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Original Article

Icariin attenuates glucocorticoid-induced bone deteriorations, hypocalcemia and hypercalciuria in mice

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Abstract: Objective: This study was performed to investigate bone deteriorations and calcium homeostasis of GIOP mice in response to the treatment of icariin. Methods: The biomarkers in serum and urine were measured, tibias were taken for the measurement on bone calcium, gene expression, histomorphology and micro-CT. Results: Glucocorticoid-treated facilitated to induce hypocalcemia and hypercalciuria in mice, and icariin-treated showed a greater increase in serum calcium and decrease in urine calcium. Icariin reversed DXM-induced trabecular deleterious effects and stimulated bone remodeling, including an increase in bone calcium, OCN and FGF-23 and a decrease in a critical bone resorption markers CTX and TRAP-5b. H&E staining and micro-CT showed the increased disconnections and separation among growth plate and trabecular bone network as well as the reduction of trabecular bone mass of primary and secondary spongiosa throughout the proximal metaphysis of tibia in DXM group. Importantly, icariin reversed DXM-induced trabecular deleterious effects and stimulated bone remodeling. Moreover, the results showed that the mRNA expression of MMP-9 and CAII was significantly increased in DXM group compared with control group. Icariin treatment could suppress the expression of MMP-9 and CAII in the tibia of mice. Conclusions: The present study demonstrated the protective effects of icariin against bone deteriorations, hypocalcemia and hypercalciuria in experimentally DIOP mice. Furthermore, these results provided further evidence to support the dual role of icariin as a bone formation enhancer and bone resorption inhibitor.

Keywords: Osteoporosis, icariin, dexamethasone, hypocalcemia, hypercalciuria

Introduction

Glucocorticoids have been widely used in clinics due to their anti-inflammatory, immunomodulatory effects, anti-shock and relief of asthma [1]. However, the therapeutic use for immunosuppression after organ transplantation or for inflammatory diseases of glucocorticoids is always accompanied by substantial adverse outcomes such as diabetes, obesity, and bone deteriorations, which is known in this case as glucocorticoid-induced osteoporosis (GIOP) [2-4]. It is a secondary osteoporosis that results in easy fracturing, and even disability. However, glucocorticoids-induced bone deleterious effects has been regarded as an important cause for osteoporosis and bone loss [5]. Clinical studies have shown that low-dose [6] or high-dose [7] glucocorticoid, especially dexamethasone (DXM), inhalatory therapy as a cause of bone loss in human. In vitro studies also show that glucocorticoids can induce osteoblasts and osteocyte apoptosis [2, 8]. Studies also show that bone mesenchymal stem cells (BMSCs) proliferation, osteogenic differentiation, and reactive activity to an osteogenic inductor are reduced in GIOP rats [1, 9]. Moreover, prolonged glucocorticoid use can induce hypercalciuria, the 24 h urinary calcium excretion is significantly increased at day 7 after the patients treatment with methylprednisolone for 10 mg/day [10]. Previous studies demonstrate that dysfunction of kidney and intestine contribute to hypercalciuria [11-13]. Interestingly, pharmacological application of glucocorticoid in patient with vitamin D insufficiency can lead to hypocalcemia in association with hypercalciuria and secondary hyperparathyroidism in the absence of hypomagnesemia [13].

Icariin has been identified as the major active ingredient in Herba epimedii, one of the most commonly used Chinese herbal medicines for

the treatment of osteoporosis [14]. When treating it, icariin is postulated to improve the function of damaged tissue such as OVX-induced marrow adiposity [15] and type II collagen-induced articular cartilage destruction [16]. In vitro studies show that icariin attenuates glucocorticoid- and hypoxia-induced osteocyte apoptosis and preserves their osteogenic differentiation potential [17, 18]. The comparing study shows that antiosteoporotic activity of icariin in ovariectomized rats has no obvious difference with estrogen [19]. Furthermore, a 24-month randomized double-blind placebo-controlled clinical trial showed that Epimedium-derived phytoestrogen is able to prevent bone loss in late postmenopausal women [20]. However, the effects of icariin in other experimental animal models, such as glucocorticoid-induced osteoporosis animal model, remains largely unknown.

