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ORIGINAL RESEARCH

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Electroacupuncture stimulation at CV4 sites prevents breast cancer-induced osteoporosis by activating the Wnt/β-catenin signaling pathway

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Abstract

Osteoporosis is a common condition among breast cancer patients, characterized by decreased bone density due to chemotherapy and hormone therapy. However, the application of electroacupuncture (EA) stimulation at Guanyuan (CV4) sites has been found to be beneficial in enhancing bone mineral density. In this study, we aimed to investigate the efficacy of EA stimulation at CV4 sites in breast cancer-induced osteoporosis and elucidate the potential underlying mechanisms. A breast cancer model in mice was established, EA stimulation at CV4 sites was performed, and hematoxylin-eosin (HE) stain was conducted to assess the bone mineral density and trabecular bone volume of femur tissues. Additionally, Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify and visualize osteoclasts. Furthermore, Western blots were used to evaluate the expression levels of various proteins, including c-src, Cathepsin K (CtSK), matrix metalloproteinase 9 (MMP9), Collagen 1A1 (COL1A1), Osteopontin (OPN), Runt-related transcription factor 2 (RUNX2), Wnt3a and β-catenin, and Enzyme Linked Immunosorbent Assay (ELISA) to measure the concentration of Alkaline phosphatase (ALP), NO synthase 2 (NOS2), glutathione reductase (GSR), superoxide dismutase (SOD), reactive oxygen species (ROS) and lactate dehydrogenase (LDH) in the serum. The results revealed that EA stimulation in the breast cancer model resulted in higher bone mineral density and trabecular bone volume, along with a reduction in the number of osteoclasts. EA stimulation at CV4 site significantly decreased the protein levels of c-src, CtSK and MMP9, while also reducing ROS and LDH production. Conversely, it promoted the expression of COL1A1, OPN, RUNX2, Wnt3a and β-catenin. Additionally, it elevated the concentration of ALP, NOS2, GSR and SOD in the serum. Overall, EA stimulation at the CV4 site may inhibit bone loss and promote bone differentiation by activating the Wnt/β-catenin pathway, indicating its potential use for breast cancer-induced osteoporosis treatment.

Keywords

Breast cancer; CV4; Electroacupuncture stimulation; Osteoporosis; Wnt/β-catenin

1. Introduction

Osteoporosis is a systemic bone disease characterized by the loss of mineral density and deterioration of bone structure. The progression of osteoporosis is often associated with an imbalance between bone formation and resorption [1]. It is more prevalent in women than men, and disruptions in bone homeostasis can lead to bone loss. Further, breast cancer (BC) is another significant cause of mortality in women [2]. Recent studies have established that osteoclasts, rather than tumor cells, play a central role in bone metastases and directly contribute to the development of bone lesions [3].

Electroacupuncture (EA) is an advanced technique derived from traditional acupuncture and moxibustion. It is based on the principles of promoting the flow of Qi and alleviating blood stasis in the meridians, as described in traditional Chinese medicine. EA has gained recognition as an effective and complementary treatment for various human ailments [4]. Clinical evidence has shown that acupuncture is beneficial for managing menopausal, perimenopausal syndromes and osteoporosis. In experimental studies, EA pretreatment has been found to increase bone mineral density (BMD) and prevent bone loss in ovariectomized rats [5, 6]. Additionally, EA stimulation of specific acupoints such as Pishu (BL20), Shenshu (BL23) and Sanyinjiao (SP6) has demonstrated the ability to delay the onset of osteoporosis induced by hindlimb unloading in rats [7]. Furthermore, EA stimulation of Guanyuan (CV4) acupoint has shown potential in preventing ovariectomy-induced osteoporosis by modulating the Wnt/β-catenin signaling pathway [8]. However, it remains to be determined whether EA stimulation

of the CV4 acupoint can improve the process of osteoporosis induced by breast cancer, thereby urging further research to elucidate the underlying mechanisms involved.

