

Original Article

Cathepsin K inhibitor, icariin, increases the healing potential and biomechanical properties of femoral neck in a mice fractured model

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Abstract: Cathepsin K inhibitors have been demonstrated to restore bone mass in both animal model and post-menopausal osteoporotic patients. In the present study, we performed to investigate the pharmacological effect of icariin in the healing potential and biomechanical properties of femoral neck in a mice fractured model. The results showed that the mRNA and protein expression of cathepsin K was markedly up-regulated in FNF group as compared to control group. However, icariin treatment could reverse the expression of cathepsin K in the proximal femur of femoral neck fractured mice. Histological analyses of icariin treated mice revealed the icariin treatment reversed fracture-induced trabecular deleterious effects, which is characterized by increasing disconnections and separation of trabecular bone. Moreover, micro-CT scanning results indicated that femoral neck fractured mice exhibited significantly lower trabecular BV/TV, Tb. N and Tb. Th and higher Tb. Sp, compared to that of the control group. Interestingly, icariin treatment for femoral neck fractured mice resulted in increasing the BV/TV ratio, Tb. N, Tb. Th and decreasing Tb. Sp. Importantly, icariin treatment could improve biomechanical properties and accelerate closure of the wounds in the femur neck. In conclusion, icariin increased the healing potential and biomechanical properties of femoral neck in mice fractured model and might represent a accessory therapeutic medicine with fracture healing.

Keywords: Cathepsin K inhibitor, icariin, fracture healing, biomechanical properties

Introduction

Bone structure is dynamically maintained by bone remodeling, which is a process balanced by osteoblast-mediated bone formation and osteoclast-mediated bone resorption [1]. Dis-equilibrium of bone remodeling and calcium homeostasis usually suppresses bone healing or lead to bone diseases such as osteoporosis and other osteopenic bone disorders [2]. A pathological bone fracture is usually associated with imbalances of bone remodeling by various diseases. In addition, postmenopausal- or osteoporosis-related bone fractures are a major risk of inducing disability and even death [3-5]. After a fracture occurs, bone healing can spontaneously take place in order to reestablish the original physical and mechanical properties of the tissue. During bone healing, many systemic and local factors are involved [6]. Cathepsin K, a family member of cysteine pro-

teases, is released from mature osteoclasts to degrade type I collagen, the major bone organic matrix protein, and allow demineralization [7]. Intriguingly, cathepsin K inhibitors suppress the degradation of type I collagen and thus enhancing bone formation. Recent preclinical and clinical trials suggest that the inhibition of resorption by cathepsin K inhibitors increases bone formation. Many of these inhibitors have passed preclinical studies and are presently in clinical trials at different stages of advancement [4]. Odanacatib is a highly potent inhibitor of human cathepsin K and is found to inhibit bone resorption by blocking degradation of demineralized collagen in the resorption lacunae and retarding transcytosis for further processing of degraded proteins [8].

Icariin, as an inhibitor of cathepsin K, has been reported that it can suppress cartilage and bone degradation in mice of collagen-induced

Icariin in a mice fractured model

Table 1. Physiological and biochemical properties

	Vehicle	FNF	Icariin
BW (g)	30.4 ± 2.3	29.3 ± 2.6	31.1 ± 2.8
Ca (mg)	3.27 ± 0.25	2.64 ± 0.21*	3.15 ± 0.28 [#]
P (mg)	1.36 ± 0.09	1.17 ± 0.21	1.24 ± 0.12
Mg (mg)	0.13 ± 0.015	0.11 ± 0.024	0.097 ± 0.043
Serum Ca (mg/dL)	9.25 ± 0.78	10.37 ± 0.97	10.29 ± 0.85
Serum TRAP-5b (pg/mL)	2.35 ± 0.32	4.14 ± 0.69**	3.37 ± 0.58 [#]
Serum OCN (ng/mL)	574 ± 52	3.67 ± 69**	493 ± 72 [#]