Thus, the present study is aimed to determine the effects of the icariin on calcium homeostasis and trabecular bone properties in glucocorticoid-induced osteoporotic mice. It is hoped that this study will further increase our understanding on the anti-osteoporotic actions of icariin, which might be useful in managing calcium balance and secondary osteoporotic patients.

Materials and methods

Animal treatment

Six-week-old male C57BL/6J mice (Slac Laboratory Animal, Shanghai, China) were allowed to acclimate to the environment for 1 week. All experimental procedures were carried out in accordance with the guidelines of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine on Animal Care. All chemicals and reagents were purchased from Sigma (Oakville, Ontario, Canada), except where noted.

The mice were randomly divided into three groups: (1) Vehicle group (6 weeks or 12 week, n = 6 in each group); (2) Mice were injected intramuscularly with 5 mg/kg body weight dexamethasone (DXM) three times a week (6 weeks or 12 week, n = 6 in each group, DXM); (3) Mice in the DI group received icariin orally at a dose of 100 mg/kg per day combined with DXM for 6weeks or 12 weeks (DI, n = 6 in each group).

Chemistries in serum and urine and bone Ca

The concentrations of calcium (Ca) and creatinine (Cre) from serum and urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The level of urine Ca was corrected by the concentration of urine Cre. Serum levels of fibroblast growth factor-23 (FGF-23), tartrate resistant acid phosphatase-5b (TRAP-5b), osteocalcin (OCN) and C-terminal telopeptide of type I collagen (CTX) were detected using rat bioactive PTH ELISA assay (Immutopics, Inc., San Clemente, CA, USA) with ELISA reader (MD SpectraMax M5, USA).

The tibias were incinerated at 800°C for 6 hours and the ash weighed. 10 mg of bone ash was then dissolved in 1 ml of 37% HCl and diluted with Milli-Q water. The calcium content was determined by the kit used for serum and urine calcium assay.

Bone histomorphology

The tibias 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 proximal metaphysis of the tibia 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 away from the distal femur growth plate. Bone morphometric parameters, including bone volume over total volume (BV/TV), trabecula number (Tb. N), trabecula thickness (Tb. Th) and bone mineral density over total volume (BMD/TV) were obtained by analyzing the VOI.

Reverse transcription-polymerase chain reaction

The tibias of each animal were crushed under liquid nitrogen conditions and RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed by reverse transcription reactions with

