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Icariin inhibits the osteoclast formation induced by RANKL and macrophage-colony stimulating factor in mouse bone marrow culture

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Icariin is a prenylated flavonol glycoside contained in the herb *Epimedium*, which has long been used to improve bone fracture healing or prevent osteoporosis because of the belief that the herb has bone-strengthening action. We have previously demonstrated that icariin enhances the osteogenic differentiation of rat bone marrow stromal cells, and partially explained the bone-strengthening mechanism of the herb. In the present study, the effect of icariin on osteoclastogenesis and bone resorption activity was investigated in mouse bone marrow culture. It was found that icariin dose-dependently inhibited the growth and differentiation of hemopoietic cells from which osteoclasts were formed. Far less $TRAP^+$ multinuclear cells appeared in the 10 μM icariin group than in the control. The bone resorption pits formed in the 10 μM icariin group was also significantly less than that of the control. RT-PCR analysis showed that the gene expression of TRAP, RANK and CTR was obviously lower than that of the control. It can be concluded that icariin has the ability to inhibit the formation and bone resorption activity of osteoclasts, which suggests that icariin should be the effective component for the bone-strengthening action of herb *Epimedium*.

1. Introduction

We have previously reported that icariin, a prenylated flavonol glycoside contained in the herb *Epimedium*, enhances the osteogenic differentiation of rat bone marrow stromal cells, which partially explain the bone-strengthening action of the herb (Chen et al. 2005). Since the bone-strengthening action can be achieved either by bone formation enhancement or by bone resorption inhibition, or by both of them as known from phytoestrogens (Dang et al. 2005), we tested the effect of icariin on osteoclastogenesis and bone-resorbing activity of osteoclasts in mouse bone marrow culture. The results indicated that icariin inhibited the formation and bone-resorbing action of osteoclasts in dose-dependent manner, providing further support for the belief that icariin is an effective component for the bone-strengthening action of *Epimedium* herb.

2. Investigations, results and discussion

To test the effect of icariin on the formation and activity of osteoclasts, *in vitro* osteoclastogenesis from mouse bone marrow was induced firstly. A single-cell suspension of bone marrow was prepared and planted on the glass coverslips or 30 μm-thickness bone slices in 24-well plates with the supplements of 10^{-8} M 1,25-$(OH)_2VD_3$, 25 ng/ml macrophage-colony stimulating factor (M-CSF) and 30 ng/ml receptor activator of NF-κB ligand (RANKL). The culture was observed every morning under the phase-contrast microscope and the osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining and bone resorption pits assay. There appeared many osteoclast-like cells after 6 days of culture (Fig. 1). TRAP staining showed the existence of typical osteoclasts. Associating with the formation of bone resorption pits after 9 days (Fig. 2), it can be concluded that osteoclastogenesis has been successfully induced in mouse bone marrow culture.

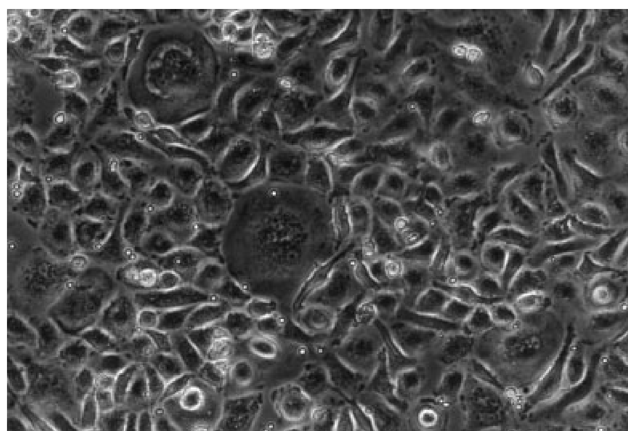

Fig. 1: The osteoclast-like cells under the phase-contrast microscope. A single cell suspension of mouse bone marrow was supplemented with M-CSF and RANKL and cultured in 24-well plates. There appeared osteoclast-like cells after 6 days of culture (magnification = 640 ×)