FNF, fracture of the femoral neck; BW, body weight; Ca, calcium; P, phosphorus; Mg, magnesium; TRAP-5b, tartrate resistant acid phosphatase-5b; OCN, Osteocalcin. Values are expressed as mean ± SD, n = 8-10 in each group. *P < 0.05, **P < 0.01 versus vehicle group; [#]P < 0.05 versus FNF group.

arthritis [9]. Icariin has been identified as the major pharmacologically active flavonol diglycoside of *Herba Epimedii*, which is the most commonly used Chinese Herbal Medicines (CHM) for the treatment of several age-related diseases, including osteoporosis, cardiovascular diseases, neurodegenerative diseases and sexual dysfunction [9, 10]. Moreover, icariin is postulated to improve the function of damaged tissue such as OVX-induced marrow adiposity, type II collagen-induced articular cartilage destruction and glucocorticoid-induced bone deteriorations [11-14]. Icariin attenuates glucocorticoid- and hypoxia-induced osteocyte apoptosis, preserves their osteogenic differentiation potential in vitro and promotes bone formation via the BMP-2/Smad4 signal transduction pathway in the hFOB 1.19 human osteoblastic cell line [14-16]. Furthermore, icariin improving bone loss in postmenopausal women has been reported in a 24-month randomized double-blind placebo-controlled clinical trial [17]. However, the effects of icariin in other experimental animal models, such as in a mice femoral neck fractured model, needs to be further investigated.

In the present study, we hypothesized that inhibition of cathepsin K by icariin will increase the healing potential and biomechanical properties of femoral neck in a mice fractured model.

Materials and methods

Animal treatment

Six-week-old male C57BL/6J mice were obtained from the Fourth Hospital of Hebei Medical University Animal Center specific-pathogen-free animal laboratory (Shijiazhuang,

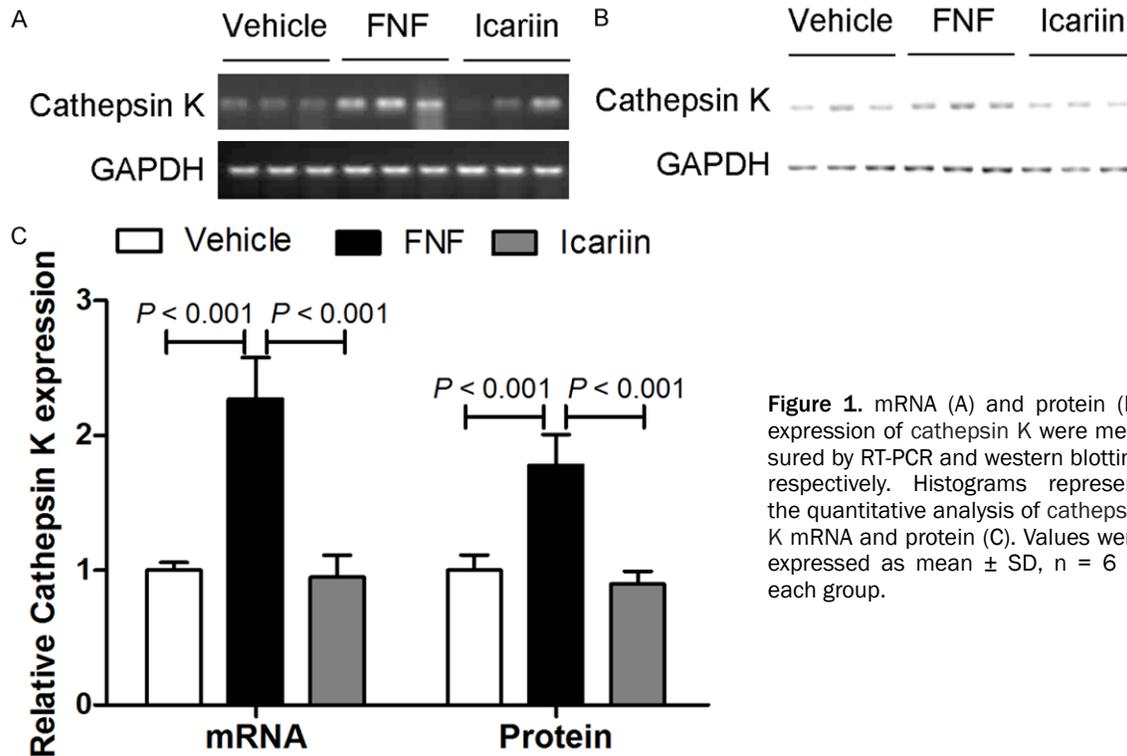
China) and allowed to acclimate to the environment for 1 week. All experimental procedures were carried out in accordance with the guidelines of the Affiliated Hospital of Hebei University on Animal Care. Surgeries were performed under continuous anesthesia, and pain was minimized using analgesics. The mice were randomly divided into three groups: (1) Vehicle group (n = 12, Vehicle); (2) Mice with femoral neck fracture (n = 12, FNF); (3) Femoral neck fractured mice received icariin orally at a dose of 200 mg/kg per day (n = 12, Icariin).

