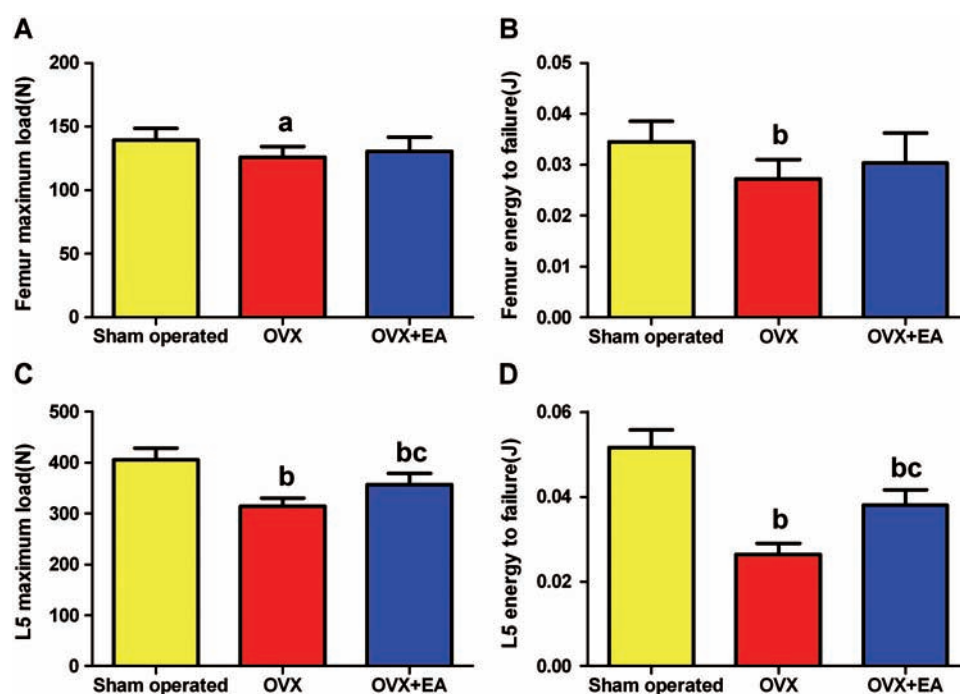


Figure 3 Biomechanical parameters for the left femur (A,B) and L5 vertebral body (C,D). The femur was tested by the three-point bending experiment and the L5 vertebral body was tested by the compression experiment. Data are expressed as mean $\pm$ SD. ^a $p<0.05$ vs sham operated group; ^b $p<0.01$ vs sham operated group; ^c $p<0.01$ vs OVX group. EA, electroacupuncture; OVX, ovariectomised.

and energy to failure (+43.9%) (both, $p<0.01$), as compared with the OVX group.

Histomorphometric examination

The static histomorphometric parameters of the trabecular bone mass and bone architecture in sections of the proximal tibia are shown in table 3 and figure 4. OVX resulted in a significant decrease in Tb.Ar and Tb.N (both, $p<0.01$) and a significant increase in Tb.Sp ($p<0.01$), as compared with the sham operated group. However, EA significantly increased the values of Tb.Ar, Tb.Wi and Tb.N (all, $p<0.01$) and significantly reduced the value of Tb.Sp ($p<0.01$), as compared with the OVX group.

mRNA expressions of LRP5 and β -catenin

The EA-induced changes in mRNA expressions were estimated using RT-PCR. As shown in figure 5, the expressions of LRP5 and β -catenin mRNAs in the OVX group were higher than those in the sham operated group, but had no statistical difference. Furthermore, this increase in the expressions of LRP5 and β -catenin mRNAs was significantly enhanced after EA treatment, as compared with the OVX group ($p<0.01$ and $p<0.05$, respectively).

Adverse events

Slight bleeding (one drop) was detected after needling in five of the 600 EA treatments administered. No other adverse events were detected.

DISCUSSION

This study was designed to evaluate the preventive effects of EA on OVX-induced osteoporosis and to explore the effects of EA on the Wnt/ β -catenin signalling pathway. We found that EA could regulate serum bone turnover indices, prevent bone loss and inhibit deterioration of bone strength and microarchitecture in OVX rats. Furthermore, we observed that Wnt/ β -catenin signalling was augmented after EA treatment in OVX rats.