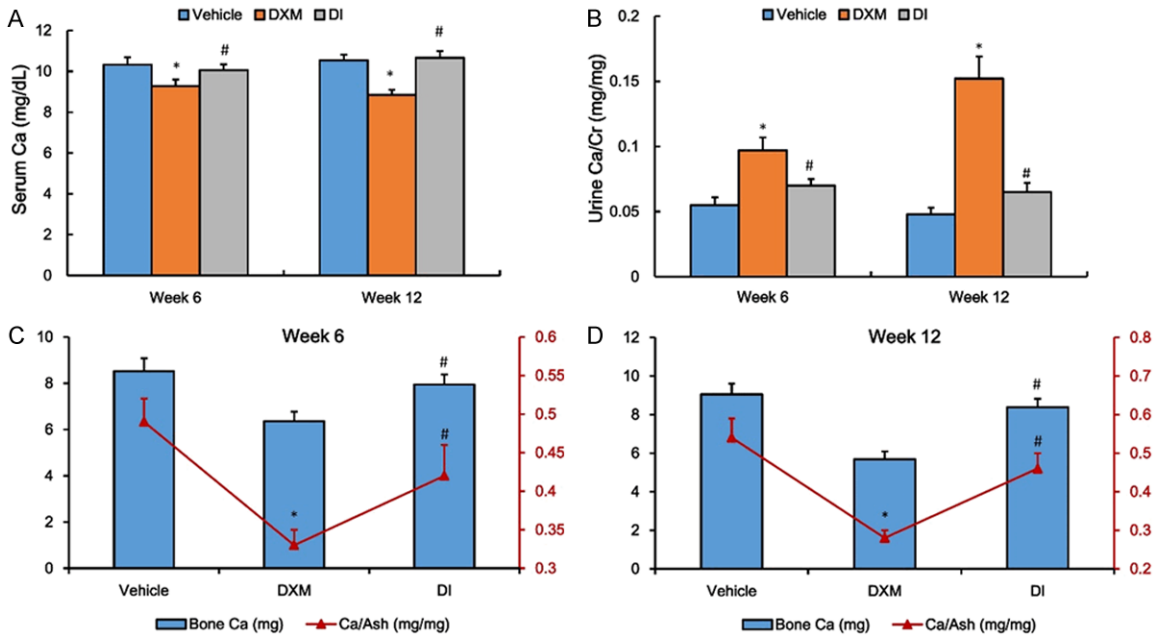

Figure 1. Calcium contents in serum, urine and tibias. Calcium level in serum (A), urine (B) and tibias (C and D). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus vehicle group; $^{\#}P < 0.05$, versus DXM group.

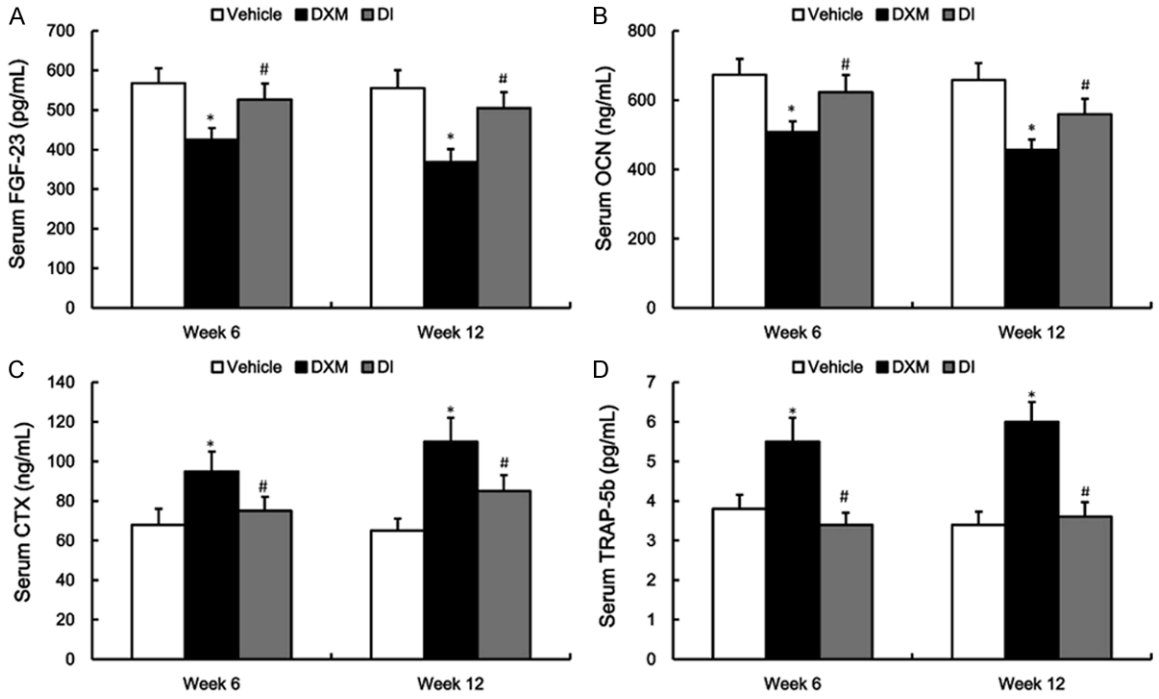

Figure 2. Bone metabolic biochemical makers. FGF-23, fibroblast growth factor-23; TRAP-5b, tartrate resistant acid phosphatase-5b; OCN, Osteocalcin; CTX, C-terminal telopeptide of type I collagen. Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus vehicle group; $^{\#}P < 0.05$, versus DXM group.