In this study, EA at the CV4 acupoint was shown to effectively prevent bone loss and stimulate bone differentiation *via* the activation of the Wnt/β-catenin pathway, ultimately leading to the prevention of osteoporosis induced by breast cancer.

2. Materials and methods

2.1 Mice

Female BALB/c mice were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and housed in a specific pathogen-free (SPF) environment with sufficient food and water and maintained under a 12-hour light/dark cycle [9].

A total of 24 six-week-old female BALB/c nude mice were included in the study and randomly assigned into four groups (n = 6/group), namely, the Sham group, where non-acupoints were treated after a sham operation; the Sham + CV4 group, where the CV4 site was stimulated with electroacupuncture after the sham operation; the BC group, where non-acupoints were treated after breast cancer modeling; and the BC + CV4 group, where electroacupuncture stimulation was applied at the CV4 site after breast cancer modeling.

For two groups, BC and BC + CV4, the mice were anesthetized using isoflurane gas and then injected with a 50 μL suspension of MDA-MB-231 cells (CRM-HTB-26; ATCC, Manassas, VA, USA) at the density of 1×10^7 cells/mL into the medullary cavity of the left tibia. In contrast, the Sham and Sham + CV4 groups received an injection of 50 μL of phosphate buffered saline (PBS). Following a one-week postoperative recovery period, electroacupuncture was administered either at the CV4 site or non-acupoints. The CV4 site is located obliquely on the midline of the mouse abdomen, 10 mm below the navel, specifically at the pubic symphysis. Sterile 0.5-inch acupuncture needles (Suzhou Medical, Suzhou, China) were inserted vertically into the acupoints to a depth of 5 mm. The SDZ-V electronic acupuncture treatment apparatus (Hwato, Shanghai, China) was utilized, connected to the acupuncture needles, and applied a dilated wave AC pulse current with an intensity of 1 mA and a frequency of 2 Hz. The acupuncture needles remained inserted at the acupoints for 20 minutes per session, conducted daily for three sessions (10 days each).

2.2 Hematoxylin and eosin (HE) staining

The tissue sections were embedded in paraffin and subsequently deparaffinized using xylene. A Hematoxylin solution was applied to stain the tissue sections, followed by treatment with an acidic solution to remove any excess hematoxylin stain. The sections were then immersed in eosin solution and dehydrated using alcohol solutions. After dehydration, the samples were cleared with xylene and mounted with a coverslip for observation.

2.3 Tartrate-resistant acid phosphatase (TRAP) staining

The tissue sections were deparaffinized using xylene and rehydrated using alcohol solutions. Next, the sections were incubated in a solution containing a substrate specific for the tartrate-resistant acid phosphatase enzyme, allowing for its detection. To visualize the tissue structure, the sections were counterstained with hematoxylin. Following counterstaining, the sections underwent dehydration using a series of alcohol solutions. They were then cleared using xylene or other appropriate clearing agents. Finally, the sections were mounted with a coverslip for further examination and analysis.

2.4 Western blot

The total protein was extracted using lysis buffer (89901, Thermo Scientific, Carlsbad, CA, USA). The lysates were subjected to immunoblot analysis using the primary antibodies listed in Table 1. Following incubation with the primary antibodies, the lysates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (B900210, ProteinTech Group, Rosemont, IL, USA; diluted 1:5000). Subsequently, the target protein bands were visualized using ECL reagents (PE0010, Solarbio Life Sciences, Beijing, China). The relative intensity of each band was quantified using ImageJ software and normalized to β-actin.

2.5 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of ALP, NOS2, GSR, SOD, ROS and LDH in serum were evaluated using ELISA kits of ALP (ab263890, Abcam, Cambridge, UK), NOS2 (ab253219, Abcam), GSR (EM0720, FineTest), SOD (MBS034842, MyBioSource), ROS (MBS2601061, MyBioSource) and LDH (MBS720560, MyBioSource). Briefly, 100 μL of the sample was added to each well of the ELISA plate and incubated for 2 hours. Then, 100 μL of the detection antibody was added and incubated for 1 hour. Subsequently, each well was treated with the enzyme working reagent, 3,3′,5,5′-Tetramethylbenzidine (TMB) reagent, and the reaction was terminated using a stop solution. The absorbance value of each well was measured at 450 nm using a spectrophotometer.