Serum biomarkers and bone mineral substances

The concentrations of calcium (Ca) in serum were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). Serum levels of tartrate resistant acid phosphatase-5b (TRAP-5b) and osteocalcin (OCN) were detected using rat bioactive ELISA assay (Immutopics, Inc., San Clemente, CA, USA) with ELISA reader (MD SpectraMax M5, USA). The femurs were incinerated at 800°C for 12 hours and the ash weighed. 10 mg of bone ash was then dissolved in 1 ml of 37% HCl and diluted with Millin-Q water. The calcium (Ca), phosphorus (P), magnesium (Mg) content was determined by the kit assay.

Bone histomorphology

The femurs were decalcified in 0.5 M EDTA (pH = 8.0) and then embedded in paraffin by standard histological procedures. Section of 5 µm were cut and stained with hematoxylin & eosin (H&E), and visualized under a microscope (Leica DM 2500). The trabecular bone microarchitecture of the femoral neck was measured using a microtomography scanner (SkyScan 1076, Kontizh, Belgium) with a slice thickness of 22 µm. The volume of interest (VOI) was trabecular compartments based on 100 consecutive slices. Bone morphometric parameters, including bone volume over total volume (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), total bone mineral content (BMC) and bone



mineral density over total volume (BMD) were obtained by analyzing the VOI. Moreover, wound size in the femur neck was measured by micro-CT scanning.

Biomechanical parameters

Femurs were placed on the Instron machine (Instron Microtester 5848, Instron Corp., USA) in a three-point bending configuration. The load was applied at the mid-diaphysis in an antero-posterior direction with a loading speed of 5 mm/min until the femur fractured. The load, stress, and strain-deflection curves were automatically calculated by the computer using the Bluehill software. The femora were kept moist at all times during the testing. The parameters measured were maximum load and stiffness.

RT-PCR and western blotting

Total RNA was extracted from femurs using TRIzol (Invitrogen, USA) and reverse transcribed into cDNA using SuperScriptIII reverse transcriptase kit (Invitrogen, USA), following the manufacturer's instructions. GAPDH served as an internal standard. RT-PCR for cathepsin K with following primers: cathepsin K, Forward

5'-CTGAAGATGCTTTCCCATATGTGGG-3' and Reverse 5'-GCAGGCGTTGTTCTATTCCGAGC-3'; GAPDH, Forward 5'-GGATTTGGTCGTATTGGG-3' and Reverse 5'-GGATTTGGTCGTATTGGG-3'.

Protein extracted from femurs were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Germany). Membranes were blocked and then incubated with primary antibodies specific for cathepsin K. GAPDH was used as protein loading control. The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with the and detected by chemiluminescence (Thermo, USA).