2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs

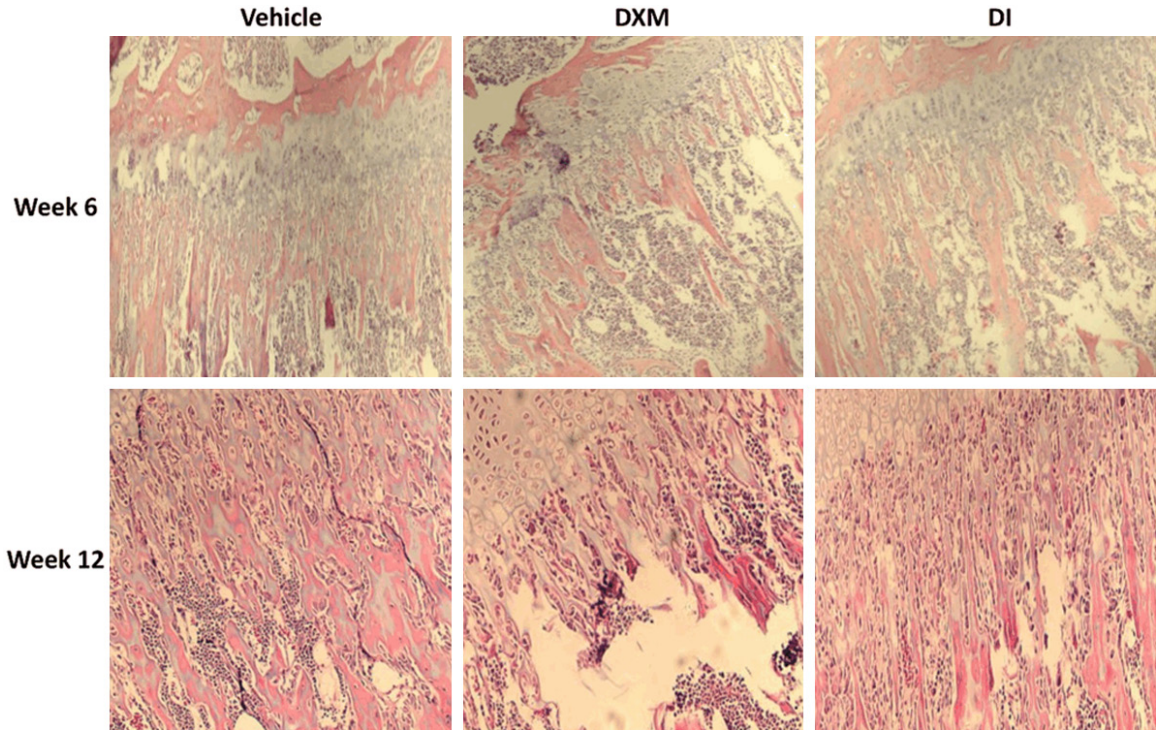

Figure 3. Hematoxylin and eosin staining of the proximal metaphysis of the tibia. Trabecular bone zone below growth plate was shown (100×).

served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 7300). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. PCR with the following primers: MMP-9, Forward 5'-TAGCCATTGTGTTATTCCT-3' and Reverse 5'-GTGGCGTCCACTGTGGG-3'; CAII, Forward 5'-TAGCGAATGTGAATTGTCCT-3' and Reverse 5'-CTGCGGTCCTCAGAGCG-3'; GAPDH, Forward 5'-TGAGCGGAGGTGAATGCATT-3' and Reverse 5'-GAGCAAAAGCGTACATACATCTC-3'.