2.6 Quantification and statistical analysis

Data analysis was performed using GraphPad Prism (8.0, Dotmatics, Boston, MA, USA). The results are presented as the mean $\pm$ standard error (SE) based on three biological replicates. Statistical comparisons between two groups were conducted using unpaired *t*-tests and multiple group comparisons using analysis of variance (ANOVA). A *p*-value less than 0.05 was considered statistically significant.

3. Results

TABLE 1. Antibodies information.

Protein	Cat. No.	Manufacturer	Dilution
c-src	ab133283	Abcam, Cambridge, MA, USA	1:3000
CtSK	ab187647	Abcam, Cambridge, MA, USA	1:5000
MMP9	ab76003	Abcam, Cambridge, MA, USA	1:3000
COL1A1	91144	Cell Signaling Technology, MA, USA	1:2000
OPN	22952-1-AP	ProteinTech Group, Rosemont, IL, USA	1:3000
RUNX2	20700-1-AP	ProteinTech Group, Rosemont, IL, USA	1:1000
Wnt3a	26744-1-AP	ProteinTech Group, Rosemont, IL, USA	1:2000
β-catenin	51067-2-AP	ProteinTech Group, Rosemont, IL, USA	1:3000
β-actin	20536-1-AP	ProteinTech Group, Rosemont, IL, USA	1:5000

CtSK: Cathepsin K; MMP9: matrix metalloproteinase 9; COL1A1: Collagen 1A1; OPN: Osteopontin; RUNX2: Runt-related transcription factor 2.

3.1 EA stimulation at the CV4 site prevents bone loss in breast cancer model

To investigate the impact of EA stimulation on osteoporosis, a breast cancer model was established in mice to examine the effect of EA stimulation at the CV4 site on bone loss. HE staining analysis revealed no significant difference when EA stimulation at the CV4 site was performed on the Sham group. However, in the BC group, mice subjected to EA stimulation exhibited higher bone mineral density and trabecular bone volume than the control group (Fig. 1a). Additionally, TRAP staining was conducted to identify and visualize osteoclasts, the cells responsible for bone tissue degradation. In the Sham group, EA stimulation at the CV4 site did not induce osteoclast production. Conversely, the BC group displayed numerous osteoclasts, which were significantly reduced following EA stimulation at the CV4 site in the BC + CV4 group (Fig. 1b). Furthermore, western blot analysis was conducted to assess the expression levels of key molecules associated with osteoporosis, such as c-src, CtSK and MMP9. The results demonstrated that these molecules were highly expressed in the BC group, while their expression was attenuated by EA stimulation at the CV4 site in the BC + CV4 group (Fig. 1c). Overall, these findings indicate that EA stimulation at the CV4 site effectively prevented bone loss in mice with breast cancer.

3.2 EA stimulation at the CV4 site promotes osteogenic differentiation in the breast cancer model

To assess the impact of EA stimulation on osteogenic differentiation, the expression levels of key molecules involved in osteogenic differentiation, including COL1A1, OPN, RUNX2 and ALP, were analyzed in the BC model. Western blot analysis was conducted to measure the protein levels of COL1A1, OPN and RUNX2 in the femur. In the Sham group with EA stimulation at the CV4 site (Sham + CV4), no significant variation in expression levels was observed compared to the Sham group. However, in the BC model, the expression levels of COL1A1, OPN and RUNX2 were significantly reduced compared to the Sham group. In contrast, EA stimulation at the CV4 site in the BC + CV4 group resulted in a significant increase in COL1A1, OPN and RUNX2 levels compared to the BC group. Notably, the expression levels in the BC + CV4 group remained lower than those in the Sham + CV4 group (Fig. 2a). Additionally, ELISA analysis was performed to evaluate the concentration of ALP in serum. The data revealed a decrease in ALP concentration in the BC group, while EA stimulation at the CV4 site increased the levels of ALP compared to the BC group (Fig. 2b). These results indicate that EA stimulation at the CV4 site promotes osteogenic differentiation in the context of breast cancer-induced osteoporosis.

