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Icariin Promotes Fracture Healing in Ovariectomized Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Osteoporotic fractures are common in postmenopausal women and associated with complications. Numerous studies have demonstrated that icariin can be used to treat fractures and osteoporosis. Herein, we evaluated the efficacy of gavage-administered icariin to promote fracture healing in postmenopausal osteoporotic fracture (POF) rats.

Material/Methods: In this study, ovariectomy-induced POF rats were treated with 600 mg/kg icariin. Micro-computed tomography (micro-CT) was used to assess fracture healing; besides, serum APK, TRACP-5b, and E_2 expression levels were detected by commercial kits, and the uterine index was calculated. In addition, the expression of osteogenesis-related proteins (Runx 2 and COL1A2) in the callus was measured by western blot, whereas the expression of OPG/RANKL pathway proteins was measured by western blot and immunohistochemical analysis.

Results: Our data revealed that icariin promoted the expression level of Runx 2 and COL1A2 and suppressed the expression level of serum bone turn over biomarkers via the OPG/RANKL pathway. Besides, a more mature callus was observed in the POF rats receiving icariin than in the untreated POF rats, while serum E_2 and uterine index were unaffected by icariin treatment.

Conclusions: These results revealed that icariin could promote fracture healing in ovariectomized rats via OPG/RANKL signaling, and that serum E_2 and uterine index were not affected by icariin treatment.

MeSH Keywords: Medicine, Chinese Traditional • Models, Animal • Osteoporosis, Postmenopausal

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Background

With the increasingly elderly population worldwide, osteoporosis has become a highly prevalent disease [1], especially in postmenopausal women. Owing to the poor bone condition of postmenopausal patients [2], postmenopausal osteoporotic fracture (POF) is becoming a global health issue, further affected by a weakened regeneration potential for fracture healing. Estrogen deficiency is an essential reason underlying osteoporosis in postmenopausal women, and the lack of estrogen plays an adverse role in fracture healing of POF [3]. Furthermore, the mortality rate of diseases caused by osteoporotic fractures has exceeded that of 3 primary gynecological tumors (breast, cervical, and endometrial cancer) combined [4], and is increasing annually [5]. Thus, the treatment of POF is expensive and poses a considerable socioeconomic burden. In the clinic, effective reduction and internal fixation combined with anti-osteoporosis treatment are beneficial to fracture healing in POF patients. However, the appropriate selection of anti-osteoporosis treatment remains critical.

Icariin, an active ingredient of Herba Epimedii, reportedly treats osteoporosis. Several studies demonstrated that icariin exerts a therapeutic effect on ovariectomized (OVX) rat models and postmenopausal women [6,7]. Icariin can stimulate bone formation, inhibit bone resorption, and promote bone reconstruction [8,9]. Therefore, icariin is an ideal anti-osteoporosis drug that may promote fracture healing in POF patients.

In the current study, we established a POF model in OVX rats to explore the efficacy of icariin to boost fracture healing, and to unravel the underlying mechanisms.

Material and Methods

Ethics Statement

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1996) and was approved by the Animal Ethics Committee of Anhui Medical University (Approval No. LLSC20190739).

Study design

Female Sprague-Dawley rats, weighing 235±15 g, were purchased from Anhui Medical University Animal Experiment Center to be used in this study. All experiments were conducted by researchers who were blinded to the experimental conditions.

Rats were allocated to 3 groups: POF (control) group, POF+icariin (experimental) group, and F (positive control) group. The POF model was established after bilateral ovariectomy. Eight weeks after ovariectomy, bone mineral density (BMD) was measured to confirm osteoporosis in OVX rats. Next, the middle-upper tibia of rats was surgically resected, followed by reduction and internal fixation by Kirschner wire. After the POF model was established, these rats were randomly allocated to the POF and POF+icariin groups. In the F group, the same weight of tissue that was removed from the POF rats was resected around the ovary, and the middle-upper tibia of rats was resected, followed by reduction and internal fixation by Kirschner wire. In this study, the dose of icariin was set to 600 mg/kg. Icariin was administered by gavage once a day in the POF+icariin group, whereas the F and POF groups were treated with an equal dose of normal saline (NS) via gavage. The experimental protocol is shown in Figure 1.