Biochemical markers of bone turnover are routinely used to estimate changes in bone formation and resorption. BALP (an indicator of osteoblast activity) and TRACP5b (an indicator of osteoclast activity) were measured by ELISA in this study. Previous studies showed that serum BALP activity²² and TRACP5b activity²³ in OVX rats were higher than that in sham operated rats. Interestingly, we

found similar results in our study, indicating that active bone formation and resorption had occurred concurrently in OVX rats. We also found that the EA-treated rats had increased serum BALP, suggesting that EA improved new bone formation. Previous studies showed that acupuncture can increase the serum E2 level,⁷ and that E2 is associated with biochemical markers.^{22,23} Therefore, we inferred that EA can similarly regulate biochemical markers by improving the serum E2 level.

Bone is continuously remodelled in a tightly regulated manner via the bone-resorbing osteoclasts and bone-forming osteoblasts acting in conjunction to maintain bone homeostasis.^{24,25} OVX can disturb this balance, biasing the process towards bone resorption.²⁶ Our study highlighted some interesting facts about changes that occur in OVX rats. The extent of bone loss of trabecular and cortical bone in OVX rats was different, as has been previously reported by others.²⁷ We also found that the BMD of L5 vertebral body (rich in trabecular bone) decreased significantly (−31.3%), whereas BMD in the femur mid-shaft (rich in cortical bone) decreased only slightly. These site-specific results might be explained by the different weight-bearing status and the rate of bone turnover between these two sites. However, EA treatment significantly increased BMD in both the L5 vertebral body (+26.0%) and the proximal femoral metaphysis (+10.3%). The extent of increase in bone mass after EA treatment was evident and confirmed by values of static trabecular bone histomorphometric parameters. These results suggest that EA can effectively prevent bone loss in the OVX rat.

Biomechanical strength is an important factor, reflecting bone fragility and fracture risk.^{28,29} Determining the ability of a pharmacological agent to increase bone biomechanical strength remains a major factor in its therapeutic utility for the treatment of osteoporosis. Therefore, we observed the effects of EA treatment on bone biomechanical properties. Our results demonstrated that OVX impaired the bone biomechanical properties, as shown by decreased maximum load and energy to failure in the femur and L5 vertebral body. These OVX-related changes were accompanied by reductions in maximum load of both the left femur (−9.5%) and L5 vertebral body (−22.4%) and energy to failure of both the left femur (−21.2%) and L5 vertebral body (−48.8%). The results of three-point bending tests of the femur diaphysis (a predominantly cortical site) showed that the maximum load and energy to failure in

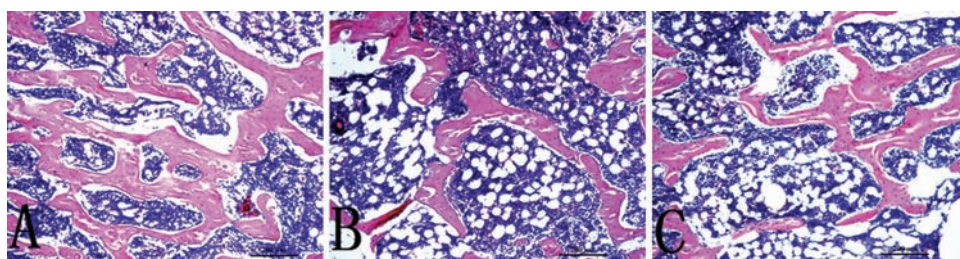


Figure 4 Histological examination of the left tibiae. The tibiae were stained with haematoxylin and eosin. (A) sham operated group; (B) OVX group; (C) OVX+EA group. All images were shown at $\times 100$ magnification. EA, electroacupuncture; OVX, ovariectomised.