3.3 EA stimulation at the CV4 site inhibits ROS production in breast cancer model

ELISA analysis was conducted to evaluate the serum concentrations of NOS2, GSR and SOD. The results showed that the levels of NOS2 were elevated in the BC model compared to the Sham group, which was further promoted by EA stimulation at the CV4 site in the BC + CV4 group. Regarding GSR and SOD, their concentrations were decreased in the BC group compared to the Sham group. However, in the BC + CV4 group, the concentrations of GSR and SOD were significantly increased compared to the BC group (Fig. 3a). Furthermore, the relative levels of ROS and LDH in the serum were analyzed using ELISA. The data demonstrated that the density of ROS and LDH was increased in the BC group compared to the Sham group but significantly reduced in the BC + CV4 group compared to the BC group (Fig. 3b). Collectively, these findings suggest that EA stimulation at the CV4 site effectively suppresses ROS production in breast cancer-induced osteoporosis.

3.4 EA stimulation at the CV4 site activates the Wnt/β-catenin signaling pathway

To investigate the activation of the Wnt/β-catenin pathway by EA stimulation at the CV4 site, the expression levels of two key molecules involved in this signaling pathway, Wnt3a and β-catenin, were assessed using western blot analysis. The results revealed that the expression of Wnt3a and β-catenin was decreased in the BC group compared to the Sham group. However, in the BC + CV4 group, where EA stimulation at the CV4 site was applied, there was a significant promotion of Wnt3a and β-catenin expression (Fig. 4), indicating that EA stimulation at the CV4 site may effectively activate the Wnt/β-catenin signaling pathway in the breast cancer model.