Statistical analysis

The data from three experiments were reported as mean \pm standard deviation (SD) for each group. All statistical analyses were performed using PRISM version 5.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post hoc test to compare the group means if overall $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

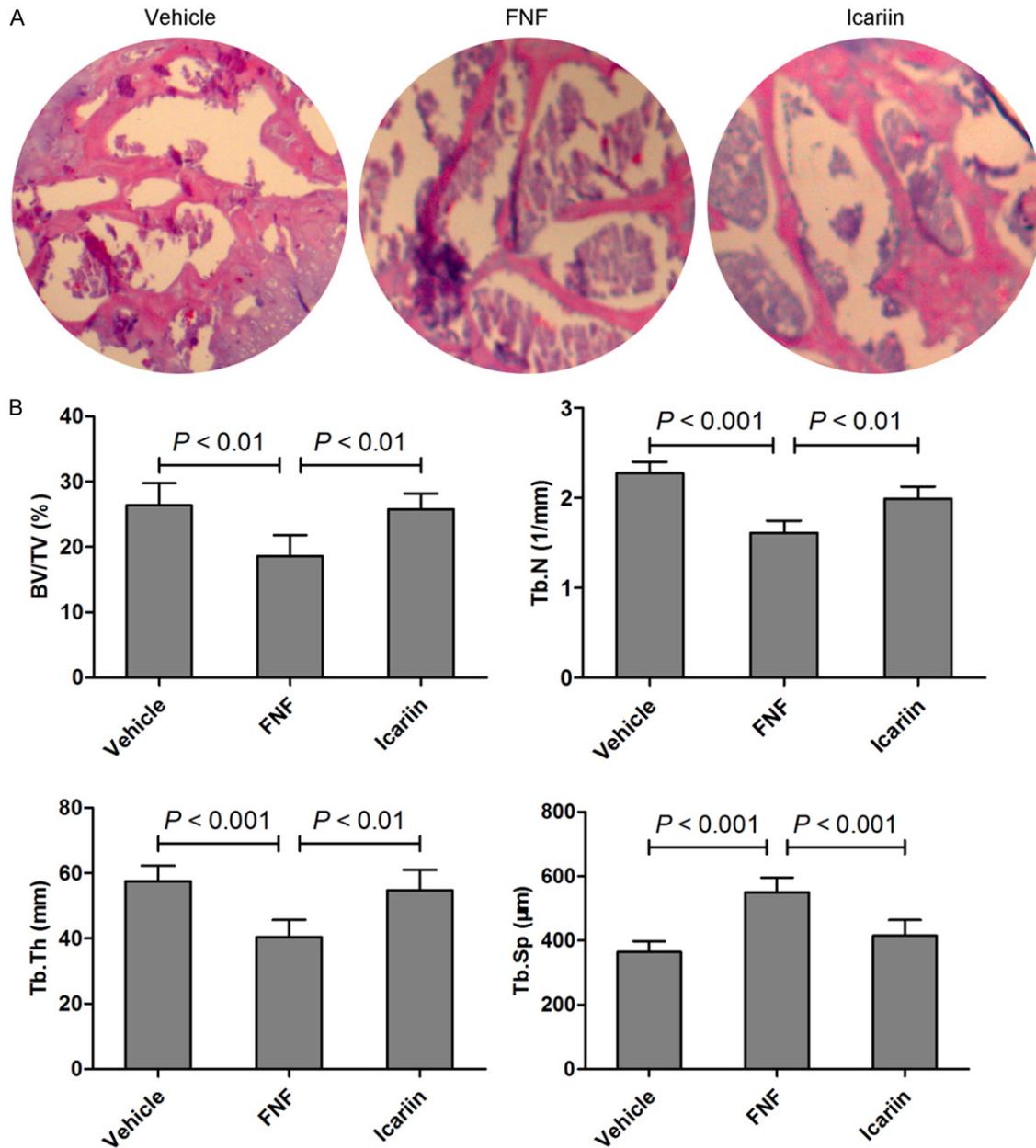


Figure 2. Hematoxylin and eosin (H&E) staining of the femoral neck in a mice fractured model (A, 100×). Bone volume over total volume (BV/TV), trabecular number (Tb. N), trabecula thickness (Tb. Th) and trabecular separation (Tb. Sp) were obtained by micro-CT assay (B). Values were expressed as mean ± SD, n = 3 in each group.