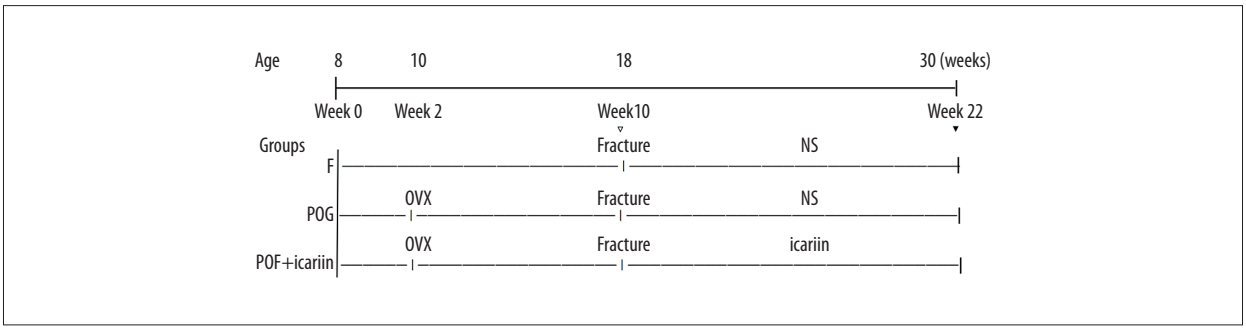

Figure 1. Experimental protocol. OVX: rats in POF group and POF+icariin group were bilaterally ovariectomized. White inverted triangles: the BMD of femur was measured to confirm if osteoporosis was happened in OVX rats. Fracture: the middle-upper tibia of rats in F group, POF group and POF+icariin group were cut off and then reduced and internally fixed by Kirschner wire. Black inverted triangles: the rats were executed at 22 weeks. NS and icariin: From 10 weeks afterwards, icariin (600 mg/kg) was administered by gavage once a day in the POF+icariin group, whereas the same dose of normal saline was administered by gavage in the POF group and F group. OVX – ovariectomized; POF – postmenopausal osteoporotic fracture; BMD – bone mineral density; F group – positive control group; NS – normal saline.