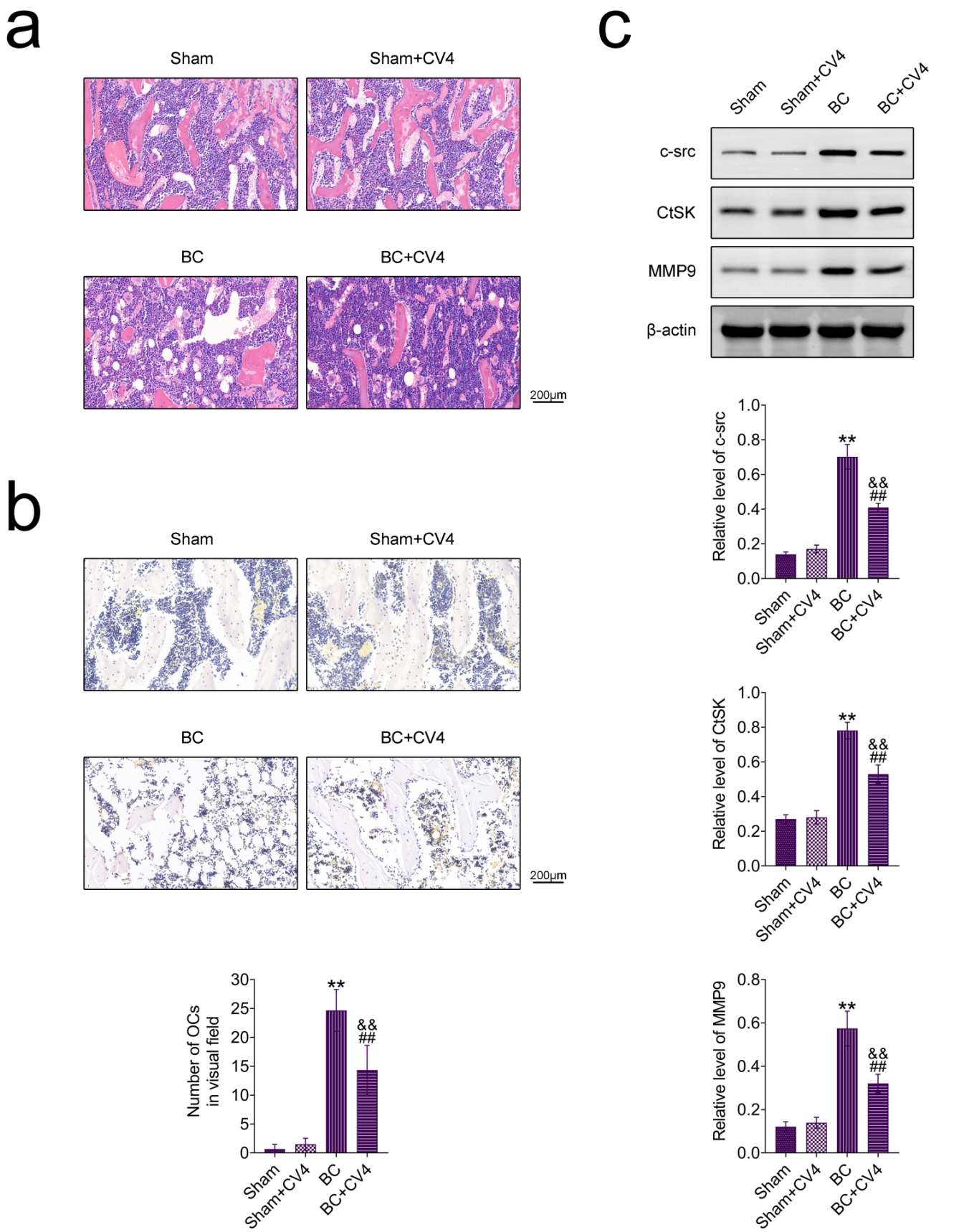

FIGURE 1. EA stimulation at the CV4 site prevents bone loss in the breast cancer model. (a) HE staining of femur tissues from breast cancer model to observe the differences in bone mineral density and trabecular bone volume. (b) TRAP staining was performed to identify and visualize osteoclasts. (c) Western blots were performed to evaluate the expression of c-src, CtSK and MMP9, which are associated with osteoporosis. Error bar, mean ± SD. *, BC *vs.* Sham; #, BC + CV4 *vs.* BC; & BC + CV4 *vs.* Sham + CV4. **/##/&&, $p < 0.01$. CtSK: Cathepsin K; MMP9: matrix metalloproteinase 9; BC: Breast Cancer; CV4: Guanyuan; OCs: osteoclasts.