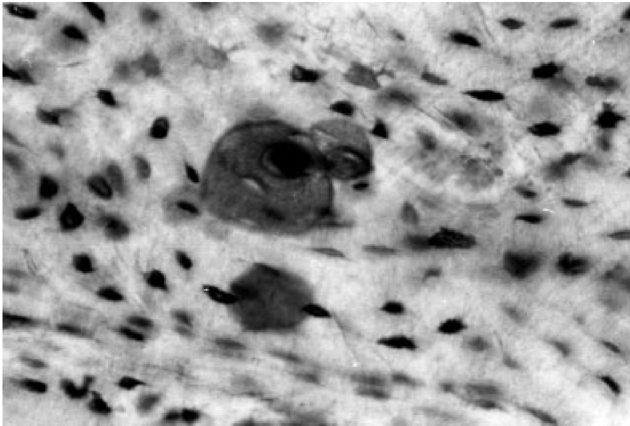

Fig. 2: The bone resorption pits formed after 12 days of culture. A single cell suspension of mouse bone marrow was supplemented with M-CSF and RANKL and planted on 30-μm bone slices in 24-well plates. The pits can be observed under the phase-contrast microscope stained by toluidine blue

Icariin was then added into the culture media at 0 (control), 1 μM, 5 μM and 10 μM at the beginning of culture (day 0). Estradial was also added at 0.1 μM and used as the positive control. The differences between the control and the icariin-supplemented groups, especially the 10 μM group, were found obvious after the first replacement of media on day 3. There were much more round hemopoietic cells in the control than in the 10 μM icariin group; the latter contained more MSCs-like cells (Fig. 3). Many osteoclast-like cells appeared in the control on day 6, while few multinuclear cells could be found in the 10 μM icariin group (Fig. 4). With the continued culture, the osteoclasts with three nuclei or more were formed, their number in the control were significantly higher than those of the icariin-supplemented groups (Fig. 5). The results of bone resorption pits assay were consistent with the morphological observation and TRAP staining (Fig. 6).

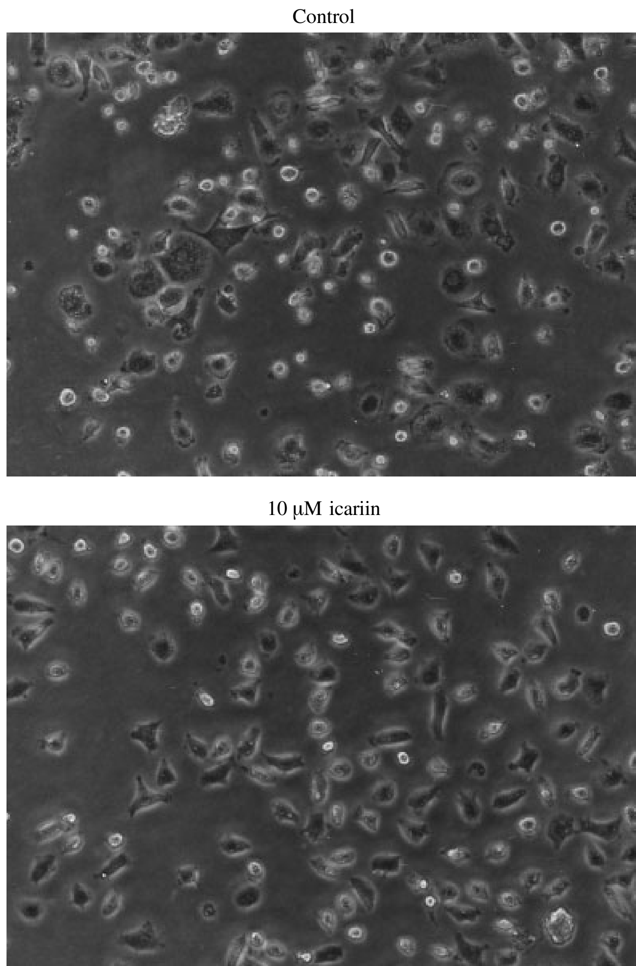

Fig. 3: The mouse bone marrow cells under the phase-contrast microscope after the first change of medium on day 3. There are obviously more round hemopoietic cells in the control than in the 10 μM icariin group. The latter contains more spindle-like cells (magnification = 400×)

