Effect of icariin on fracture healing in an ovariectomized rat model of osteoporosis

HONG CAO[1*], YING ZHANG[2*], WEI QIAN[1], XIAO-PENG GUO[3], CHEN SUN[1], LEI ZHANG[1] and XIN-HUA CHENG[1]

[1]Department of Orthopedic Surgery; [2]Reproductive Medicine Center, Renmin Hospital, Hubei University of Medicine; [3]Department of Orthopedic Surgery, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

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Abstract. Osteoporosis is frequently asymptomatic, presenting a significant clinical and economic burden, particularly following an osteoporosis-associated fracture. Icariin has been reported to inhibit osteoporosis *in vitro*, and the present study investigated whether icariin also promoted bone fracture healing in ovariectomized osteoporotic (OVX) rats *in vivo*. A total of 30 female rats were randomly divided into three groups (n=10 per group): i) Sham surgery; ii) OVX; and iii) OVX with icariin (OVX + ICA) groups. At 3 months after the ovariectomy, a unilateral cross-tibia fracture was made at the proximal right tibia. Animals were then sacrificed after 5 weeks of oral treatment. X-rays were taken at 1 week, 3 weeks and 5 weeks of treatment, and dual energy X-ray absorptiometry was used to measure the bone mineral density (BMD). Changes to the osteocalcin (BGLAP), alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP) and estradiol levels in blood were measured. Callus formation and bone union were observed, the BMD was significantly higher and the BGLAP, ALP and TRAP levels were reduced, but no significant increase was observed in the blood estradiol level in the OVX + ICA group compared with the OVX group. The present findings indicate that icariin has potential as a novel alternative therapeutic agent for fracture healing in postmenopausal osteoporosis.

Correspondence to: Dr Xin-Hua Cheng, Department of Orthopedic Surgery, Renmin Hospital, Hubei University of Medicine, 39 Chaoyang Middle Road, Shiyan, Hubei 442000, P.R. China
E-mail: 33556511@qq.com

*Contributed equally

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Introduction

Osteoporosis is a disease that frequently presents asymptomatically, and is typically characterized by low bone mass, microarchitectural deterioration of bone tissue and decreased bone strength (1,2). Osteoporosis presents with symptoms such as low bone density and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (3), and with a T-score of -2.5 standard deviations (SD) below the bone mineral density (BMD) of a healthy person of the same gender (4), which is most widely measured using dual energy X-ray absorptiometry (DXA) (5). In the United States, osteoporosis affects 2% of men and 10% of women aged ≥50 years old (6). Furthermore, 49% of older women and 30% of older men have osteopenia (7). Estimates indicate that 50% of women and 20% of men aged >50 years old will experience an osteoporosis-associated fracture. Among these osteoporosis-associated fractures, hip fracture is the most devastating of these, due to the consequent disability, mortality and costs (8). Owing to the aging of the global population, estimates suggest that the incidence of osteoporosis will double in the next 20 years (2). Consequently, an exponential increase in the numbers of fractures is anticipated, with an inevitable increased clinical and economic burden for healthcare systems.

Most strategies for treating bone loss have focused on pharmacological interventions (9); however, drug treatments may have adverse effects and poor long-term adherence, despite their effectiveness (10). Chinese herbal medicine has been used for thousands of years for the treatment of bone diseases (11). For postmenopausal women, *Epimedium pubescens* flavonoids are one of the most frequently used herbal compounds that is prescribed for the treatment of osteoporosis (12). *Epimedium*-derived phytoestrogenic flavonoids inhibit bone resorption, stimulate bone formation and prevent ovariectomy-induced osteoporosis, without resulting in uterine hyperplasia. It is suggested that these compounds have an anabolic effect on osteoporotic bone by concomitantly promoting the osteogenic differentiation of bone marrow stromal cells while suppressing adipogenic differentiation (13). Icariin ($C_{33}H_{40}O_{15}$; molecular weight: 676.65; Fig. 1), one of the primary active compounds within *Epimedium*, reportedly has an anabolic effect on the bone; it stimulates the

proliferation of rat bone marrow stromal cells, increases the number that stain positive for osteocalcin (BGLAP) secretion, alkaline phosphatase (ALP) and enhances ALP activity, and calcium deposition levels in a dose-dependent manner (14,15). In previous work, we reported that icariin inhibits osteoporosis *in vitro*, potentially owing to its role in increasing bone morphogenetic protein-2 (BMP-2) protein expression (16), and that icariin promotes bone formation via the BMP-2/Smad4 signal transduction pathway in the hFOB 1.19 human osteoblastic cell line (17).