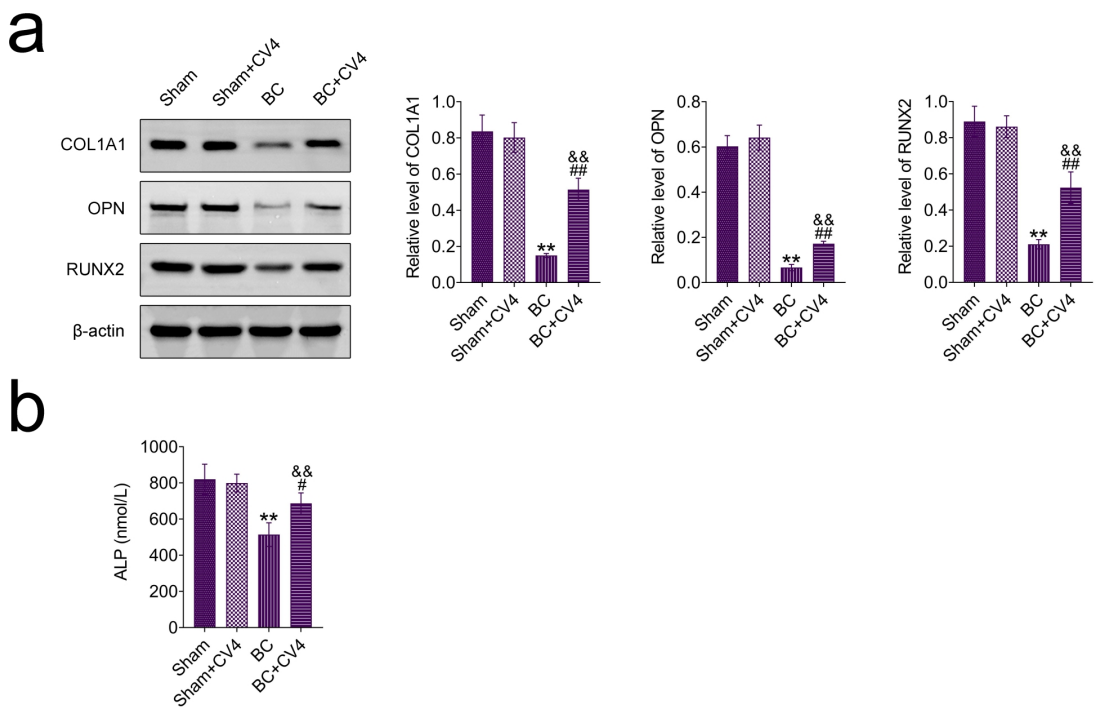

FIGURE 2. EA stimulation at the CV4 site promotes osteogenic differentiation in breast cancer. (a) Western blot was performed to measure the protein expression of COL1A1, OPN and RUNX2 in the femur. (b) ELISA was performed to evaluate the concentration of ALP in serum. All the experiments were repeated three times. Error bar, mean ± SD. *, BC *vs.* Sham; #, BC + CV4 *vs.* BC; & BC + CV4 *vs.* Sham + CV4. #, $p < 0.05$; **/##/&&, $p < 0.01$. COL1A1: Collagen 1A1; OPN: Osteopontin; ALP: Alkaline phosphatase; RUNX2: Runt-related transcription factor 2; BC: Breast Cancer; CV4: Guanyuan.