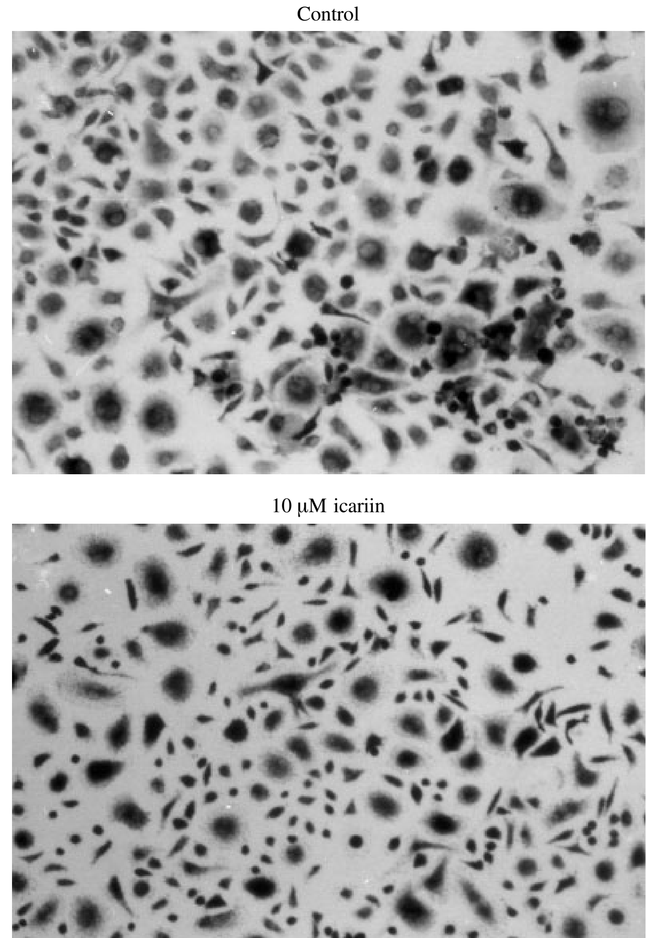

Fig. 4: The osteoclasts identified by TRAP staining. There are more multinuclear cells stained positive for TRAP in the control than in the 10 μM icariin group (magnification = 400×)

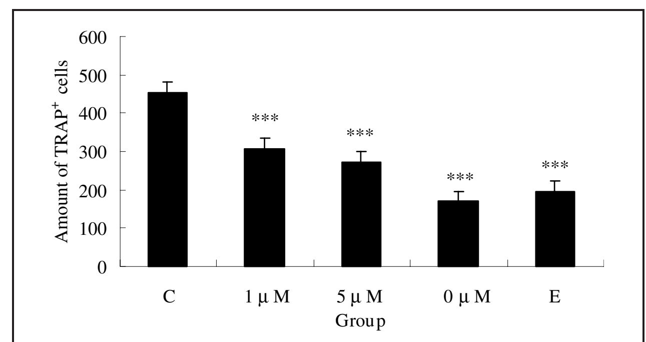

Fig. 5: The amount of TRAP-stained positive cells in the control and icariin-supplemented groups after 9 days of culture. C refers to the control, E represents 0.1 μM estradial-supplemented group. *** $P < 0.001$ vs control

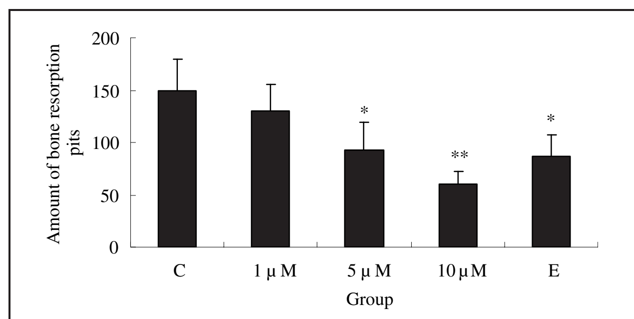

Fig. 6: The amount of bone resorption pits formed in the control and icariin-supplemented group after 12 days of culture. C refers to the control, E represents 0.1 μM estradial-supplemented group. * $P < 0.05$, ** $P < 0.01$ vs control

