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# Effects of electroacupuncture at GB points on markers of osteoporosis and bodyweight in ovariectomised rats

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## ABSTRACT

**Background** Based on a description of acupuncture to treat a bone disease resembling osteoporosis in the ancient text of *Huangdi Neijing*, we aimed to assess the effects of electroacupuncture (EA) at GB points in ovariectomised (OVX) rats.

**Methods** 40 female Wistar rats were randomly divided into four groups (n=10 each): ovariectomised model group (OVX); ovariectomised group treated with EA at GB points (OVX+GB); ovariectomised group treated with EA at non-GB points (OVX+N) in the hindlimb; and a sham surgery group (Sham). Three months after ovariectomy, rats in the OVX+GB and OVX+N groups received EA treatment for 3 months. Urine, blood and femur samples were collected from each animal for analysis.

**Results** Bodyweight (BW) in the OVX+GB group decreased after EA treatment, reaching a minimum of ~12% below the OVX and OVX+N groups at 1 month. Concentrations of urine deoxypyridinoline, a bone resorption marker, were significantly elevated in the OVX and OVX+N groups but not the OVX+GB group. Concentrations of serum bone specific alkaline phosphatase, a bone formation marker, were significantly higher in the OVX+GB group versus the Sham and OVX groups. Bone mineral density (BMD) did not differ between the OVX, OVX+GB and OVX+N groups, but was ~10% lower than the Sham group. However, BMD/BW in the OVX+GB group was significantly higher than in the OVX and OVX+N groups and similar to the Sham group. Histological assessment of the femur showed that EA at GB points improved the bone architecture.

**Conclusions** EA treatment at GB points had anti-osteoporotic effects in a rat model of osteoporosis.

## INTRODUCTION

Bone remodelling is a dynamic process of bone formation and bone resorption that repairs microfractures and replaces old bone with new bone. Osteoporosis is a bone disease caused by a decrease in the rate of bone formation to that of bone resorption, that results in a decrease in bone mass and increased risk of bone fracture; it is characterised by reduced bone mineral density (BMD), deteriorations in bone macro-architecture, and alterations in the protein content of the bone.<sup>1</sup> Overwhelming evidence indicates that oestrogens play a crucial role in the maintenance of normal bone homeostasis. Depletion of oestrogen occurs either spontaneously after menopause or by surgical ovariectomy, and often results in severe osteoporosis, which can be largely prevented by oestrogen replacement therapy.<sup>2–3</sup> However, in 2002 the Women's Health Initiative published data that showed an increased risk of heart attacks and breast cancer secondary to long-term treatment with hormone replacement therapy,<sup>4</sup> and there has subsequently been a large reduction in the use of oestrogen and growing interest in alternative treatments. Bisphosphonate compounds have moderate potency on bone resorption.<sup>5</sup> More recently, osteoprotegerin (OPG) has been used to block RANKL (receptor activator of nuclear factor- $\kappa$ B ligand), which ultimately leads to the inhibition of bone resorption by reducing the production, maturation, and activity of osteoclasts.<sup>5</sup> This treatment has since been substituted by the use of antibodies against RANKL.<sup>6</sup> Sclerostin

(SOST) and Dickkopf-related protein 1 (DKK1) are endogenous inhibitors of the canonical Wnt/ $\beta$ -catenin pathway specific to bone. Antibodies against SOST or DKK1 should therefore have anabolic effects on bone<sup>5,7</sup> and are in development.

Acupuncture has been used successfully to treat a wide range of human diseases, and its efficacy for the treatment of certain conditions is now widely accepted. Recently the clinical effects of acupuncture on osteoporosis have been studied,<sup>8,9</sup> and there is a growing volume of basic science research into its underlying mechanisms of action, particularly in ovariectomised (OVX) animals, which are well established models of osteoporosis.<sup>10–13</sup> One particular research group has published a series of papers reporting that electroacupuncture (EA) at BL, ST, and SP points in OVX rats and rabbits had significant anti-osteoporotic effects including increased femoral BMD, elevated serum oestradiol concentrations, and decreased body-weight (BW), and has suggested that these changes are at least partly due to EA stimulation of endogenous sex hormones.<sup>11–13</sup>