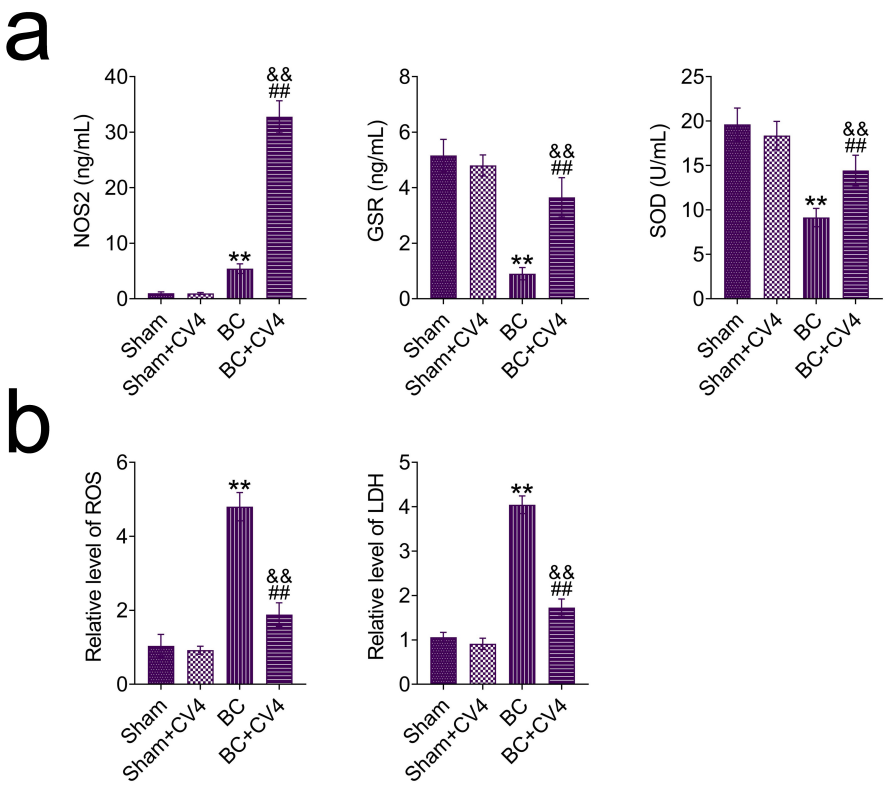

FIGURE 3. EA stimulation at the CV4 site inhibits ROS production in the breast cancer model. (a) ELISA was performed to evaluate the serum concentration of NOS2, GSR and SOD. (b) The relative levels of ROS and LDH in serum analyzed using ELISA. All the data was from at least three repeated experiments. Error bar, mean ± SD. *, BC *vs.* Sham; #, BC + CV4 *vs.* BC; & BC + CV4 *vs.* Sham + CV4. **/##/&&, $p < 0.01$. NOS2: NO synthase 2; GSR: glutathione reductase; SOD: superoxide dismutase; ROS: reactive oxygen species; BC: Breast Cancer; CV4: Guanyuan; LDH: Lactate dehydrogenase.

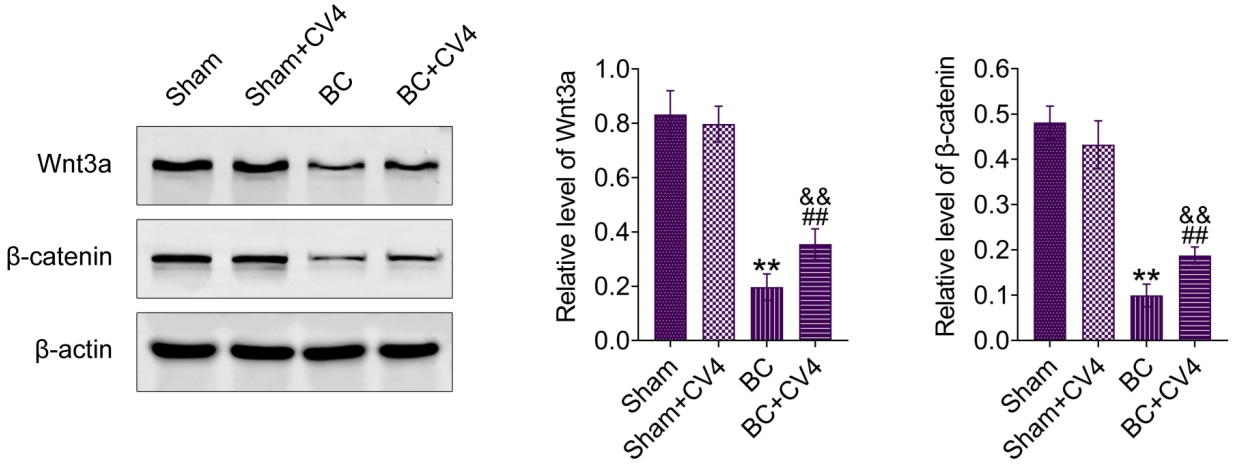

FIGURE 4. EA stimulation at the CV4 site activates the Wnt/β-catenin signaling pathway. Western blots were performed to evaluate the expression levels of Wnt3a and β-catenin. All the analysis was based on at least three repeated experiments. Error bar, mean $\pm$ SD. *, BC *vs.* Sham; #, BC + CV4 *vs.* BC; & BC + CV4 *vs.* Sham + CV4. #, $p < 0.05$; **/##/&&, $p < 0.01$. BC: Breast Cancer; CV4: Guanyuan.

4. Discussion

Osteoporosis is characterized by a decrease in bone density, resulting in a heightened susceptibility to fractures. It is particularly prevalent among older women, including breast cancer patients, who often experience decreased bone density due to chemotherapy and hormone therapy, increasing their risk of developing osteoporosis. EA applied at the CV4 point has been suggested to have potential benefits in regulating hormone levels and improving menstrual cycle regularity. By complementing traditional acupuncture techniques, EA may provide additional pain relief and therapeutic advantages for individuals with osteoporosis, including breast cancer patients [10].

