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Icariin, a flavonoid from the herb *Epimedium* enhances the osteogenic differentiation of rat primary bone marrow stromal cells

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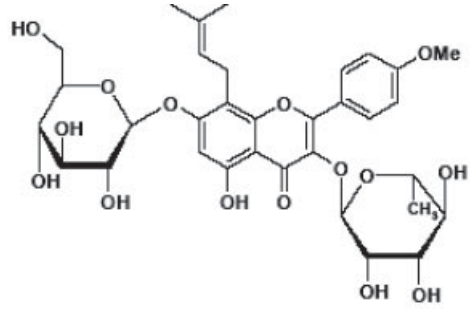
The herb *Epimedium* has long been used in Traditional Chinese Medicine to treat bone fracture and prevent osteoporosis. Researchers believe that the flavonoids contained in the herb are the effective component for this activity. However, no single flavonoid has been studied for its effect on bone-related cells. In the present study, icariin, one of the major flavonoids of the herb, supplemented the primary culture medium of rat bone marrow stromal cells (rMSCs) at 0.1 μM , 1 μM and 10 μM respectively. It was found that icariin stimulated the proliferation of rMSCs and increased the number of CFU-F stained positive for alkaline phosphatase in a dose-dependent manner. Icariin also dose-dependently increased the alkaline phosphatase activity, osteoalcin secretion and calcium deposition level of rMSCs during osteogenic induction. The addition of 10 μM icariin caused four times more mineralized bone nodules to be formed by rMSCs than in the control. The results demonstrated that icariin should be an effective component for bone-strengthening activity, and one of the mechanisms is to stimulate the proliferation and enhance the osteogenic differentiation of MSCs.

1. Introduction

The herb of the genus *Epimedium* has long been used in Traditional Chinese Medicine to treat bone fractures and prevent osteoporosis, as it is believed that the herb can strengthen bones and tendons. The practice has now been partially identified as rational as a result of phytochemical extraction and animal experiments in rats (Li et al. 1996; Ji et al. 2001; Ma et al. 2002). Their work revealed that the flavonoids contained in the herb are highly related to its therapeutic effects. We have previously reported that the serum of rats administered a total flavonoid extract of *Epimedium sagittatum* enhances the development of rat calvarial osteoblasts while the extract itself had no appreciable effect (Chen et al. 2004). In the present study, however, we found that icariin, a prenylated flavonol glycoside contained in the herb, strongly promotes the osteogenic differentiation of rat marrow stromal cells (rMSCs) in primary culture.



2. Investigations, results and discussion

A single-cell suspension of whole bone marrow from tibias and femurs of a rat were divided into 4 equal parts and plated into 6-well plates in MEM medium supplemented with icariin at 0 (Control), 0.1 μM, 1 μM and 10 μM respectively. After 3 days, the erythrocytes and unattached cells were removed by two washes with PBS and the adherent cells were observed under the phase contrast microscope. The adherent cells in the icariin-supplemented groups were mostly the spindle-shaped MSCs, while in the control they were mixed with many hemopoietic cells (Fig. 1). With continued culture, MSCs expanded quickly and became predominant in all cultures. The cell numbers in each treatment were counted over the course of time. There were obviously more cells in the 1 μM and 10 μM icariin-supplemented groups than in the control group from day 6, indicating the stimulating effect of icariin on rMSC proliferation (Fig. 2).

The colonies stained positive for alkaline phosphatase (CFU-F_{ALP}) were an early marker of osteogenic differentiation of MSCs. It was found that there was more CFU-F_{ALP} in the icariin-supplemented cultures than in the control, especially in the highest concentration group (Fig. 3). To further study the effect of icariin on osteogenic differentiation, osteogenic supplements (10^{-8} M dexamethasone, 100 mM AsAP and 10 mM β-glycerophosphate) were added to the medium beginning from day 9 (day 0 for the induction of osteogenic differentiation). The intracellular alkaline phosphatase (ALP) activity and other osteogenic differentiation markers were measured every 4 days. As a result, it was found that icariin increased intracellular ALP activity in a