The present study investigated whether icariin promotes bone fracture healing in ovariectomized osteoporotic (OVX) rats *in vivo*, with the intention of determining a novel method to treat osteoporosis-associated fracture.

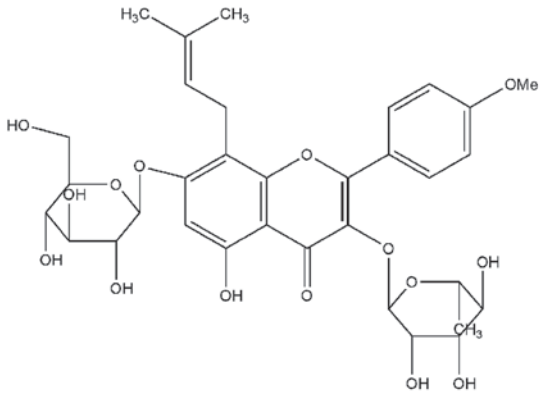

Figure 1. Chemical structure of icariin ($C_{33}H_4O_{15}$, molecular weight 676.65).

Materials and methods

Animals and modeling method. For the present study, 30 6-month-old Sprague-Dawley (SD) female rats were obtained from Hubei University of Medicine (Shiyan, China). The rats were housed in a temperature-controlled room (25˚C) with constant humidity (40-50%) and received food and water *ad libitum*. Rats were left for 1 week to acclimatise to their environment, which was subject to a 12/12 h light/dark cycle. These rats were randomly divided into three groups consisting of 10 rats per group, as follows: i) Sham surgery (SS); ii) OVX; and iii) OVX and icariin (OVX + ICA) groups.

Bilateral ovariectomy was performed in 20 female rats through an incision in the back, under general anesthesia with an intraperitoneal injection of 10% chloral hydrate at a dose of 3 ml/kg (Chemical Reagent Co., Shanghai, China). Approximately 1.5 cm of the skin, the abdominal cavity and the muscles were incised, and the ovaries were exposed (Fig. 2A). The oviduct was ligated with a silk thread and the ovariectomy was performed bilaterally (Fig. 2B), while the remaining 10 animals underwent a sham surgery in which the bilateral ovaries were examined and returned to the original position under the same protocol.

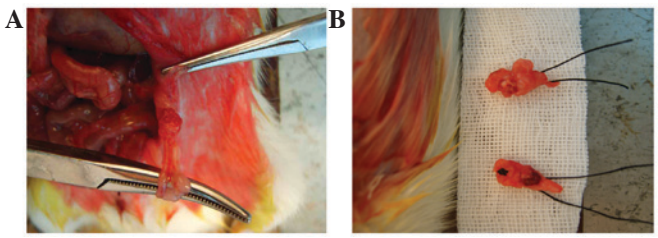

Figure 2. Following anesthetization, a bilateral ovariectomy was performed in rats. (A) Ovary was exposed and (B) each fallopian tube was completely ligated using 2-0 silk thread; bilateral ovariectomy was performed.

Three months after the ovariectomy, a unilateral cross-tibial fracture was made at the proximal right tibia and fixed with intramedullary nailing (diameter, 1 mm, length, 50 mm; Wego Medical Systems Co., Ltd, Weihai, China; Fig. 3), performed under anesthesia. All procedures were approved by the Animal Research Ethics Board at Hubei University of Medicine (Shiyan, China).