Previous studies have indicated that EA stimulation at the CV4 point can positively impact enhancing bone mineral density. It has been observed that EA stimulation enhances the expression of transmembrane calcium transport-related receptors and facilitates intestinal calcium absorption [11]. In the present study, HE and TRAP staining demonstrated that EA stimulation at the CV4 site led to an improvement in bone mineral density and a reduction in the quantity of osteoclasts in the breast cancer model. Furthermore, EA stimulation at the CV4 site weakened the expression of osteoporosis-related molecules, including c-src, CtSK and MMP9. c-src is known to play a role in bone resorption by regulating osteoclast activity [12]. CtSK is involved in the resorption of calcified cartilage during endochondral bone formation [13], and MMP9 is associated with tissue remodeling, wound healing and angiogenesis [14]. Based on these findings, it might be reasonable to conclude that EA stimulation at the CV4 site can effectively prevent bone loss in the context of breast cancer-induced osteoporosis.

Several studies have investigated the effects of EA stimulation at the CV4 site on osteogenic differentiation, a process in which stem cells differentiate into bone-forming cells. It has been observed that EA stimulation at CV4 promotes osteogenic differentiation in bone marrow mesenchymal stem cells (BMSCs). Specifically, BMSCs treated with EA stimulation exhibited increased expression of osteogenic markers and higher mineralization rates compared to control groups [15]. Furthermore, in postmenopausal women with osteoporosis, EA stimulation at the CV4 site has been shown to significantly improve bone mineral density, particularly in the lumbar spine and hip regions [16]. These findings indicate that EA stimulation at the CV4 site holds promise as a beneficial intervention for promoting osteogenic differentiation and improving bone health.

EA stimulation has indeed demonstrated antioxidant effects and the ability to inhibit the production of ROS, which are known to cause cellular and tissue damage, and their accumulation has been implicated in various diseases, including cancer, cardiovascular disorders and neurodegenerative conditions. Studies have shown that EA stimulation can significantly reduce ROS levels in organs such as the liver and brain. Additionally, it has been found to enhance the activity of antioxidant enzymes, such as SOD and catalase (CAT). Thus, EA stimulation represents a valuable therapeutic approach for preventing and treating diseases associated with oxidative stress, as it can potentially mitigate the detrimental effects of oxidative stress-related conditions by inhibiting ROS production and bolstering antioxidant defenses.

The Wnt/β-catenin pathway is known to play a pivotal role in cell proliferation, differentiation, and tissue regeneration, and its dysregulation has been associated with various physiological and pathological processes. Activation of this pathway can lead to increased cell proliferation and tissue regeneration [17]. Previous research has demonstrated that EA can regulate the Wnt/β-catenin signaling pathway, leading to the reduction of inflammatory factors such as IL-1β and improvement in cartilage morphology and structure [18]. In another study conducted on rats, EA stimulation at the CV4 site was shown to significantly increase the expression of Wnt3a, a ligand that activates the Wnt/β-catenin pathway [8]. These findings suggest that EA stimulation has the potential to activate the Wnt/β-catenin signaling pathway in specific

experimental models and may hold therapeutic implications for tissue regeneration. However, further investigation is required to fully elucidate the underlying mechanisms and determine the clinical relevance of these effects.

5. Conclusions

In conclusion, EA stimulation at the CV4 site may effectively inhibit bone loss and promote bone differentiation by activating the Wnt/β-catenin signaling pathway, thereby preventing breast cancer-induced osteoporosis. Thus, this proposed treatment approach might be a promising strategy for treating breast cancer-induced osteoporosis.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

SXW and XX—designed the study and carried them out; SXW, XX and LYY—supervised the data collection, analyzed the data, and interpreted the data; SXW, XX, GQL and JYX—prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of the Affiliated Hospital of Xiangnan University (Approval no. DW2022-002-01), and all experiments were performed according to the guidelines and rules of the Institutional Animal Care and Use Committee.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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