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The beneficial effect of Icariin on bone is diminished in osteoprotegerin-deficient mice

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ABSTRACT

Background: Osteoprogeterin (OPG) plays an important role in regulating bone homeostasis by inhibiting osteoclastogenesis and bone resorption. Icariin is the major ingredient of *Herba Epimedii*, which exerts anabolic and anti-resorptive effects on bone, but the mechanism remains unknown. In this study, we evaluated the role of OPG in Icariin-mediated beneficial effects on bone.
Materials and methods: Twelve-week-old *Opg* knockout (KO) male mice and their wild type (WT) littermates were orally administered with Icariin (0.3 mg/g) everyday for 8 weeks. Bone mass and microstructure in the right proximal tibiae were analyzed with micro-computed tomography (μCT). Bone remodeling was evaluated with serum biochemical analyses and bone histomorphometry. The colonies of fibroblast and osteoblast from bone marrow derived cells were quantified. The mRNA expressions of osteoblast and osteoclast related genes in trabecular bone from the femora were analyzed by real-time PCR.
Results: Icariin treatment led to greater trabecular bone volume and trabecular number compared with vehicle treatment in WT mice. Icariin treatment increased bone formation parameters while it decreased bone resorption parameters in WT mice; however, the anabolic response of trabecular bone to Icariin treatment was diminished in KO mice. At cellular and molecular levels, Icariin significantly increased the formation of osteoblast colonies from bone marrow derived cells and the *Opg* gene expression in trabecular bone of WT mice.
Conclusions: These data suggest that Icariin treatment exerted anabolic and anti-resorptive effects on trabecular bone of WT mice, in which the effects were diminished in KO mice. The effects of Icariin treatment on bone are dependent on up-regulation of *Opg*, therefore, OPG plays an essential role in Icariin-mediated beneficial effects on trabecular bone.

Introduction

Postmenopausal osteoporosis is a disease characterized by bone loss and increased risks of bone fracture because of an imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Currently, the drugs for prevention and treatment of osteoporosis approved by the US Food and Drug Administration (FDA) either reduce bone resorption (anti-catabolic agents) or increase bone formation (anabolic agents) [1]. Due to the disadvantages of serious side-effects or high cost of these drugs, it is desirable to develop new alterative agents with minor side-effects and lower cost. The traditional Chinese herbs may be good candidates to meet these requirements.

Herba Epimedii (HEP) is commonly used in traditional Chinese medicine for "strengthening the kidney" and providing nutrition to bone. Icariin, a marker flavonoid glycoside in HEP, is believed to be the major active ingredient that accounts for its skeletal beneficial effects. In a randomized, double-blind and placebo-controlled trial, HEP (containing 60-mg Icariin, 15-mg daidzein and 3-mg genistein) was reported to reduce bone loss and increase bone mineral density (BMD) in late postmenopausal women [2]. Our previous study suggested that the same herb formula could ameliorate deterioration of microarchitecture of the proximal tibiae induced by ovariectomy, and it could increase bone formation and decrease bone resorption in ovariectomized rats [3]. Another *in vivo* study showed that Icariin treatment could improve BMD and bone strength in ovariectomized rats [4]. Other *in vitro* studies demonstrated that Icariin could increase cell proliferation, differentiation and activity in osteoblastic cells [5].

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Meanwhile, it also has the potential to inhibit osteoclast differentiation and bone resorption [6,7]. The results of these studies implied that Icariin could exert anabolic and anti-catabolic effects on bone. However, the mechanisms for the skeletal beneficial effect of Icariin remain to be elucidated.

Osteoclasts are multinucleated cells that differentiate from monocyte–macrophage lineage of hematopoietic precursors [8]. Osteoblasts regulate osteoclastogenesis and bone resorption through expression of receptor activator of nuclear factor κ B ligand (RANKL), which is a member of tumor necrosis factor (TNF) family. RANKL binds to the receptor, RANK, which is expressed in pre-osteoclasts/osteoclasts, leading to the activation of osteoclastogenesis. RANKL–RANK interaction is essential for the differentiation and activity of pre-osteoclasts [9–13]. Osteoprotegerin (OPG), a decoy receptor for RANKL, is expressed by osteoblasts and blocks the interaction of RANKL–RANK. Therefore, the OPG/RANK/RANKL axis plays an important role in bone metabolism.

Previous studies showed that flavonoids (including genistein and daidzein) had the potential to modulate gene expression of *Opg* and *Rankl* in osteoblasts [14–16] or their protein levels in the serum [17]. It was recently demonstrated that Icariin could increase *Opg* gene expression in osteoblastic cells [5]. Based on these observations, we hypothesized that the dual effect of Icariin on bone was possibly associated with its regulation of *Opg* expression. An *Opg* gene deficient mice model was adopted to test this hypothesis in this study.