The gene expression of marker proteins for osteoclasts was also compared by RT-PCR between the 10 μM icariin group and the control (Blair et al. 2004; Kudo et al. 2002). The mouse bone marrow suspension was cultured in 100-mm dish this time without coverslip or bone slice in order to obtain enough Total RNA for reverse transcription. As a result, the gene expression levels including TRAP, RANK (receptor activator of NF-κB) and CTR (calcitonin receptors) were all found much lower in the 10 μM icariin group than in the control (Fig. 7). However, there was no obvious difference between the two groups for the expression of GAPDH, which was used as internal control.

The osteoclastogenesis induced by M-CSF and RANKL from hemopoietic precursor cells of bone marrow has been extensively applied to study the origin of osteoclasts or to evaluate anti-resorption drugs (Duong et al. 2001; Sakiyama et al. 2001). When icariin was added into the culture, the hemopoietic cells were reduced compared to the control, and the remaining hemopoietic cells seemed to be inhibited in growth and differentiation. This phenomenon was noticed in previous studies (Chen et al. 2005), but could not be found in the estrodial-supplemented group, although 0.1 μM estrodial also reduced the bone resorption pits significantly (Fig. 5). However, it is not sure that the bone resorption inhibition at late stage was the natural result of early inhibition on hemopoietic cells by icariin. In fact, we found that icariin inhibited the resorption activity of mature osteoclasts isolated from the femurs of newborn rabbits (Fig. 8).

We attributed the bone-strengthening action of *Epimedium* herb to the flavonoids contained in the herb, and further

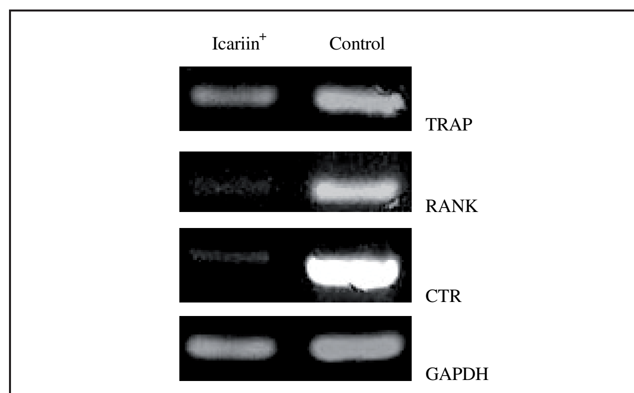

Fig. 7: Comparison of marker gene expression of osteoclasts by RT-PCR between the control and the 10 μM icariin group. GAPDH was used as internal control

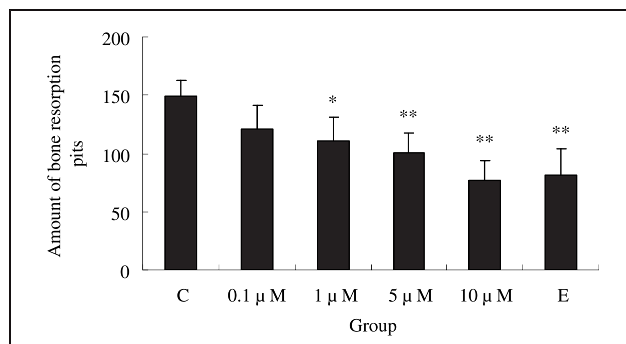

Fig. 8: The amount of bone resorption pits formed in the culture of rabbit mature osteoclasts on day 7. C refers to the control, E represents 0.1 μM estradial-supplemented group. * $P < 0.05$, ** $P < 0.01$ vs control

traced to icariin (Chen et al. 2003, 2005). Now we can conclude that icariin should be an effective component with bone-strengthening activity, as it owns the abilities to inhibit the formation and bone resorption activity of osteoclasts and enhance the osteogenic differentiation of marrow mysenchymal stem cells.