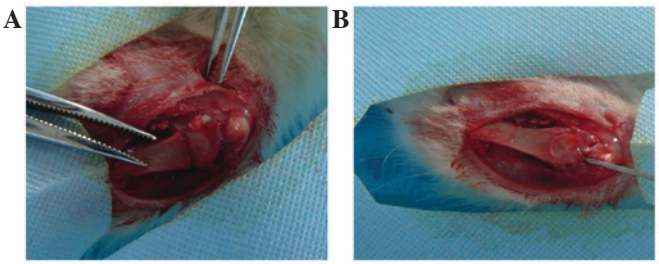

Figure 3. Three months after the ovariectomy: (A) A unilateral cross-tibial fracture was made at the proximal end of the right tibia and (B) fracture of the right tibia was fixed with an intramedullary nailing.

Treatment method. Icariin was obtained from the Institute of Pharmaceutical Research (Beijing, China) with a purity of 99%, dissolved with 0.9% sodium chloride at a concentration of 100 mg/ml. The OVX + ICA group was treated with a daily 150 mg/kg icariin, administered orally following the intramedullary fixation and right tibial fracture procedure. The SS group and OVX group received equal amounts of 0.9% sodium chloride orally.

Specimen collection. X-rays (800 mA, 150 kV, R-500; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) were taken at 1, 3 and 5 weeks after oral treatment, and dual energy X-ray absorptiometry (DSC-3000, Aloka, Tokyo, Japan) was used to measure the BMD (mg/cm^2) prior to sacrifice, 5 weeks after oral treatment. The rats were sacrificed by cervical dislocation. Blood was drawn from the inferior vena cava following sacrifice, added to anticoagulant, and was stirred at a rate of 3,000 rpm for 20 min to extract the blood plasma. The extracted blood plasma was stored at -70˚C until analysis.

Blood variable analysis. BGLAP and ALP, as bone formation markers, were each measured with an ELISA kit (BGLAP, cat. no. DSTCN0; ALP, cat. no. DY725; R&D Systems, Inc., Minneapolis, MN, USA). Tartrate-resistant acid phosphatase (TRAP), used as a bone resorption marker, and blood estradiol levels were also measured using ELISA kit (cat. no. KGE014; R&D Systems, Inc.).

Statistical analysis. Data are expressed as mean ± standard deviation, and statistical analyses were performed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to assess differences

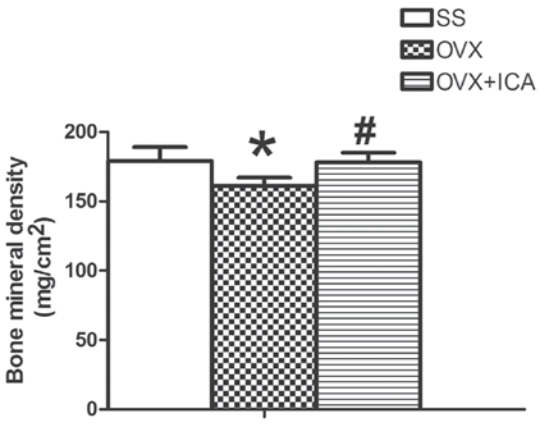

Figure 4. Bone mineral density, measured from the trabecular bone of the distal femur of the female rats. *P<0.05 vs. SS group; #P<0.05 vs. OVX group. SS, sham surgery; OVX, ovariectomized osteoporotic; ICA, icariin.

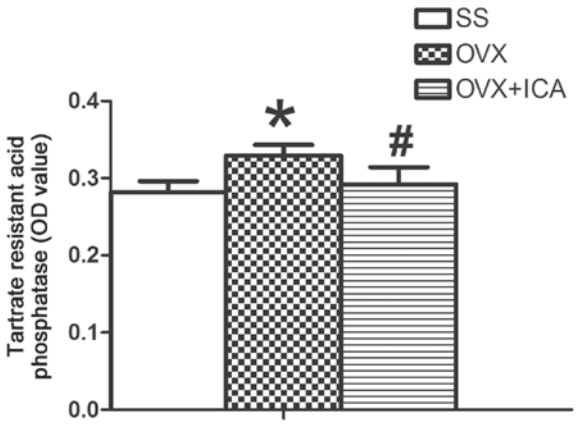

Figure 6. Effects after 5 weeks of icariin treatment on the bone resorption marker tartrate resistant acid phosphate. *P<0.05 vs. SS group; #P<0.05 vs. OVX group. SS, sham surgery; OVX, ovariectomized osteoporotic; ICA, icariin; OD, optical density.