The ancient classical text of acupuncture, *Huangdi Neijing* (edited during the Southern and Northern Dynasties (420–589AD)<sup>14</sup>), included a description of the “Gallbladder Meridian of Foot Shaoyang” for treatment for bone diseases including ‘bone-shaking and fragility’, which was described as a systemic illness with multifocal bone pain and brittle bones that resembles contemporary osteoporosis. Regrettably, this description was lost in later editions after the Tang Dynasty and consequently this acupuncture protocol has remained obscure for a thousand years. We used this ancient description as the basis for point selection in the present study, the aim of which was to examine the effects of EA at GB points on BMD, bone macro-architecture, and markers of bone formation and resorption in an ovariectomised rat model. This study has already been published in Chinese.<sup>15</sup>

## METHODS

### Experimental animals

Forty female Wistar rats (12 weeks old, weighing 210  $\pm$  10 g) were purchased from the Experimental Animal Centre of Chongqing Medical University (Chongqing, China). The experiments were approved by the ethics committee of Luzhou Medical College and the animals were cared for in accordance with institutional guidelines. Rats were housed in cages maintained at a controlled temperature of 20–22°C and air humidity of 80–85% under a 12 h /12 h light/dark schedule. They were fed a standard diet (Chengdu Dashuo Bioscience Inc) and received filtered drinking water ad libitum. The BW of the animals was measured approximately every 15 days. The rats were randomly divided into four groups (n=10 each): an ovariectomised model group (OVX);

an ovariectomised group treated with EA at GB points (OVX+GB); an ovariectomised group treated with EA at non-GB points (OVX+N) in the hindlimb; and a sham surgery group (Sham). After 1 week of acclimatisation, the rats were anaesthetised using an intraperitoneal injection of 3% pentobarbital sodium (0.3 mL/100 g BW). Ovariectomy was performed by ligating and excising the ovaries at laparotomy, as previously described.<sup>15</sup> In the Sham group, a small fat mass close to the ovaries was dissected out. After surgery, a single intramuscular injection of penicillin (2500 U) was administered for 3 days. At 90 days post-surgery, rats in the OVX+GB and OVX+N groups were treated with EA for a further 90 days.

### Electroacupuncture

For EA treatment, sterilised stainless-steel acupuncture needles 0.3 mm in diameter and 25 mm in length (Huatuotuo, Suzhou Medical Instruments Factory, Suzhou, China) were inserted unilaterally without anaesthesia. Laterality was alternated at every other treatment. In the OVX+GB group, needles were inserted at GB25 (*Jingmen*), GB30 (*Huantiao*), GB34 (*Yanglingquan*), and GB39 (*Xuanzhong*), to a depth of 2–5 mm from the body surface depending on the individual acupuncture point. Alternating current (15 Hz) was applied across the needles at GB30 and GB39 using an EA apparatus (Model G6805-I, Xin Sheng Ltd, Qingdao, China) and gradually increased until local muscle twitches were observed (intensity 0.2–0.6 mA). The rats were quiet and relaxed during the EA treatment. Starting at 90 days post-surgery, each rat received six courses of EA, each of which consisted of 10 treatments (given once daily for 10 min between 08:00 and 10:00) followed by a 5-day rest (90 days of treatment in total). In the OVX+N group, EA was applied across two non-acupuncture points on the inner aspect of the upper hindlimbs using the same stimulation parameters as the OVX+GB group.

### Examinations of bone properties

After completion of six courses of EA treatment (at 180 days post-surgery), individual animals were placed into metabolic cages for 1 day. Urine was collected for 24 h and stored at –20°C. After terminal anaesthesia using an intraperitoneal overdose of sodium pentobarbital, the animals were weighed and then euthanased by cardiac puncture. Collected blood was centrifuged (2000 rpm for 10 min) and the resultant serum was stored at –80°C. Both femora were excised and cleared of the overlying soft tissues. The right femur was stored at –80°C pending measurement of BMD. The left femur was fixed in 4% formaldehyde then the lower third of the bone was decalcified in 14% EDTA at 4°C for 4 weeks. After dehydration, specimens were embedded in resin pending histological assessment.

Urinary concentrations of urine deoxypyridinoline (uDPD), a marker of bone resorption, and serum bone specific alkaline phosphatase (sBAP), a marker of bone formation, were measured using commercially available ELISA kits (uDPD: K-E30030R 2009-04-07; and sBAP: CK-E30492R 2009-03-04; ADL Company, USA).