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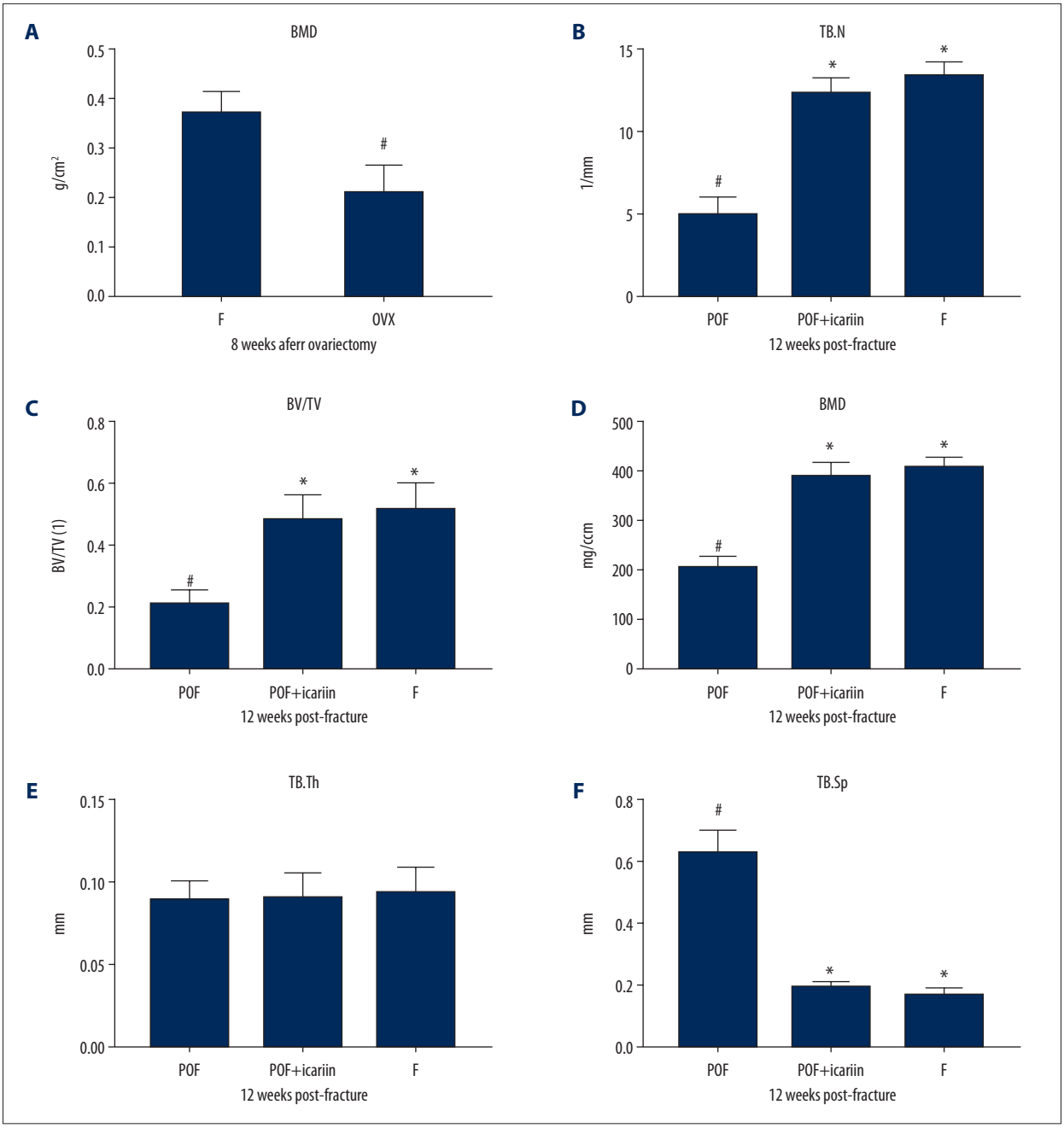

Figure 2. (**A**) The femoral BMD of rats was measured by dual energy x-ray absorptiometry at 8 weeks after ovariectomy, and the results indicated that osteoporosis had developed in OVX rats. Data are expressed as mean±SD (n=12). # *P*<0.05 versus F group. At 12 weeks after fracture, micro-CT analysis was used to determine callus conditions. (**B**) Micro-CT analysis of TB.N. (**C**) Micro-CT analysis of BV/TV. (**D**) Micro-CT analysis of BMD. (**E**) Micro-CT analysis of TB.Th. (**F**) Micro-CT analysis of TB.Sp. Data are expressed as mean±SD (n=20). * *P*<0.05 versus POF group, # *P*<0.05 versus F group. BMD – bone mineral density; OVX – ovariectomized; SD – standard deviation; F group – positive control group; CT – computed tomography; TB.N – trabecular number; POF – postmenopausal osteoporotic fracture.