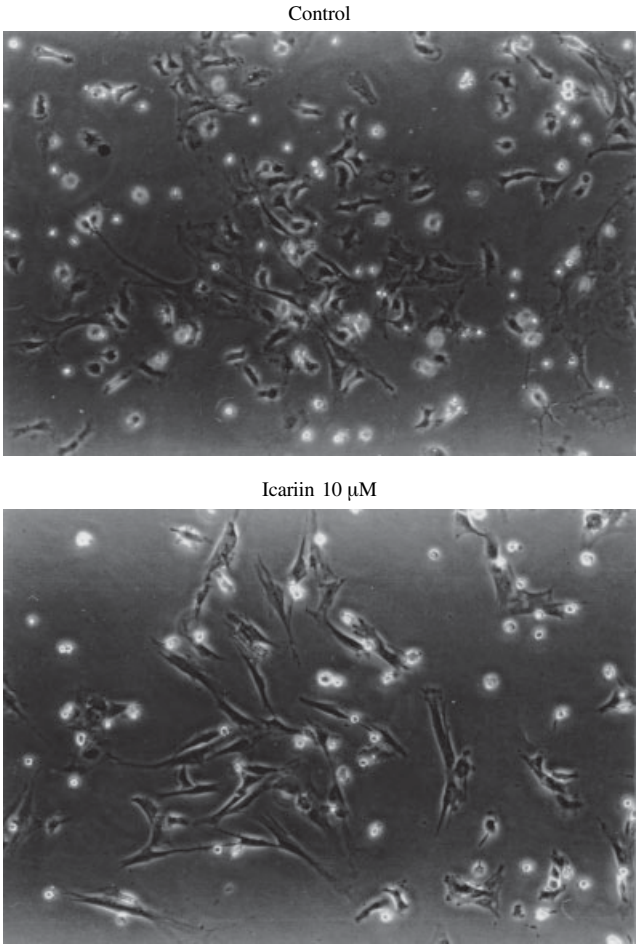


Fig. 1: The adherent cells under phase contrast microscope after the first change of medium on day 3. The cells are mostly spindle-shaped MSCs in 10 μM-icariin supplemented group, while they are mixed with many hemopoietic cells in the control (Manification = 400×)

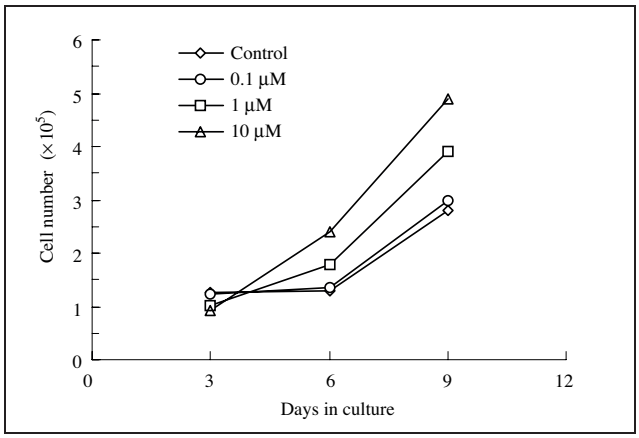


Fig. 2: Cell number growth of rat bone marrow stromal cells (rMSCs) in primary culture supplemented with different concentrations of icariin. The cells were released by treatment with 0.25% trypsin and 1 mM EDTA in PBS and counted using a hemocytometer. Results are the average of triplicate cultures

dose-dependent manner. The activity the in 10 μM icariin-supplemented group was 5 times higher than that in the control group on day 12 (Fig. 4). Icariin also improved osteocalcin secretion and the calcium deposition level in a dose-dependent manner (Figs. 5, 6). The mineralized bone nodules were first formed in the 10 μM icariin-supplemented group, and their total number on day 16 was about 4 times more than that in the control group (Fig. 7).