BMD of the right femur was measured at the Fourth Hospital Affiliated to Sichuan University (Chengdu) using a dual-energy X-ray absorptiometry (DEXA) analyser (QDR-4500A, Hologic, Bedford, New York, USA) with specialised software (Host Software V.3.9.4 and Scanner Software V.1.2.0, QDR-4500A, Hologic) at a scanning rate of 10 mm/s and a resolution of 0.2 mm.

The left femur was sectioned in the longitudinal plane into slices of 5  $\mu$ m thickness using a microtome (Reichert-Jung 2040 Leica, Germany) and stained with haematoxylin and eosin (H&E). Histomorphometry was performed using a light microscope (Olympus-BX51, Japan). Histological assessment was performed last of all and only in a subgroup of animals (five of eight in the Sham group, eight of 10 in the OVX group, and seven of nine in the OVX+GB group). Samples from the OVX+N group were not examined as there had been no effect of this sham treatment on any other parameters.

#### Statistical analysis

Data are expressed as mean $\pm$ SEM and were compared between the four groups using one-way analysis of variance followed by post-hoc test of least significant difference. For sBAP, the Kruskal-Wallis test was applied. All statistical comparisons were performed

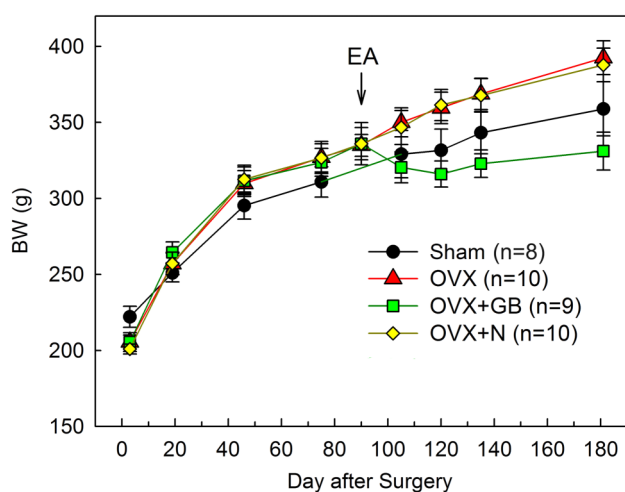
using SPSS V.13.0 (Chicago, Illinois, USA). A value of  $p < 0.05$  was considered to be statistically significant.

## RESULTS

Three rats (one in the OVX+GB group and two in the Sham group) died during anaesthesia. Data were obtained for 10 rats in each of the OVX and OVX+N groups, nine rats in the OVX+GB group, and eight rats in the Sham group.

#### Bodyweight

Figure 1 plots the BW of the four groups of rats (Sham, OVX, OVX+GB, OVX+N) against the number of days after surgery. Postoperatively, BW increased quickly during the first 40 days and then continued more slowly. The rate of BW increase in the ovariectomised rats was higher than in the Sham group, and the differences became statistically significant ( $p = 0.030$ ) at 50 days post-surgery. After starting EA treatment, the BW of rats in the OVX and OVX+N groups continued to increase in a similar manner. In the OVX+GB group, however, BW decreased quickly after starting EA treatment and actually fell below the level of the Sham group during the first course of treatment (15 days). BW reached a minimum at 1 month and then increased gradually. At the 1 month stage, the BW of OVX+GB rats was  $\sim 12\%$  lower than that of the OVX and OVX+N groups ( $p = 0.004$ ), and  $\sim 3\%$  lower than that of the Sham group ( $p = 0.21$ ). At the end of the sixth course of treatment, BW of the OVX+GB rats was  $\sim 16\%$  lower than that of OVX and OVX+N rats ( $p = 0.002$ ), and  $\sim 8\%$  lower than that of the Sham group ( $p = 0.057$ ). There were no noticeable differences in the behaviour or physical conditions of the OVX+GB rats compared to the other groups, except that the OVX+GB rats drank more water than those in the other groups after each EA treatment.



**Figure 1** Changes in bodyweight over time in 37 Wistar rats undergoing sham surgery (Sham group,  $n = 8$ ) or ovariectomy followed by treatment with electroacupuncture (EA) at GB points (OVX+GB group,  $n = 9$ ), EA at non-GB points in the hindlimb (OVX+N group,  $n = 10$ ), or no treatment (OVX group,  $n = 10$ ). Data are mean and SE (SEM).