3. Experimental

3.1. Reagents

Soluble human recombinant RANKL was obtained from Alexis Biochemical (Australia, ALX-522-012). Mouse recombinant M-CSF (M9170), $1,25(OH)_2VD_3$ (D1530) and estrodial (E2758) were the products of Sigma. α-MEM was supplied by Gibco, Invitrogen Corporation. Fetal bovine serum (FBS) was produced by Lanzhou National HyClone Bio-Engineer Co., LTD (China). Icariin was isolated in our lab and the purity was ≥96%.

3.2. Cell culture

Femora and tibiae were obtained from 4-week-old BABL/c mice and the connective soft tissues were removed from the bones. Bone marrow cells were flushed out from the bone marrow cavity, suspended in α-MEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The suspension was adjusted to 10^7 cells/ml and supplemented with 10^{-8} M $1,25\text{-}(OH)_2VD_3$, 25 ng/ml M-CSF and 30 ng/ml RANKL, and then planted on the aseptic glass coverslips or 30 μm-thickness bone slices in 24-well plates at 0.5 ml/well. The bone slices were prepared by a low-speed diamond saw (Leica SP1600) from bovine cortical bone and sterilized in 10 × antibiotic (1000 units/ml penicillin and 1000 μg/ml streptomycin in PBS). Icariin was dissolved into DMSO and added into the medium at 1 μM, 5 μM and 10 μM. The final concentration of DMSO was less than 0.05% (Zhang et al. 2000). Estradial was separately added into the medium at 0.1 μM and used as positive control (Michael et al. 2005). All solutions were incubated at 37 ° in 5% humidified CO_2. After 72 h, half of the medium was replaced with fresh medium; thereafter the medium was changed every 3 days in half.

3.3. TRAP stainging

Four glass coverslips in every group were taken out on day 3, 5, 7 and 9, fixed in 10% formalin at least 10 min, and then stained for TRAP activity with a leukocyte acid phosphatase kit (Sigma 387A) according to the manufacturer's instruction. The multinucleate cells with more than three nuclei were considered to be osteoclastic cells.

3.4. Pit formation assay

Bone resorption pits were assayed on day 9 or 12. 4 slices of every group were fixed in 2.5% glutaraldehyde for 7 min, washed with ultrasonication in 0.25 M ammonium hydroxide three times to remove attached cells and subsequently stained with toluidine blue (0.1%, w/v). The number of pits formed on the slices was determined under a light microscope with 100 × magnification.

3.5. RT-PCR analysis

Total cellular RNA was extracted from the marrow cells cultured in 100-mm plates on day 6, 9 and 12 with TaKaRa RNAiso Regent (TaKaRa Biotechnology Co., Ltd China). The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm. 2.5 μg

RNA was reversely transcribed to cDNA using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Aliquots of the cDNA were then amplified in 50 ml of a PCR reaction mixture which contained 20 pmol primer sets, 1 × PCR buffer, 0.2 mM dNTP mix and 1 U Taq DNA polymerase (all provided by TaKaRa). Primers for TRAP (sense: 5′-CAGCCCTTACTACCGTTT C-3′, antisense: 5′-GTTCTCGTCCTGAAGA-TACTGC-3′), RANK (sense: 5′-GGCTGCACACTGGAACT-′3′, antisense: 5′-TCCTGTAGTAAACGCCGAAG A-3′), CTR (sense: 5′-TACCGAC-GAGCAACGCCTAC-3′, antisense: 5′-ACAGCCCGAGGAGCACAAC-3′) and GAPDH (sense: 5′-CGGTGCTGAGTATGTCGT-3′, antisense: 5′-CTTCTGGGTGGCAGTGAT-3′) were prepared based on published DNA sequences (Gene bank accession numbers BC029644, AF019046, NM007588 and BC083080 respectively). GAPDH was used as internal control. DNA amplification included an initial denaturation at 95 °C for 5 min, followed by 30 (TRAP and GAPDH), 35 (RANK and CTR) cycles of denaturation at 94 °C for 45 S, annealing at 56 °C for 45 S, and extension at 72 °C for 1 min. The final cycle included 7 min for extension. The PCR products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. The 100 bp DNA Ladder (Takara) was used as a molecular weight marker.

3.6. Satastical analysis

Values are presented as mean ± S.D. The analysis was conducted by Dunnett's multiple test with SPSS10.0 software.

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