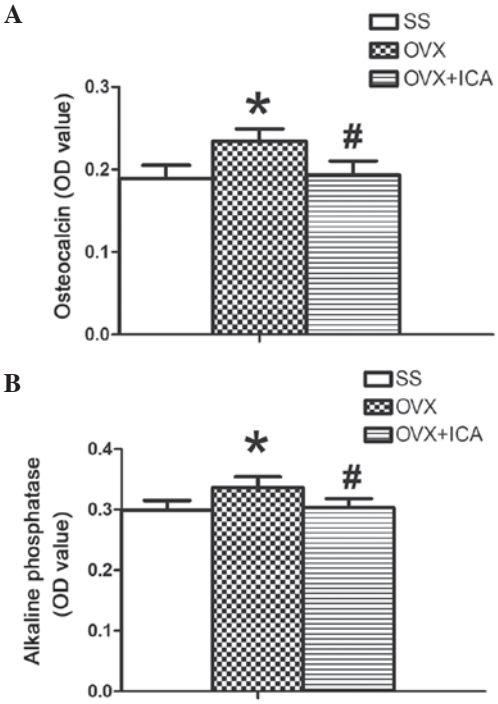

Figure 5. Effects after 5 weeks of icariin treatment on the bone formation markers (A) blood osteocalcin and (B) alkaline phosphatase. *P<0.05 vs. SS group; #P<0.05 vs. OVX group. SS, sham surgery; OVX, ovariectomized osteoporotic; ICA, icariin; OD, optical density.

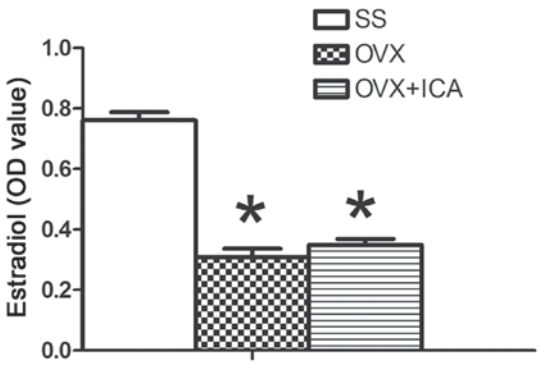

Figure 7. Blood estradiol levels after 5 weeks of icariin treatment. *P<0.05 vs. SS group. SS, sham surgery; OVX, ovariectomized osteoporotic; ICA, icariin; OD, optical density.

between the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Bone mineral density is altered following icariin treatment of OVX rats. The BMD of the SS group, measured from the trabecular bone of the distal femur of the rats, was significantly higher than that of the OVX group (P<0.05). The BMD of OVX + ICA group was significantly higher than that of the OVX group (P<0.05), but was not significantly different from the BMD of the control group (P>0.05; Fig. 4).

BGLAP, ALP and TRAP levels increase in OVX rats, and are rescued by icariin treatment. After 5 weeks of oral treatment, the OVX group demonstrated significantly increased BGLAP, ALP and TRAP levels compared with the SS group (P<0.05). The OVX + ICA group demonstrated a reduction of BGLAP, ALP and TRAP levels compared with the OVX group (P<0.05; Figs. 5 and 6).

Changes are observed in blood estradiol levels in OVX rats, which are not rescued following icariin treatment. OVX rats exhibited a significant reduction in their blood estradiol level compared with that in the SS group (P<0.05). However, the OVX + ICA group exhibited no significant increase in the blood estradiol levels compared with those in the OVX group (P>0.05; Fig. 7).

X-rays of rat tibias reveal incomplete remodeling in OVX rats, which is improved by icariin treatment. The effects of icariin on callus formation, remodeling and bone union were observed at 1, 3 and 5 weeks after treatment. Callus formation and bone union were observed in the OVX + ICA group at 5 weeks but, in the OVX group, a small callus was formed and remodeling remained incomplete, with no union of bones (Fig. 8).