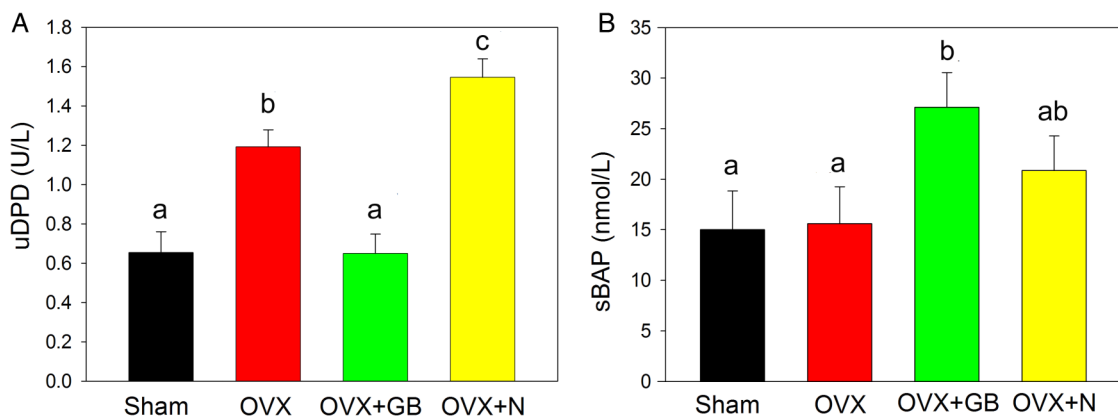
#### Bone turnover

Figure 2 illustrates the effects of EA treatment on the concentration of uDPD, a bone resorption marker, and sBAP, a bone formation marker. As shown in figure 2A, uDPD concentrations in the OVX and OVX+N groups were markedly elevated compared with the Sham group. In contrast, the uDPD concentration in the OVX+GB group did not increase, and was maintained at the same value as the Sham group.

The concentration of sBAP in the OVX+GB group was significantly higher than in the Sham and OVX groups (figure 2B), but there was no significant difference in the concentration of sBAP between the OVX and Sham groups. The concentration of sBAP in the OVX+N rats was intermediate but was not significantly different compared to the other three groups.

#### Bone mineral density

BMD of the right femur in the OVX rats was  $\sim 10\%$  lower than that of sham-operated rats, but there were



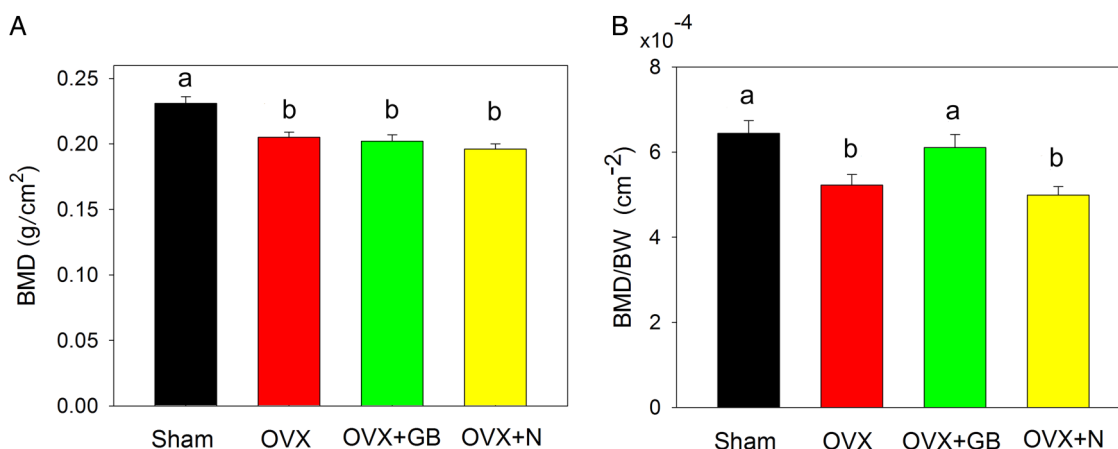
**Figure 2** Concentrations of urine deoxypyridinoline (uDPD), a bone resorption marker (A), and serum bone specific alkaline phosphatase (sBAP), a bone formation marker (B), in 37 Wistar rats undergoing sham surgery (Sham group, n=8) or ovariectomy followed by treatment with electroacupuncture (EA) at GB points (OVX+GB group, n=9), EA at non-GB points in the hindlimb (OVX+N group, n=10), or no treatment (OVX group, n=10). Data are mean and SE (SEM). Significant differences between groups are indicated by *differing* superscripts and are detailed as follows. uDPD: Sham versus OVX+N (all  $p<0.001$ ); and OVX versus OVX+GB, and OVX+GB versus OVX+N (all  $p<0.001$ ); and OVX versus OVX+N ( $p=0.02$ ). sBAP: Sham versus OVX+GB ( $p=0.017$ ) and OVX versus OVX+GB ( $p=0.007$ ).