Results

Effect of icariin on physiological and biochemical properties in the femoral neck fractured mice model

The body weight, bone P, bone Mg and serum Ca had no significant difference in the three experimental groups (Table 1). However, the bone Ca contents in the femur of femoral neck

fractured mice was markedly decreased, and icariin treatment increased the bone Ca contents in the femur of femoral neck fractured mice. Serum concentrations of bone turnover markers, like TRAP-5b as a bone resorption marker, OCN as a bone formation marker, were determined. The results showed that the serum TRAP-5b level in FNF group was significantly increased, and the serum OCN level was significantly decreased when compared to that of the

Icariin in a mice fractured model

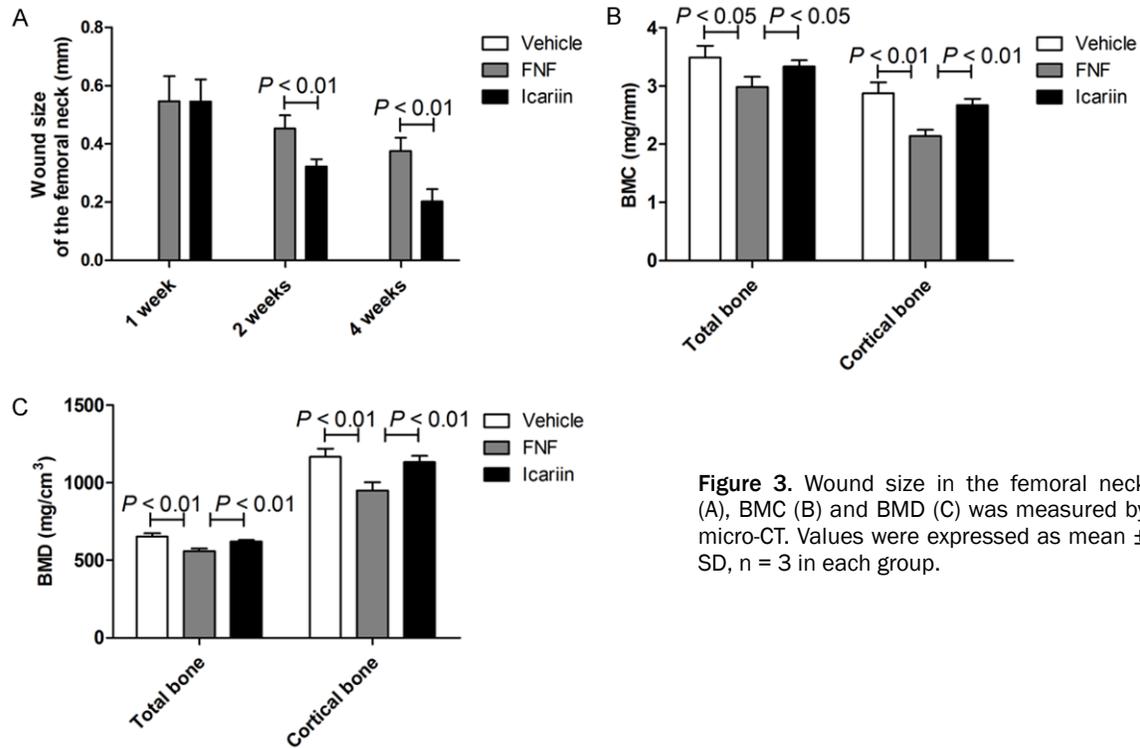


Figure 3. Wound size in the femoral neck (A), BMC (B) and BMD (C) was measured by micro-CT. Values were expressed as mean \pm SD, n = 3 in each group.

Table 2. Effects of icariin on femoral neck cancellous bone histomorphometry dynamic parameters

	Vehicle	FNF	Icariin
MS/BS (%)	40.6 \pm 4.5	23.6 \pm 5.9*	36.8 \pm 6.2#
MAR (μ m/day)	0.74 \pm 0.12	0.51 \pm 0.09*	0.67 \pm 0.10#
BFR/BS (μ m/day 100)	27.8 \pm 5.5	18.3 \pm 4.6*	25.1 \pm 5.1#
BFR/BV (%/year)	28.5 \pm 4.9	18.7 \pm 5.2*	26.3 \pm 5.7#

FNF, fracture of the femoral neck; MS/BS, the ratio of mineralizing surface to bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate per unit of bone surface; BFR/BV, bone formation rate per unit of bone volume. Values are expressed as mean \pm SD, n = 5 in each group. *P < 0.05 versus vehicle group; #P < 0.05 versus FNF group.

control group. Icariin treatment could inhibit serum TRAP-5b level and increase serum OCN level in the femoral neck fractured mice (**Table 1**).