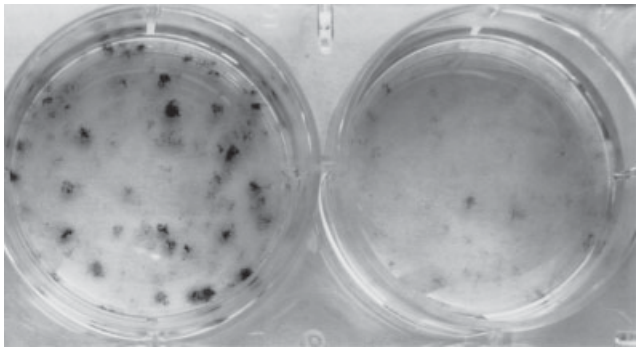


Fig. 3: Colonies stained positive for alkaline phosphatase (CFU-F_{ALP}) formed by rat primary bone marrow stromal cells. There are obviously more colonies in the 10 μM-icariin supplemented group (left) than in the control (right)

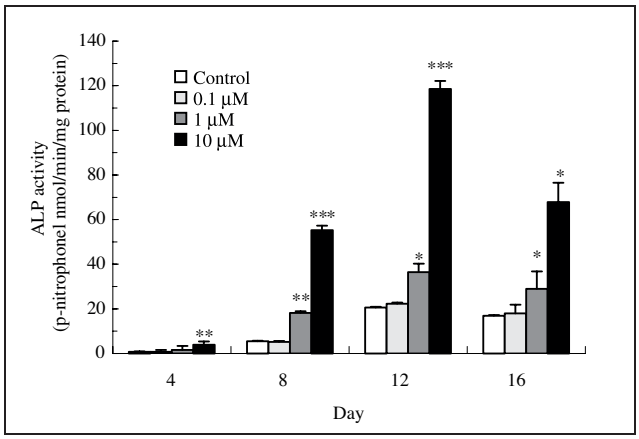


Fig. 4: Times course of intracellular alkaline phosphatase activity of rat primary bone marrow stromal cells during osteogenic induction supplemented with different concentrations of icariin. Results are the mean of triplicate cultures. Standard deviation (S.D.) of mean is shown by vertical bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control

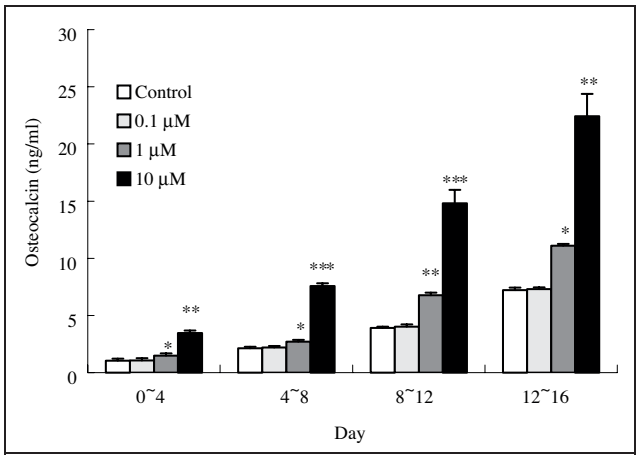


Fig. 5: Time course of osteocalcin concentration in culture medium of rat primary bone marrow stromal cells during osteogenic induction supplemented with different concentrations of icariin. Results are the mean of triplicate cultures. Standard deviation (S.D.) of mean is shown by vertical bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control

Icariin is a major flavonoid of the herb *Epimedium* that has been extensively studied. It has been reported that icariin has antihepatoxic, immunoenhancing and neurite outgrowth activity as well as being a cGMP-specific PDE5 inhibitor that might develop into an agent for the treatment of erection dysfunction (Kuroda et al. 2000; Lee et al. 1995; Liang et al. 1995; Xin et al. 2003). However,

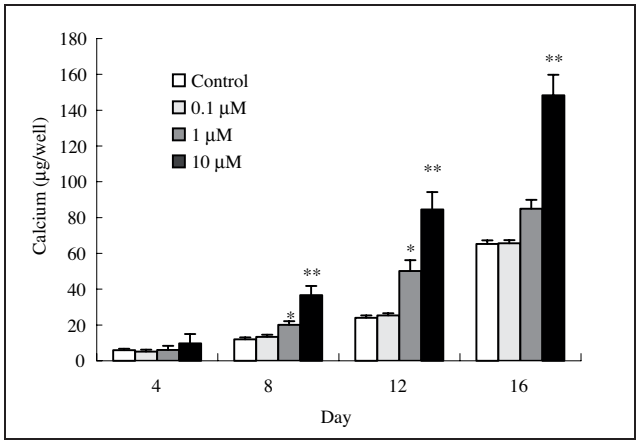


Fig. 6: Time course of calcium deposition in rat primary bone marrow stromal cells during osteogenic induction supplemented with different concentrations of icariin. Results are the mean of triplicate cultures. Standard deviation (S.D.) of mean is shown by vertical bars. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control