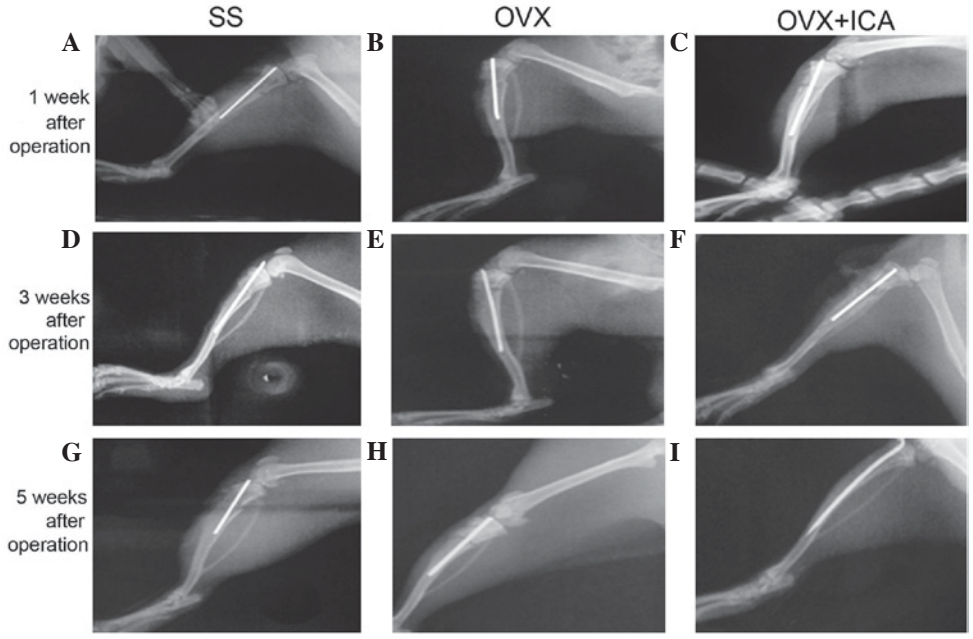

Figure 8. X-rays at (A-C) 1 week, (D-F) 3 weeks and (G-I) 5 weeks after ovariectomy in the (A, D and G) SS; (B, E and H) OVX; and (C, F and I) OVX + ICA groups, demonstrating the degree of callus formation and bone union following fracture. SS, sham surgery; OVX, ovariectomized osteoporotic; ICA, icariin

Discussion

Osteoporosis, which results from a disturbance in normal bone remodeling by increasing bone resorption relative to bone formation, is identified by a low bone mass and leads to a high risk of fractures (18). Osteoporosis is often an undiagnosed disease prior to the occurrence of fracture. Bone fragility occurs due to an excess of resorption and reduced bone formation, resulting in an increased risk of hip and vertebral fractures (19). It is reported that osteoporosis affects about 25 million individuals in the United States alone, and it is estimated that women >50 years old possess an 11-18% risk of suffering a hip fracture (20). Therefore, to prevent and treat the osteoporosis, medication, exercise and additional treatments such as hormone replacement therapy are commonly used (21,22). Amongst these, hormone replacement therapy is the most widely used; however, previous evidence indicates that long-term treatments with these drugs may cause adverse reactions, such as an increased risk of ovarian and endometrial cancer (23,24). Thus, an alternative therapeutic strategy with a proven efficacy and safety is required to prevent and treat osteoporosis. Icariin, one of the primary active ingredients of *Epimedium*, reportedly has an anabolic effect on bones; this may contribute to its role in the induction of osteoblast proliferation and differentiation, which results in bone formation (25,26).

The ovariectomy model is a well-established animal model in osteoporosis studies (27). The OVX rat model was selected for the present study as it shares numerous similarities with postmenopausal bone loss and is recommended by the US Food and Drug Administration as a test species for evaluating the long-term skeletal safety and efficacy of osteoporosis therapies (20). Previous laboratory studies have reported that osteoporosis impairs fracture healing in early and late stages (28,29). In the present study, it was investigated whether icariin promotes bone fracture healing in OVX rats *in vivo*; this was determined 3 months after the ovariectomy, the unilateral cross-tibia fracture was made and the fracture had been fixed with an intramedullary nailing.