Statistical analysis

The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. All statistical analyses were performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a posttest to compare the group means if overall $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

Results

Calcium contents in serum, urine and tibias

The calcium levels in serum, urine and tibias were comparable in the three experimental groups. At week 6 or week 12, when comparing the results of serum calcium and urine calcium between vehicle and DXM groups, we could easily see that DXM-treated decreased serum calcium (10.32 ± 0.37 to 9.28 ± 0.32 mg; 10.54 ± 0.27 to 8.85 ± 0.25 mg/dL) and increased urine calcium (0.055 ± 0.006 to 0.097 ± 0.01; 0.048 ± 0.005 to 0.152 ± 0.017) (**Figure 1A** and **1B**). Glucocorticoid-treated facilitated to induce hypocalcemia and hypercalciuria in mice. We also found that increasing duration time of glucocorticoid-treated significantly upregulated the urine calcium level and downregulated the serum calcium level, hypocalcemia and hypercalciuria were dramatically inhibited in GIOP mice in response to icariin administration (**Figure 1A** and **1B**). At week 6 or week 12, when comparing the results of bone calcium content between vehicle and DXM groups, we could easily see that DXM-treated decreased bone calcium content (8.52 ± 0.49 to 6.36 ± 0.33 mg; 9.05 ± 0.54 to 5.68 ± 0.28

Table 1. Bone parameters of proximal tibia in mice

	Week 6			Week 12		
	Vehicle	DXM	DI	Vehicle	DXM	DI
BV/TV (%)	39 ± 3.7	31 ± 2.5*	36 ± 3.1#	41 ± 4.3	24 ± 2.7*	33 ± 3.8#
Tb. N (mm^{-1})	9.4 ± 0.68	6.9 ± 0.53*	8.6 ± 0.62#	8.9 ± 0.62	5.4 ± 0.51*	7.7 ± 0.65#
Tb. Th (μm)	50 ± 4.6	39 ± 3.3*	45 ± 2.5#	55 ± 4.6	30 ± 3.7*	47 ± 4.4#
BMD/TV (mg HA/cm^3)	136 ± 10	96 ± 6.5*	120 ± 8.7#	128 ± 11	81 ± 7.4*	118 ± 13#

BV/TV, bone volume over total volume; Tb. N, trabecula number; Tb. Th, trabecula thickness; BMD/TV, bone mineral density over total volume. Values are expressed as mean ± SEM, n = 6 in each group. *$P < 0.05$, versus vehicle group; #$P < 0.05$, versus DXM group.

mg). Six weeks or twelve weeks after the icariin treatment, the bone calcium content was increased in the combination group when compared to that of the DXM single group ($P < 0.05$) (**Figure 1C** and **1D**). Moreover, Ca/Ash was decreased in GIOP mice, and icariin treatment could reverse DXM-induced bone deteriorations (**Figure 1C** and **1D**). From these calcium metabolic data, it was well shown that icariin exerted protective effects on maintaining calcium balance of DXM-induced bone deteriorations in mice.

Bone metabolic biochemical makers

Serum concentrations of bone turnover markers, like TRAP-5b and CTX as a bone resorption marker, OCN and FGF-23 as a bone formation marker, were determined. The results showed that the serum TRAP-5b and CTX level in DXM group were significantly increased, and the serum FGF-23 and OCN level were significantly decreased when compared to that of the control group (**Figure 2A-D**). The serum TRAP-5b and CTX level in the DI group were lower than DXM group ($P < 0.05$), and the serum FGF-23 and OCN level were significantly elevated in DI group after icariin treatment (**Figure 2A-D**).

Bone histology and micro-CT

Histological analysis on trabecular bone in proximal metaphysis of mice was performed by H&E staining (**Figure 3**). The histology of trabecular bone below growth plate was markedly different in the three experimental groups. H&E staining showed the increased disconnections and separation among growth plate and trabecular bone network as well as the reduction of trabecular bone mass of primary and secondary spongiosa throughout the proximal metaphysis of tibia in DXM group. Importantly, icariin reversed DXM-induced trabecular deleterious effects and stimulated bone remodeling. The loss of trabecular bone mass at the proximal metaphysis of the tibia was quantified using micro-CT scanning. Analyses of the data from the proximal metaphysis of the tibia revealed that GIOP mice exhibited significantly lower trabecular BMD/TV, BV/TV, Tb. N and Tb. Th, compared to that of the control group (**Table 1**). Notably, treatment with icariin for GIOP mice resulted in increasing the BV/TV ratio, Tb. N, Tb. Th and BMD/TV (**Table 1**).