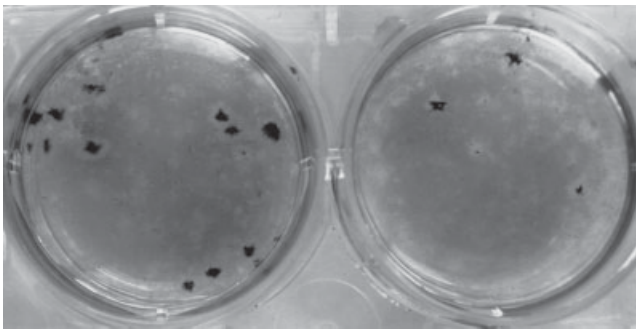


Fig. 7: Mineralized bone nodules formed by rat primary bone marrow stromal cells on day 16 during osterogenic induction. There are about 4 times more nodules in the 10 μM-icariin supplemented group (left) than in the control (right)

its effect on bone-related cells has rarely been studied. Since MSCs are well known to be the precursor of osteoblasts and their osteogenic differentiation is a key factor for bone formation (Aubin 1997), we were able to study the effect of icariin on the proliferation and osteogenic differentiation of MSCs *in vitro*. The addition of icariin seemed to reduce the attachment of hemopoietic cells to the culture dish and stimulate the proliferation of MSCs in a dose-dependent manner. The increased alkaline phosphatase activity and other osteogenic differentiation markers indicated that icariin also enhances the osteogenic differentiation of MSCs. These results demonstrated that icariin should be an effective component for the bone-strengthening activity of the herb *Epimedium*, and one of the possible mechanisms for this activity is to stimulate the proliferation and enhance the osteogenic differentiation of MSCs.

3. Experimental

3.1. Preparation and purification of icariin

The dried aerial parts of *Epimedium saggitattum* were crushed and soaked in ten times weight of distilled water. The water was twice boiled for 1.5 h to obtain an aqueous extract. The aqueous extracts were filtered and loaded onto a macroporous polymer-packed column for hydrophobic chromatography. Ethanol (60% in water) was used to obtain an icariin-containing eluate. After concentration, the preparation was further processed by a second chromatography on a polyamide resin column. A 1 : 1 mixture of methanol and ethanol was used to obtain the eluate, which was then evaporated and crystallized. The final product contained more than 96% icariin, according to analysis by HPLC.

3.2. Cell culture

A male or female Wistar rat weighing about 150 g was sacrificed by dislocation of the cervical spine. Its tibias and femurs were immediately dissected from the attached muscles and tissues with an aseptic technique. The ends of the bones were removed, and marrow plugs were flushed out from one end of the bony tube by injection of MEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). The plugs were dispersed by repeated pipetting and forcefully drawn through 8- and 4-gauge needles successively in order to obtain a single cell suspension (Chen et al. 2003). The suspension was adjusted to 10^7 cells/ml and divided into 4 equal parts. Icariin was added to 3 of them at 0.1 μM, 1 μM and 10 μM respectively. The final concentration of DMSO as the solvent of icariin was less than 0.05%, a concentration that did not interfere with the test system (Zhang et al. 2000). One part had no icariin added and was used as a control. Each suspension was plated out in 6-well culture plates at 1.5 ml/well in triplicate and incubated at 37 °C in 5% humidified CO_2. After 72 h, the medium was removed and all wells were washed two times with PBS. Thereafter the cell proliferation and osteogenic differentiation were assayed one after another.

3.3. Cell proliferation and CFU-F_{ALP} assay

The adherent cells in each group were released by treatment with 0.25% trypsin and 1 mM EDTA in PBS (pH 7.8) and cell numbers were determined using a hemocytometer on days 3, 6 and 9 respectively. To analyze for MSC colonies stained positive for alkaline phosphatase (CFU-F_{ALP}), the cells were first fixed in 3.7% formaldehyde-90% ethanol solution for 5 min, and then stained histochemically by placing in the incubation solution (20 ml Michalis buffer, pH 8.9, containing 10 mg 1-naphthyl phosphate sodium and 10 mg fast blue RR salt) for 15 ∼ 20 min at 37 °C.