no significant differences in BMD values between the OVX, OVX+GB, and OVX+N groups (figure 3A). In view of the significant differences in BW between groups (figure 1), BMD values were corrected for BW (BMD/BW), as shown in figure 3B. BMD/BW was significantly higher in the OVX+GB rats than in the OVX or OVX+N rats, and was increased to a level comparable to that of the Sham group.

#### Bone architecture

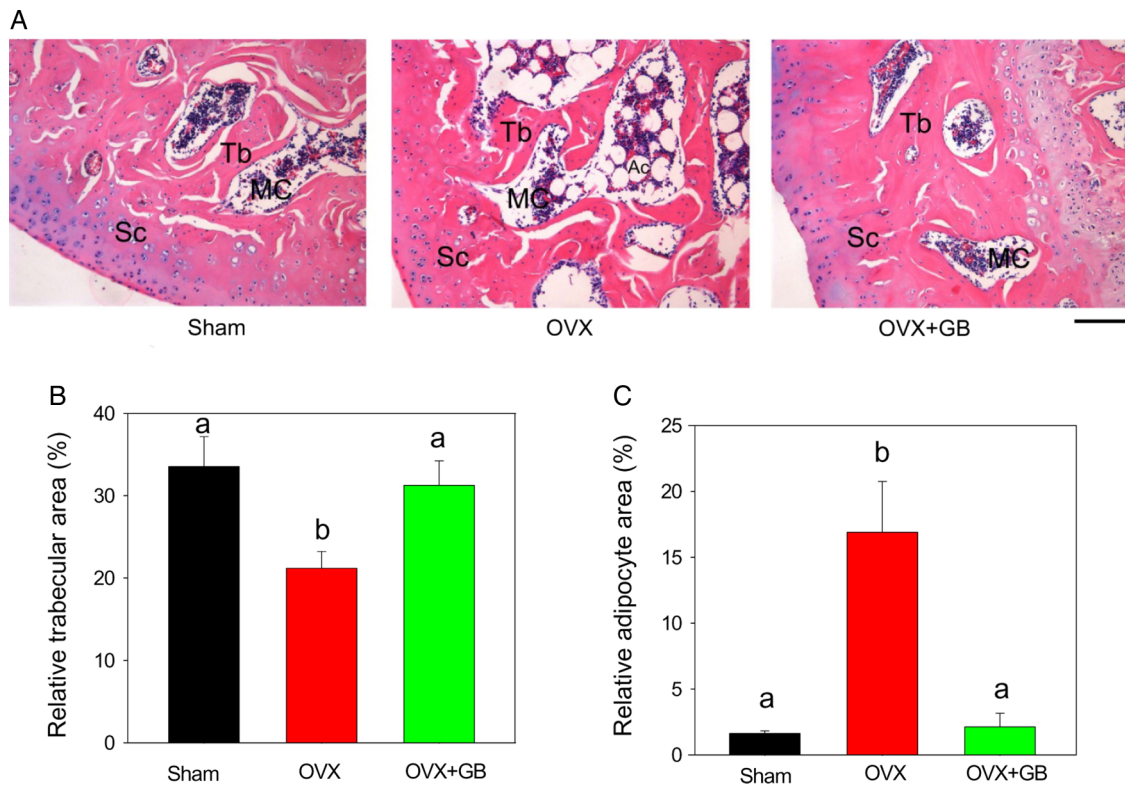
Figure 4A exhibits representative micrographs from three of the four experimental groups (Sham, OVX, OVX+GB) comparing the macrostructure of H&E stained sections of the distal femur. This region in the Sham group demonstrated normal bone structure

consisting of wide sclerotin, trabeculae and marrow cavities. In the bone of the OVX rats, there were stigmata of osteoporosis including thinner sclerotin and thinner trabeculae, and larger marrow cavities with the appearance of abundant adipocytes within them. These deteriorations in bone architecture were not seen in the bone of the OVX+GB rats. Sclerotin was regular and wide, representing matured differentiation, marrow cavities were smaller, and adipocytes were greatly decreased. Quantitative data analyses confirmed these observations. Figure 4B, C show the trabecular area and adipocyte area relative to the total bone area for each micrograph. The relative trabecular area of the OVX rats was >30% smaller than that of rats in the Sham and OVX+GB groups, which were