Decreased BMD is one of the major factors jeopardizing bone strength, resulting in increased susceptibility to fractures (30). The present study revealed that OVX reduced BMD in the distal femurs of female SD rats, which are rich in trabecular bone, whilst treatment with icariin prevented these decreases in BMD.

BGLAP is closely bonded with hydroxyapatite and calcium in the bone, and is an established bone formation marker; this was used to predict the bone loss rate as it is indirectly involved in the activation of osteoblasts during bone generation (31). ALP is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids (32). ALP increases during active bone formation, as ALP is a byproduct of osteoblast activity (heightened levels of which appear in Paget's disease) (33). TRAP is a glycosylated monomeric metalloprotein enzyme expressed in mammals, which may be used as a bone resorption marker. Osteopontin and bone sialoprotein, which are bone matrix phosphoproteins, are highly efficient *in vitro* TRAP substrates that bind to osteoclasts when phosphorylated. Upon partial dephosphorylation, osteopontin and bone sialoprotein are incapable of binding to osteoclasts (34). From this effect, it has been hypothesized that TRAP is secreted from the ruffled membrane of osteoclasts, where it dephosphorylates osteopontin and allows osteoclast migration and additional resorption to occur (35). The OVX group significantly increased the blood BGLAP, ALP and TRAP levels, while the OVX + ICA group rescued these. These results may be due to decreased estrogen increasing the number of osteoblasts and osteoclasts, or altered activity of these cells.

The most common type of osteoporosis is the post-menopausal bone loss associated with ovarian hormone deficiency (36). Several previous studies report that estrogen is the most important hormone in maintaining bone mass and that a deficiency of this hormone is a major cause of bone loss associated with age in both genders (37-39). Notably, when the circulating estrogen level decreases, calcium in the bones rapidly decreases, and calcium loss occurs via an increase in its urinary excretion (40). In the current study, rat blood estradiol level was gauged using an ELISA kit. Those rats which experienced the menopause induced by ovariectomy demonstrated a significant decrease in blood estradiol compared with the SS group. Nian *et al* (41) suggested that icariin has an antiosteoporotic effect, similar to estrogen, and that it may be effective for prevention of bone fractures induced by estrogen deficiency. In the present study, the OVX + ICA group that was treated with icariin intragastrically for 5 weeks demonstrated a non-significant increase in blood estradiol compared with the OVX group. Ye *et al* (42) previously reported that nonconjugated forms of icaritin and desmethylicaritin, two derivatives of icariin, possess estrogen-like activity; however, icariin appeared to have no estrogenicity in the MCF-7 cell line model *in vitro*. Mok *et al* (43) revealed that icariin exerts anabolic effects in bone, possibly by activating the endoplasmic reticulum in a ligand-independent manner. In previous work, we reported that icariin inhibits osteoporosis *in vitro*, potentially owing to its role in increasing BMP-2 protein expression (16), and that icariin promotes bone formation via the BMP-2/Smad4 signal transduction pathway in the hFOB 1.19 human osteoblastic cell line (17).

The present study investigated the effects of icariin on callus formation, remodeling and bone union at 1, 3 and 5 weeks after treatment by examining X-rays. Callus formation and bone union increased every 2 weeks in the SS group and the fracture line was fuzzy at 5 weeks after sham surgery. In the OVX group, small calluses were formed and the fracture line was evident, revealing an absence of union. Callus formation and bone union increased temporally following icariin treatment, the fracture line was almost absent, and bone union and remodeling were observed 5 weeks after intragastric administration.

In conclusion, the present *in vivo* study reported that icariin attenuates the decrease in BMD in rats with osteopenia and that postfracture administration of icariin accelerates mineralization and osteogenesis and is associated with improved fracture healing. The current findings indicate that icariin has the potential to be developed as an alternative for fracture healing in postmenopausal osteoporosis. However, it should be noted that the mechanism by which icariin performs these roles remains to be examined.

Acknowledgements

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