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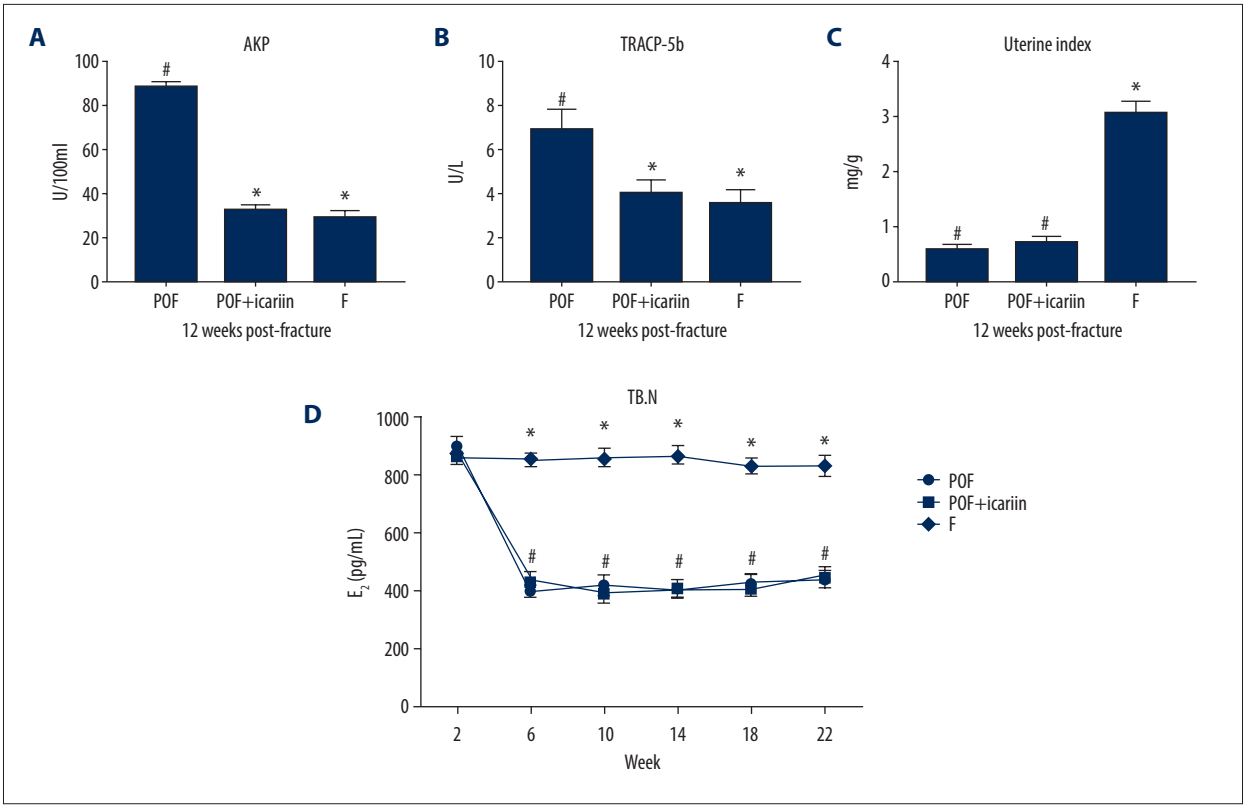

Figure 3. Effects of icariin on bone resorption markers in OVX rats' serum. (**A**) The serum AKP level in rats. (**B**) The serum TRACP-5b level in rats. (**C**) The uterine index in rats. (**D**) The serum E_2 level in rats. Data are expressed as mean±SD (n=20). * $P<0.05$ versus POF group; # $P<0.05$ versus F group. OVX – ovariectomized; SD – standard deviation; POF – postmenopausal osteoporotic fracture; F group – positive control group.

Dual-energy x-ray and micro-computed tomography (CT) analysis

Dual-energy x-ray absorptiometry (InAlyzer, Biotimes Technology Limited, Zhejiang, China) was used to gauge the femoral BMD to determine the degree of osteoporosis in OVX rats, in line with the manufacturer's instructions. Micro-CT (LCT200, United Well Technology, Shanghai, China) was used to assess fracture healing, according to the instructions provided. The microarchitecture parameters of the measured callus included BMD, trabecular thickness (TB.Th), mineralized volume fraction (BV/TV), trabecular number (TB.N), and trabecular separation (TB.Sp).

Serum parameter and uterine index assay

The serum AKP level was measured with a commercial assay kit (A059-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The serum TRACP-5b level was determined with an ELISA kit (XY-E11090, Shanghai Xinyu Biological Technology Co., Ltd., Shanghai, China), and the serum E_2 level was measured by an enzyme-linked immunosorbent assay (ELISA) kit (XY-E30608, Shanghai Xin Yu Biotech Co., Ltd., Shanghai, China), in line with the manufacturer's instructions. The uterus was removed, and the uterus index (uterus weight/body weight) was calculated.