Materials and methods

Animal treatment

Twelve-week-old male *Opg* heterozygous mice with C57BL/6J background were provided by the Shanghai Research Center for Biomodel Organisms. Heterozygous offspring were intercrossed to generate homozygous mice and backcrossed to 129S1/Sv strain. The genotyping was identified by PCR with OPG WT primers (5′-GTAA CGCCCT TCCTCACACTCACA-3′), OPG5′ JDU primers (5′-ACCCA CCAT CCTTCACCTCACTTCTG-3′) and OPG5′ JDD primers (5′-GCATCGCCTT CTATCGCCTTCTTG-3′) in the same reaction system. 4.0 kb and 4.5 kb fragments can be detected separately in wild type ($Opg^{+/+}$), homozygous ($Opg^{-/-}$) mice, and both fragments in heterozygous ($Opg^{+/-}$) mice. *Opg* expression was examined by routine RT-PCR and Western blot. All the mice were housed under specific environment (22 °C, 12 h/12 h light/dark, and 50% humidity) with free access to food and tap water. They were randomly assigned to the following 4 groups (n = 8/group): (1) WT mice treated with either vehicle (0.9% saline) (WT + Veh) or (2) Icariin (3 mg/g) (WT + Icariin), (3) KO mice treated with either vehicle (KO + Veh) or (4) Icariin (3 mg/g) (KO + Icariin). The dosage used in this study has been previously demonstrated to exert skeletal beneficial effects on mice [5]. Both Veh and Icariin were orally administrated daily to the mice and lasted for 8 weeks. Double fluorescence labeling with calcein (10 mg/kg s.c.) was performed at days 7 and 2 before sacrificing the mice. All procedures were reviewed and approved by the Animal Research Committee at Tongji Medical School, Huazhong University of Science and Technology.

Icariin preparation

A dual-mode high-speed counter-current chromatographic method was developed for separation and purification of bioactive flavonoids from the medicinal herb Epimedii. The crude flavonoids were obtained by extraction with ethyl acetate and ethanol from the dried aerial parts of Epimedii under sonication. High-speed counter-current chromatography with a two-phase solvent system composed of n-butanol-ethyl acetate–water (3:7:10, v/v) was performed using a dual-mode method, which yielded Icariin at a purity of 96.8% based on HPLC analysis.

Biochemical analyses

Serum bone formation marker (osteocalcin, OC) and bone resorption marker (tartrate-resistant acid phosphatase 5b, TRACP 5b) were analyzed with biochemical assays. Serum samples were centrifuged at 3600 rpm for 5 min at 4 °C, and the serum was stored at −80 °C until use. The expression levels of these markers were measured with ELISA kits (IDS Inc., Fountain Hills, AZ) according to the manufacturer's instructions.

MicroCT measurement

The microarchitecture of trabecular bone in the right proximal tibiae was analyzed by a μCT scanner (*viva*CT 40, Scanco Medical, Brüttisellen, Switzerland) using a 10.5-μm isotropic voxel size in all three spatial dimensions. Trabecular bone within the proximal tibiae was extracted by semi-automatically drawn contour at each two-dimensional (2-D) section. Direct 3D measurement methods were used to calculate the following parameters: trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and connectivity density (Conn.D).

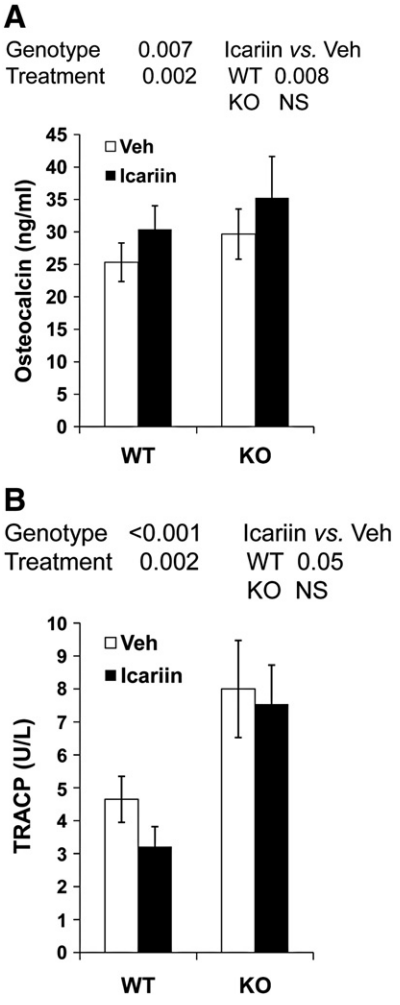

Fig. 1. Serum bone formation markers (osteocalcin, OC) and bone resorption markers (tartrate-resistant acid phosphatase 5b, TRACP 5b) in the WT and KO mice. The markers were measured with the ELISA kits. (A) Osteocalcin (ng/ml), (B) TRACP 5b (U/l). Data are represented as mean ± SEM.