**Figure 3** Bone mineral density (BMD) of the left femora (A) and BMD corrected for bodyweight (BW) (B) in 37 Wistar rats undergoing sham surgery (Sham group, n=8) or ovariectomy followed by treatment with electroacupuncture (EA) at GB points (OVX+GB group, n=9), EA at non-GB points in the hindlimb (OVX+N group, n=10), or no treatment (OVX group, n=10). Data are mean and SE (SEM). Significant differences between groups are indicated by *differing* superscripts and detailed as follows. BMD: Sham versus OVX, Sham versus OVX+GB, and Sham versus OVX+N (all  $p<0.001$ ). BMD/BW: Sham versus OVX, Sham versus OVX+N, OVX versus OVX+GB, and OVX+GB versus OVX+N (all  $p<0.001$ ).





**Figure 4** Representative light micrographs of haematoxylin and eosin (H&E) stained sections of the distal femur region (A) and calculations of relative trabecular area (B) and relative adipocyte area (C) in 37 Wistar rats undergoing sham surgery (Sham group,  $n=5$ ) or ovariectomy followed by treatment with electroacupuncture at GB points (OVX+GB group,  $n=7$ ) or no treatment (OVX group,  $n=8$ ). Data are mean and SE (SEM). Significant differences between groups are indicated by differing superscripts and detailed as follows. Relative trabecular area: Sham versus OVX ( $p=0.009$ ) and OVX versus OVX+GB ( $p=0.039$ ). Relative adipocyte area: Sham versus OVX ( $p=0.003$ ) and OVX versus OVX+GB ( $p=0.001$ ). Ad, adipocytes; MC, marrow cavity; Sc, sclerotin; Tr, trabeculae. Scale bar indicates 100  $\mu\text{m}$ .

not significantly different from one another. Relative adipocyte area in the OVX rats was more than eight times higher than in the Sham group, while levels in the OVX+GB rats were equivalent to rats in the Sham group.

## DISCUSSION

The results of the present study demonstrated that EA at GB points for 3 months had a number of effects on ovariectomised rats, including: (1) a decrease in BW; (2) prevention of the increase in the bone resorption marker uDPD; (3) an increase in the bone formation marker sBAP; and (4) apparent recovery of the bone macro-architecture. Collectively, the results indicate that EA at GB points exerts an anti-osteoporotic effect in ovariectomised rats.

Some of our results, including the effects on BW and BMD, are inconsistent with recent reports of EA at BL, ST and SP points in animal models of ovariectomy,<sup>11–13</sup> which may reflect differences attributable to acupuncture point location. The reduction in rat BW beyond that of the sham-operated controls following EA at GB but not at hindlimb points was perhaps surprising; however, a similar pattern of BW decrease in OVX+GB rats after starting EA treatment

has been observed in another series of experiments which used 1-month-old Wistar rats (H Jiang, unpublished data). Ovariectomy per se can lead to an increased BW due to greater fat cell differentiation in place of lower skeletal mass; therefore, it is feasible that EA could attenuate this phenomenon via effects on bone formation and turnover. However, why BW fell to levels lower than the Sham group remains unclear. In this study, BMD did not differ between EA treated and untreated ovariectomised groups. However, as it is a quantity measured by two-dimensional scanning at the bone surface, BMD per se does not reflect bone strength, which is heavily influenced by body mass.<sup>16</sup> To assess bone strength better, BMD has been adjusted for various body mass parameters such as body width<sup>17</sup> or BW.<sup>17–19</sup> As BMD/BW was significantly greater in the OVX+GB groups, we would expect that bone strength would be greater in this group. By contrast, EA treatment at non-GB points appeared to have no effect.