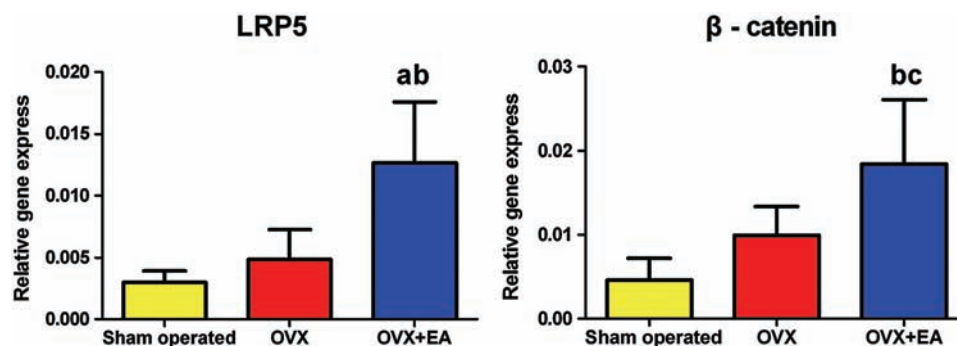


Figure 5 Effects of electroacupuncture on expressions of LRP5 and β -catenin mRNAs. RNA was isolated from the bone marrow cells obtained from the right femur and evaluated by reverse transcription polymerised chain reaction assays. Data are expressed as mean $\pm$ SD. ^a $p < 0.01$ vs OVX group; ^b $p < 0.01$ vs sham operated group; ^c $p < 0.05$ vs OVX group. EA, electroacupuncture; LRP5, low-density lipoprotein receptor-related protein 5; OVX, ovariectomised.

the OVX+EA group did not increase significantly, as compared with the OVX group. However, in the trabecular-rich vertebra, EA significantly increased maximum load (+13.6%) and energy to failure (+43.9%) in the L5 vertebral body, which was consistent with the positive changes seen in trabecular bone microarchitecture and BMD.

The results of static histomorphometric analysis demonstrated that EA significantly increased the values of Tb.Ar (+66.9%), Tb.Wi (+29.2%) and Tb.N (+30.3%) and decreased the value of Tb.Sp (−31.9%), as compared with the OVX group. Because trabecular microarchitecture plays an important role in maintaining normal bone strength,^{30–34} we inferred that the observed improvements in microarchitecture of trabecular bone may, at least in part, lie behind the ability of EA to increase bone strength.

Previous studies have demonstrated that the Wnt/ β -catenin signalling pathway plays a key role in the regulation of bone growth and remodelling. Activation of the pathway promotes proliferation and differentiation of osteoblast precursor cells and increases osteoblast activity, which favours the deposition of new bone and increases bone density.^{12 13 35} In our study, we investigated EA-induced changes in the factors involved in Wnt signalling and found that the mRNA expressions of LRP5 and β -catenin were upregulated in the bone marrow of OVX rats, as compared with those in sham operated rats. Furthermore, EA was found to have increased the mRNA expressions of LRP5 and β -catenin, as compared with the OVX group, which may be the possible mechanism responsible for favourable results from this treatment. The RT-PCR test presented evidence that EA can prevent bone loss in OVX rats, at least partly, through increasing bone formation via stimulation of the Wnt signalling pathway.

There are some limitations to our study. First, EA treatment was performed immediately after OVX. In the majority of clinical settings, however, EA treatment is given after obvious bone loss has occurred. Thus, it may be argued that the beneficial effects seen in this study may not translate into clinical benefits. Although we agree that further studies may be required before conclusive decisions can be made, the fact that EA activates the Wnt signalling pathway supports its positive role even during

Summary points

- ▶ Thirty Sprague–Dawley rats were randomised to three groups.
- ▶ Electroacupuncture (EA) preserved bone mass in ovariectomised (OVX) rats.
- ▶ EA activated the Wnt/ β -catenin signalling pathway in OVX rats.

advanced or late stages of osteoporosis when actual bone loss would have taken place. Second, we did not observe the dynamic histomorphometric parameters of the trabecular bone architecture. However, we believe that the basic data obtained from this study will provide a foundation for future studies that are likely to deal with this important aspect.

CONCLUSION

EA treatment had beneficial effects on the skeletal mass, structural architecture and biomechanical integrity of trabecular bone in OVX rats. Furthermore, EA upregulated LRP5 and β -catenin mRNA expressions in OVX rats. We conclude, therefore, that augmentation of the Wnt signalling pathway might be key to the beneficial effects of EA on OVX-induced osteoporosis. EA may represent a promising adjunct or alternative to existing treatment for the prevention of osteoporosis in clinical settings.

Contributors JZ conceived the study, performed the statistical and data analysis and drafted the manuscript. SC, HG, LX, HL, YQ participated in the experimental design of the study and data acquisition. CH conceived the study and is the primary author of the manuscript.

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Competing interests None.

Ethics approval This study was approved by the ethics committee at Sichuan University.

Provenance and peer review Not commissioned; externally peer reviewed.

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