mRNA expression of key regulators for bone metabolism

To determine the changes of the osteoclast-specific genes which are responsible for osteoclasts-involved bone resorption, the mRNA expression of carbonic anhydrase II (CAII) and matrix metalloproteinase (MMP)-9 was measured. The results (**Figure 4A-D**) showed that the mRNA expression of MMP-9 and CAII was significantly increased in DXM group compared with control group. Icariin treatment could suppress the expression of MMP-9 and CAII in the tibia of mice.

Discussion

Short-term or long-term of glucocorticoid treatment is suggested to influence bone physiology and remodeling and disturbance of calcium homeostasis [21]. In vivo studies, in contrast to the non-treated rabbits or mice, the dexamethasone-injected exhibited the typical features of GIOP as shown by the basic biomechanical parameters, including the decreased of bone mineral density, and the increased disconnections and separation of trabecular bone network [22, 23]. These pathological changes of bone metabolism are correlated with distur-

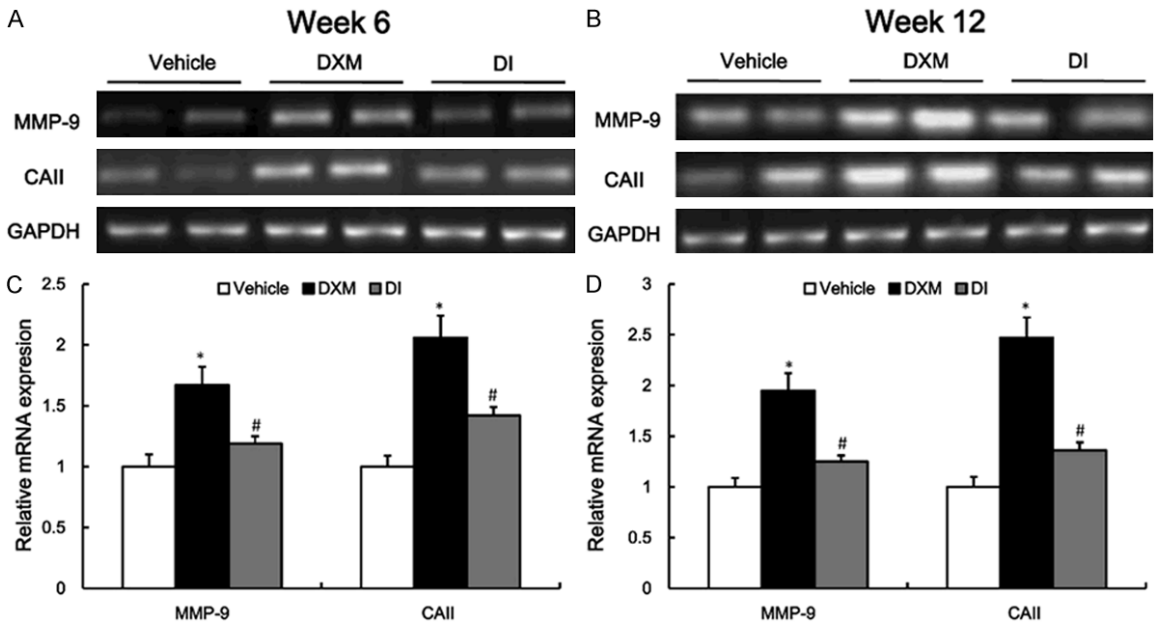

Figure 4. mRNA expression of key regulators for bone metabolism. The mRNA expression of matrix metalloproteinase (MMP-9) and carbonic anhydrase II (CAII) in the tibia with icariin treatment for 6weeks (A) and 12 weeks (B). Densitometric quantification was measured for 6weeks (C) and 12 weeks (D). Values are expressed as mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, versus vehicle group; $^{\#}P < 0.05$, versus DXM group.

bance of calcium homeostasis [24]. In MG-63 human osteosarcoma cells, cytokine-stimulation to increase [Ca^{2+}] which is prevented by dexamethasone [25]. As expected, the dexamethasone-injected significantly upregulated the content of urine calcium and downregulated the levels of serum calcium in mice. Consequently, leading to marked bone deteriorations of trabecular bone at the proximal metaphysis of tibia as shown by H&E staining and the micro-CT quantitative data. To our surprise, the opposite results are demonstrated in premature infants, and dexamethasone shows no significant influence on the urinary excretion of calcium, but dexamethasone treatment might increase the risk of osteopenia by enhancing phosphate excretion [26, 27].