Western blot

The expression of collagen type I alpha 2 chain (COL1A2), Runx 2, OPG, and RANKL in callus was determined by western blot analysis. Total proteins were exacted by a bone tissue protein extraction kit (BB-31813, BestBio Biotechnology Co., Ltd., Shanghai, China), and protein concentration was evaluated using a bicinchoninic acid (BCA) protein quantification kit (Beyotime Institute of Biotechnology, Shanghai, China), in line with the instructions provided. Equal amounts of protein (40 μg) were separated by electrophoresis and electroblotted onto a 0.45 μm membrane. COL1A2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Runx 2 (Abcam, Cambridge, UK), OPG (Abcam), and RANKL (Abcam) were detected with specific primary and secondary antibodies. The dilutions of the primary antibodies were as follows: COL1A2, 1: 500; Runx 2, 1: 500; OPG, 1: 1000; RANKL, 1: 500. The dilutions of the secondary antibodies were as follows: COL1A2, 1: 10 000; Runx 2, 1: 10 000; OPG, 1: 10 000; RANKL, 1: 10 000. Western blot analysis was performed according to standard procedures [10].

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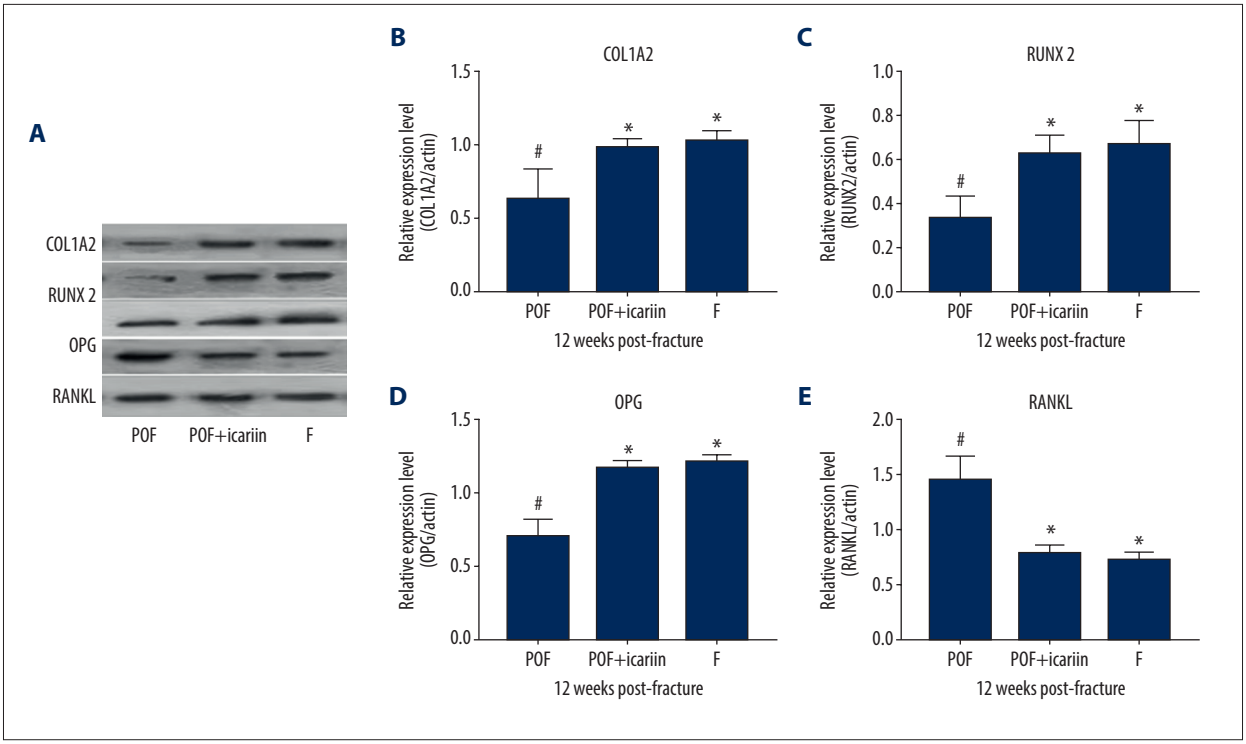