Effect of icariin on cathepsin K expression in the proximal femur of femoral neck fractured mice model

Pharmacological inhibition of cathepsin K on fracture repair has been reported in a mice model, and cathepsin K inhibitors have been developed and established to restore bone mass in both animal models of bone loss and postmenopausal osteoporotic patients [3]. Previous study demonstrates that icariin as a

cathepsin K inhibitor can suppress cartilage and bone degradation in mice of collagen-induced arthritis [9]. To further characterize the expression of cathepsin K in the proximal femur of femoral neck fractured mice in response to icariin treatment, we performed RT-PCR and western blotting analyses and found that mRNA and protein expression of cathepsin K were markedly up-regulated in FNF group as compared to control group. However, icariin treatment could reverse the expression of

cathepsin K in the proximal femur of femoral neck fractured mice (**Figure 1A-C**).

Effect of icariin on the histopathology and biomechanical properties in the femoral neck of fractured mice model

Histological analysis on trabecular bone in femoral neck was performed by H&E staining. The results showed that there was markedly different of the micro-architecture in trabecular bone. Fracture-induced increased disconnections and separation of trabecular bone network as well as the reduction of trabecular bone mass of primary and secondary spongiosa were observed in the femoral neck of frac-

Icariin in a mice fractured model

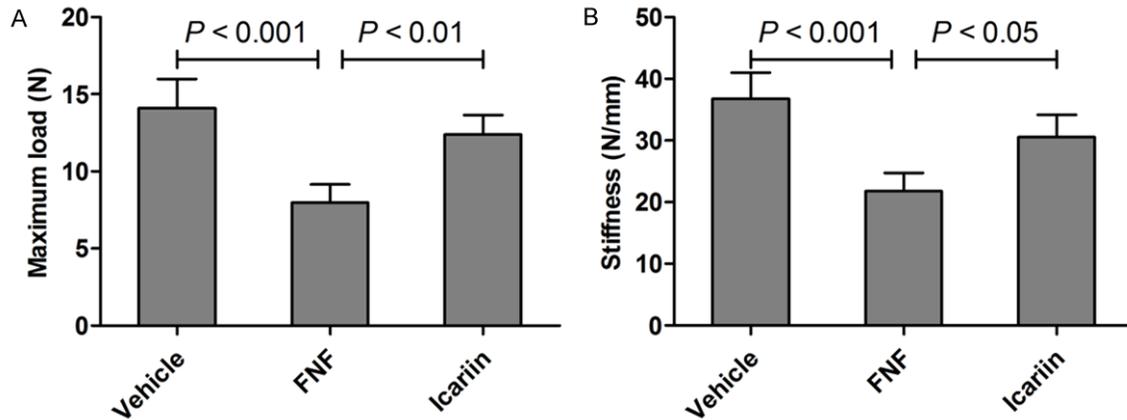


Figure 4. Biomechanical parameters. Maximum load values (A) and stiffness (B). Values were expressed as mean \pm SD, $n = 5$ in each group.