In this study, glucocorticoid injection successfully led to bone deleterious effects. The glucocorticoid-induced increasing in bone resorption was confirmed by the increased level of urine calcium, CTX and TRAP-5b, and the decreased level of serum calcium, OCN and FGF-23 in the serum. Moreover, histomorphology staining also confirmed the results. Results from previous studies suggest that the osteoprotective effect of icariin results from the ability of icariin to suppress bone absorption [17, 28]. Our findings showed that icariin induced a decrease in a critical bone resorption markers CTX and TRAP-5b. A recently identified phosphatonin, known as fibroblast growth factor 23 (FGF-23), disclosed new pathways in the pathophysiology of mineral metabolism [29]. Clinical studies had shown that the downregulation of serum FGF-23 levels in Crohn disease appeared as a secondary compensatory effect on the bone and mineral metabolism induced by chronic intestinal inflammation [30]. In patients with trochanteric and femoral neck osteoporotic fractures, not only an age-related decline of renal function but also the type of skeletal injury may contribute to the circulating concentrations of cFGF-23 [31]. Intriguingly, the FGF 23 levels were correlated with the age of menopause, FGF-23 is found to be significantly higher in the group of menopausal age < 5 years compared to the group of menopausal age >10 and to the group of menopausal age 5-10 years [32]. It was found in this study that the treatment with dexamethasone resulted in the decreased level of FGF-23 in serum, which could be, at least partially, attributed to the development of GIOP in mice. In addition, dexamethasone-treated could increase osteoclast-involved resorptive activity as it further induced the up-regulation of matrix metalloproteinase

(MMP-9) and carbonic anhydrase II (CAII), which could act on CO_2 and H_2O to generate the hydrogen ions that are secreted extracellularly by H^+-ATPase in osteoclasts to dissolve bone inorganic substance [33]. Icariin-treated could reverse dexamethasone-induced osteopenia or osteoporosis through suppression the mRNA expression of bone metabolic key regulators such as MMP-9 and CAII in the tibia of mice.

In the present study, we evaluated the osteoprotective effects of icariin against deteriorating effect of glucocorticoid treatment in mice model. Icariin is widely employed in preventing ovariectomy-reduced boss loss and therapy of postmenopausal osteoporosis [15, 34]. Increased BMD and suppression of urinary deoxypyridinoline are observed in menopausal women when treated with combined herbal preparation containing 60 mg Icariin, 15 mg daidzein, and 3 mg genistein [20]. In our study, osteoporotic mice treated with icariin for 6 or 12 weeks showed a significant increase in BMD and equilibrium of calcium homeostasis. In vitro studies show that Icariin is more potent than genistein in promoting osteoblast differentiation and mineralization [35]. It has also been reported that icariin could inhibit the formation and differentiation of osteoclasts as well as their bone resorption activities [36]. In our study, icariin-treated could increase the serum OCN and FGF-23 when compared to that of the DXM group. These studies provide further evidence to support the dual role of icariin as a bone formation enhancer and bone resorption inhibitor.

In conclusion, the present study clearly demonstrated that icariin protected against osteoporosis associated with glucocorticoid use. On the basis of the present results, icariin might represent a therapy with bone-forming as well as an anti-resorptive activity in GIOP mice. Our findings provide evidence to support the role of icariin as an effective therapeutic approach in the management of glucocorticoid-induced bone loss and disequilibrium of calcium homeostasis.

Disclosure of conflict of interest

None.

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