Figure 4. (**A**) The expression of COL1A2, Runx 2, OPG, and RANKL in callus was determined by western blot analysis at 12 weeks after fracture. (**B**) The relative expression of COL1A2. (**C**) The relative expression of Runx 2. (**D**) The relative expression of OPG. (**E**) The relative expression of RANKL. Data are expressed as mean±SD (n=10). * *P*<0.05 versus POF group; # *P*<0.05 versus F group. SD – standard deviation; POF – postmenopausal osteoporotic fracture; F group – positive control group.

The optical density of the western blot bands was then determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Immunohistochemistry was performed in serial callus sections to detect OPG and RANKL protein expression, as previously described [11]. The primary OPG antibody (Abcam) and primary RANKL antibody (Bioworld, Hefei, China) were diluted 1: 100, while the secondary OPG antibody (Abcam) and secondary RANKL antibody (Bioworld) were diluted 1: 200. To quantify the expression levels of OPG and RANKL, we randomly selected 3 images (400x) for each group, and the average optical density of each protein signal was determined using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Statistical analysis was conducted using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad, La Jolla, CA, USA). The data were expressed as the mean±standard deviation (m±SD) and analyzed using one-way ANOVA. *P*<0.05 was considered to indicate statistically significant differences.

Results

Establishment of an OVX rat model

Eight weeks after ovariectomy, the femoral BMD of 12 rats randomly selected from both the OVX and F groups was measured by dual-energy x-ray absorptiometry. The femoral BMD in the OVX group was significantly lower than that in the F group (*P*<0.05), which indicated that OVX rats developed osteoporosis (Figure 2).

Micro-CT

At 12 weeks after fracture, we conducted a micro-CT analysis to determine callus conditions. Notably, a more mature callus was observed in the POF rats receiving icariin than in untreated POF rats. The BMD, BV/TV, and TB.N in the POF+icariin group were significantly higher than those in the POF group but did not significantly differ from those in the F group. The TB.Th in the POF+icariin group was not significantly different from that in the POF group and F group. The TB.Sp in the POF+icariin group was significantly lower than that in the POF group but not significantly different from that in the F group (Figure 2).