3.4. Osteogenic differentiation assay

Beginning from day 9, the primary cultures of MSCs were changed to a medium supplemented with 10^{-8} M dexamethasone, 100 mM AsAP and 10 mM β-glycerophosphate (day 0 for the induction of osteogenic differentiation). The medium was changed every 4 days until day 16. The following osteogenic differentiation markers were assayed every time when the medium was changed.

3.4.1. Cellular alkaline phosphatase activity

Cells were rinsed twice with PBS and then lysed in 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$ and 0.1% Triton X-100 at 4 °C. Subsequently, cells were sonicated for 1 min and centrifuged at 10000 × g. ALP activity was measured as the rate of conversion of *p*-nitrophenyl-phosphate using Sigma kit 104-LL as specified by the manufacturer. Total protein was then measured using a BCA kit (Pierce). The enzyme activity was expressed as nmol *p*-nitrophenol/15min/mg protein. (Heim et al. 2003)

3.4.2. Osteocalcin

The amount of osteocalcin secreted into the culture medium was determined by immunoradiometric assay (IRMA) using a rat osteocalcin kit (Immutopics, Inc.San Clemente, CA 92673 USA). 500 μl of culture medium were harvested when it was replaced by fresh medium and stored at −30 °C until the assay. The amount of osteocalcin accumulated during every 4-day culture was measured.

3.4.3. Calcium and mineralized nodules

Both the calcium deposition level and the mineralized nodules were assayed as previously described (Chen et al. 2004). The former was determined using Sigma kit 587-A, and the latter on day 16 by staining by the von Kossa method.

3.5. Statistics

Values are presented as mean ±S.D. Statistical analyses were performed by Student's t-test.

References

Aubin JE (1998) Bone stem cells. J Cell Biochem 31 (suppl): 73–82.

Chen KM, Ge BF, Ma HP et al. (2004) The serum of rats administered flavonoid extract from *Epimedium sagittatum* but not the extract itself enhances the development of rat calvarial osteoblast-like cells *in vitro*. Pharmazie 59: 61–64.

Chen KM, Ge BF, Liu XY et al. (2003) Directed differentiation of rat bone marrow stromal cells into osteoblast *in vitro*. J Lanzhou University (Natural Sci) 39: 69–73.

Heim M, Frank O, Kampman G et al. (2004) The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. Endocrinology 145: 848–59.

Ji H, Liu K, Gong, XJ et al. (2001) Effects of *Epimedium koreanum* flavoids on osteoporosis in ovariectomized rats. Chin J Osteoporos 7: 4–8.

Kuroda, Mi, Mimaki Y, Sashida Y et al. (2000) Flavonol glycosides from *Epimedium sagittatum* and their neurite outgrowth activity on PC12h cells. Planta Med 66: 575–577.

Lee MK, Choi YJ, Sung SH et al. (1995) Antihepatotoxic activity of icariin, a major constituent of *Epimedium koreanum*. Planta Med 61: 523–526.

Li QN, Liao J, Wu T et al. (1996) *Epimedium sagittatum* Maxim preventing hormone-induced osteoporosis. Chin Pharm J 31: 467–470.

Liang, HR, Vuorela P, Vuorela H et al. (1997) Isolation and immunomodulatory effect of flavonol glycosides from *Epimedium hunanense*. Planta Med 63: 316–319.

Ma HP, Jia ZP, Bai MH et al. (2002) Studies on the therapeutic effect of total flavonoids of Herba Epimedii on experimental osteoporosis in rats. West China J Pharm Sci 17: 163–167.

Xin ZC, Kim EK, Lin CS et al. (2003) Effects of icariin on cGMP-specific PDE5 and cAMP-specific PDE4 activities. Asian J Androl 5: 15–18.

Zhang YW, Morita I, Shao G et al. (2000) Screening of anti-hypoxia/reoxygenation agents by an *in vitro* method. Part 2: Inhibition of tyrosine kinase activation prevented hypoxia/reoxygenation-induced injury in endothelial gap junctional intercellular communication. Planta Med 66: 119–23.