Bone remodelling consists of three phases: an osteoclastic phase (osteoclast activation and resorption of microscopic portions of bone); a reversion phase (osteoclast replacement by so-called post-osteoclastic cells); and an osteoblastic phase (osteoblastic

reconstruction of the resorbed bone matrix until the initial volume has been regained). Bone remodelling is regulated by a number of systemic and local factors, in which oestrogen plays a major role.<sup>20–21</sup> Oestrogen deficiency enhances osteoclast formation by increasing haematopoietic progenitors and by providing a larger recruited osteoclast progenitor pool.<sup>22</sup> In addition, oestrogen depletion increases the lifespan of osteoclasts, leading to prolonged bone loss, deeper resorption cavities, and trabecular perforation that advances the fragility of bone.<sup>23</sup> Up-regulated formation and activation of osteoclasts lead to cortical porosity and enlarged resorption areas in the trabecular surfaces.<sup>24</sup> The present study suggests that EA may activate an intrinsic mechanism that can, at least partially, compensate for the lack of oestrogen.

EA stimulation at GB points prevented the increase in the concentration of the bone resorption marker uDPD that occurred in the ovariectomised rats, which resembles the effect of oestrogen. However, our results revealed that the EA treatment also elevated the concentration of the bone formation marker sBAP, while ovariectomy itself did not change the concentration of sBAP compared to that of the Sham group. These results suggest that, although oestrogen can up-regulate OPG and promote bone mass, EA stimulation at GB points might activate a metabolic pathway outwith the oestrogen pathway to accelerate the process of bone formation, and balance bone remodelling by bidirectional regulation, that is, inhibiting bone resorption and promoting bone formation. We have measured OPG, RANKL, SOST, DKK1, and core binding factor  $\alpha 1$  (Cbfa1) mRNA expression in rat bone tissue, and calculated the ratio to RANKL (H Jiang, unpublished data). These results further suggest that the net effect on bone turnover in ovariectomised rats treated with EA at GB points involves both down-regulation of bone resorption and up-regulation of bone formation.

Oestrogen also regulates adipocyte differentiation and fat depot localisation,<sup>25–27</sup> and depletion of oestrogen causes obesity.<sup>28–29</sup> As already mentioned, this helps to explain why BW increased in OVX rats. In the present study, we found that EA treatment at GB points in OVX rats reduced BW by ~12% at 1 month and ~16% at 3 months, which is notable considering that oestrogen replacement in OVX rats prevented increases in BW but did not reduce it below the levels of non-OVX controls.<sup>30</sup> Histological observations of the H&E stained sections of distal femur showed that EA treatment at GB points recovered the bone architecture, which had progressively deteriorated after ovariectomy, and this was accompanied by a decrease in the number of adipocytes. It may be postulated that EA at GB points stimulates a signalling pathway that inhibits the differentiation of mesenchymal stem cells into adipocytes, and promotes their differentiation into osteoblasts within bone. This suggests that EA

therapy might simultaneously prevent obesity and improve bone quality, which would be of great interest for human health.

In summary, the present study showed that EA to GB points exerted anti-osteoporotic effects in a rat model. Ovariectomy-induced increases in BW and the bone resorption marker uDPD were prevented, and there was a measurable increase in the bone formation marker sBAP together with histological evidence of improved bone architecture relative to untreated ovariectomised rats. The findings support the potential role of EA in the management of osteoporosis, although robust clinical trials are needed to confirm putative beneficial effects in postmenopausal women.

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**Contributors** H-DW focused on the ancient TCM theory about GB meridian, arranged the funding support, designed the experiment and drafted the report. ZC conducted the experiments and trained the students. II wrote and submitted the paper. S-JF, X-LS and LT performed the specimen detection. F-ZZ and YJ preformed the animal experiment, conducted the electro-acupuncture and collected the animals' samples such as bone, blood and urine. HJ monitored the whole experiment and revised the report and is responsible for publishing work.

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**Competing interests** None declared.

**Ethics approval** This study was prospectively approved by the ethics committee of Luzhou Medical College (reference number: 2008DW-0003) and was conducted in accordance with local and national guidelines for animal welfare equivalent to the National Research Council 'Guide for the Care and Use of Laboratory Animals'.

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**Data sharing statement** For details of unpublished data, please contact Ms Hua Jiang, the corresponding author of this article; jhflower2586546@sina.com.

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