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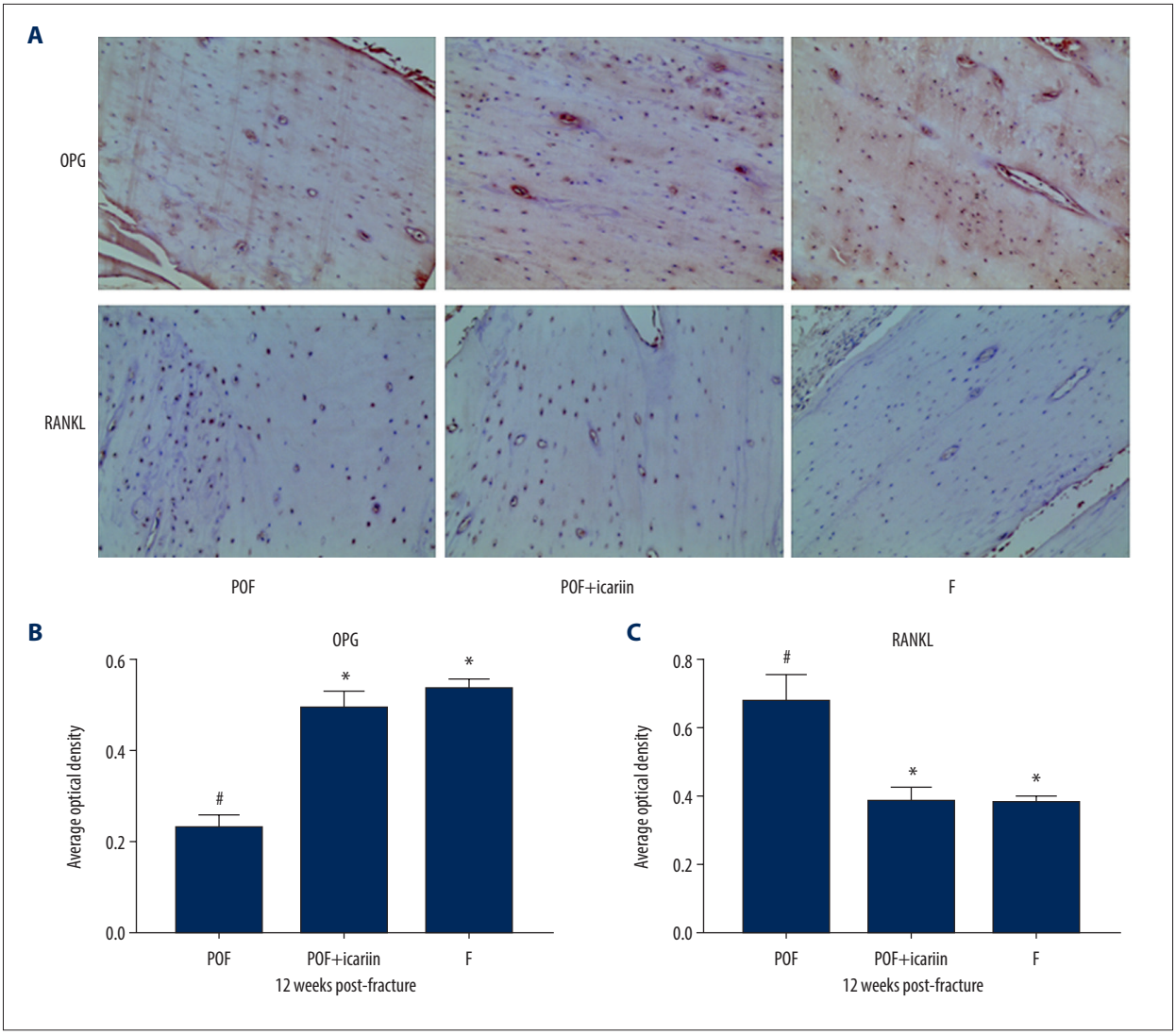

Figure 5. (**A**) The expression of OPG and RANKL in callus was determined by immunohistochemical analysis (n=10). (**B**) The average optical density of OPG (n=3). (**C**) The average optical density of RANKL (n=3). Data are expressed as mean±SD. * $P<0.05$ versus POF group; # $P<0.05$ versus F group. SD – standard deviation; POF – postmenopausal osteoporotic fracture; F group – positive control group.

Serum biochemistry and uterine index

The serum AKP and TRACP-5b levels in the POF+icariin group were significantly lower than those in the POF group but not significantly different from those in the F group (Figure 3). The serum E_2 level and uterine index in the POF+icariin group were significantly lower than those in the F group but did not significantly differ from those in the POF group (Figure 3).

Western blot

At 12 weeks post-fracture, we detected the expression of COL1A2, Runx 2, OPG, and RANKL. The expression of COL1A2, Runx 2, and OPG in the POF+icariin group was not significantly different from that in the F group but was significantly higher than that in the POF group. The expression of RANKL in the POF+icariin group was not significantly different from that in the F group but was significantly lower than that in the POF group (Figure 4).

Immunohistochemistry

To further explore the efficacy of icariin on the expression level of OPG and RANKL proteins in the callus, we conducted an immunohistochemical analysis 12 weeks after the fracture. The expression level of OPG in the POF+icariin group was significantly higher than that in the POF group ($P<0.05$) but was not significantly different from that in the F group (Figure 5).