tured mice model. However, icariin treatment reversed fracture-induced trabecular deleterious effects and stimulated bone healing potential (Figure 2A). Moreover, the micro-architecture in trabecular bone at the femoral neck was quantified using micro-CT scanning. The results indicated that femoral neck fractured mice exhibited significantly lower trabecular BV/TV, Tb. N and Tb. Th and higher Tb. Sp, compared to that of the control group. Interestingly, icariin treatment for femoral neck fractured mice resulted in increasing the BV/TV ratio, Tb. N, Tb. Th and decreasing Tb. Sp (Figure 2B). Furthermore, micro-CT scanning also displayed an accelerated closure of the wounds in the femur neck by icariin treatment (Figure 3A). Evaluation of bone quality with micro-CT scanning revealed that icariin treatment increased BMC and BMD at the wound site of femoral neck (Figure 3B and 3C). Total BMC was 10.9% higher in icariin treatment group than that of in FNF group. An increase of 25% in cortical BMC was detected in icariin treatment group compared with FNF group. Total BMD was elevated by 11% in icariin treatment group compared with FNF group, while cortical BMD was elevated by 19% in icariin treatment group as compared to FNF group (Figure 3B and 3C). The dynamic parameters (MS/BS, MAR, BFR/BS, and BFR/BV) of trabecular bone significantly decreased at the femoral neck mice as compared to control group. However, icariin treatment could reverse decreased dynamic parameters (MS/BS, MAR, BFR/BS, and BFR/BV) in the femoral neck of fractured mice (Table 2). Mechanical test showed that icariin treatment increased maximum load (Figure 4A) and stiff-

ness (Figure 4B) of femur in icariin group as compared to FNF group.

Discussion

There is mounting evidence that inhibition of cathepsin K can protect bone via restoring bone mass in both animal models of osteoporosis and osteoporotic patients [18, 19]. Transgenic mice that overexpressed cathepsin K has reduced trabecular bone volume as a result of accelerated bone turnover [20]. Interestingly, cathepsin K-knockout mice display high numbers of non-resorbing osteoclasts as well as osteoblasts on bone surfaces associated with a high bone formation rate (BFR) in both trabecular and cortical bones [21]. Moreover, odanacatib provides full protection and restores lumbar vertebral and femoral bone loss induced by estrogen deficiency in rabbits' model [22]. Recent research has shown that cathepsin K inhibitor, L-006235, can inhibit osteoclastic activity without changing bone formation, and the inhibition of cathepsin K delays but does not abrogate callus remodeling during bone repair [3]. Inhibition of cathepsin K reduces bone and cartilage degradation evoked by collagen-induced arthritis in mice [7, 9]. In this study, we found that the mRNA and protein expression of cathepsin K were markedly up-regulated in the proximal femur of femoral neck fractured mice as compared to normal control. These results suggested that the levels of cathepsin K could be evoked by fracture trauma. Based on above phenomenon, we up-regulated cathepsin K might be involved in fracture healing. So, we

designed a pharmacological experiment to inhibit the expression of cathepsin K in the femoral neck fractured mice model.

Icariin is a prenylated flavonol glycoside contained in the herb Epimedium, which is thought to have bone-strengthening properties and has long been used to promote healing of bone fractures or prevent osteoporosis [23]. Based on the growing evidence that icariin has been reported to inhibit osteoclast differentiation, bone resorption and attenuates glucocorticoid-induced bone deteriorations in mice [13, 23]. Moreover, icariin can increase OPG and decrease RANKL expression and reduce the number and activity of osteoclasts [24]. These findings suggest that icariin may have potential in the treatment of bone deteriorations. However, there is no literature reported regarding if icariin can increase the healing potential and biomechanical properties of femoral neck in a mice fractured model. In the present study, we demonstrated that icariin treatment could suppress the expression of cathepsin K in the proximal femur of femoral neck fractured mice. Our histological analyses of icariin treated mice revealed the icariin treatment reversed fracture-induced trabecular deleterious effects, which is characterized by increasing disconnections and separation of trabecular bone. Moreover, micro-CT scanning results indicated that femoral neck fractured mice exhibited significantly lower trabecular BV/TV, Tb. N and Tb. Th and higher Tb. Sp, compared to that of the control group. Interestingly, icariin treatment for femoral neck fractured mice resulted in increasing the BV/TV ratio, Tb. N, Tb. Th and decreasing Tb. Sp. Importantly, icariin treatment could improve biomechanical properties and accelerate closure of the wounds in the femur neck.

In conclusion, the present study clearly demonstrated that icariin, cathepsin K inhibitor, increases the healing potential and biomechanical properties of femoral neck in a mice fractured model. On the basis of the present results, icariin may represent a accessory therapeutic medicine with fracture healing.

Disclosure of conflict of interest

None.

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Icariin in a mice fractured model

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