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The expression of RANKL in the POF + icariin group was significantly lower than that in the POF group ($P<0.05$) but was not significantly different from that in the F group (Figure 5). These findings were consistent with the western blot results.

Discussion

To date, many anti-osteoporosis drugs are commercially available, including parathyroid hormone (PTH), bisphosphate, and selective estrogen receptor modulators. Although these drugs are effective, long-term use is expected to result in side effects, such as renal function damage and increased incidence of breast cancer [12]. Considering that estrogen plays a vital role in postmenopausal osteoporosis, estrogen replacement therapy is undoubtedly a promising therapeutic alternative. However, estrogen can increase the risk of female reproductive system cancer, which hinders the full implementation of estrogen replacement therapy [13]. Phytoestrogen flavonoids reportedly exert a therapeutic effect on osteoporosis and do not affect endometrial hyperplasia [14]. Icariin is one of the known phytoestrogen flavonoids, and negligible side effects have been reported after its long-term use for osteoporosis treatment. In this study, the serum E_2 level and the uterus index were unaffected by icariin treatment. Besides, icariin is inexpensive and may reduce the cost of treatment. Therefore, effective reduction and internal fixation combined with icariin may be an optimal choice to promote fracture healing in postmenopausal women.

Collagen type I is abundant in the bone matrix and is essential for bone formation. COL1A2 is a collagen chain of collagen type I [15]. A previous report revealed that the COL1A2 gene is associated with BMD and is often mutated in women with severe osteoporosis [16]. Moreover, COL1A2 was recently found to be involved in POF [17]. In the current study, we revealed that the expression of COL1A2 in the POF+icariin group was higher than that in the POF group but was similar to that in the F group, consistently with previous reports. Furthermore, Runx 2, a pivotal factor for bone formation [18], and the expression of Runx 2 in the POF+icariin group were higher than in the POF group, indicating that icariin treatment could boost bone formation in patients with POF. TRACP-5b is secreted into the blood during bone resorption, and therefore, can be used as biomarkers of bone resorption [19]. Besides, the serum AKP and TRACP-5b levels in osteoporosis patients are commonly elevated due to the enhanced bone turn over, which is detrimental to bone formation [20]. The decrease in serum AKP and TRACP-5b levels indicates that bone resorption and bone turn over decreased in the POF+icariin group. These results may explain why the callus in the POF+icariin group was more prominent than that in the POF group, as observed by micro-CT.

OPG and RANKL play an essential role in recalibrating bone reconstruction [21,22]. When RANKL binds to RANK located on the surface of osteoclasts [23], bone resorption capacity is enhanced. OPG is a natural antagonist of RANKL [24]; when OPG competitively binds to RANKL, the binding of RANK to RANKL is blocked, which inhibits the differentiation of osteoclasts and affects osteoblasts formation and maturation, thus regulating bone metabolism [25]. Estrogen deficiency-mediated osteoporosis is primarily caused by increased bone resorption [26], in which the OPG/RANKL pathway is involved [27]. Xu et al. reported that icariin could suppress RANKL-induced osteoclastogenesis [28]. In addition, Lv et al. reported that the expression level of COL1A2 could be regulated through the OPG/RANKL pathway [29]. Furthermore, Jiang et al. found that icariin could bind to OPG/RANKL targets and plays an anti-osteoporosis role [30]. Therefore, we hypothesized that icariin promotes fracture healing in OVX rats through the OPG/RANKL pathway. In the current report, the expression level of OPG and RANKL proteins in the callus in the POF+icariin group supported our hypothesis.

The main limitation of this study was that the model animals were young, whereas osteoporosis typically occurs in postmenopausal women. However, similarly to 2 previous studies [30,31], we selected young rats to create the OVX model, as the weight and physiological condition of older rats would pose technical issues and bias with modeling.

Conclusions

Our findings demonstrated that effective reduction and internal fixation, combined with icariin treatment, promotes fracture healing in OVX rats via OPG/RANKL signaling.

Conflicts of interest

None.

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