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Icariin enhances the osteogenic differentiation of bone marrow stromal cells but has no effects on the differentiation of newborn calvarial osteoblasts of rats

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Since the total flavonoid extract (TFE) of *Epimedium* herb was found to prevent osteoporosis induced by ovariectomy in rats, we have been attempting to identify the exact compound responsible for the bone-strengthening activity. In this experiment, four flavonoid extracts were obtained from *Epimedium sagittatum* (Siebold & Zucc.) Maxim, which contained 25.3%, 51.2%, 82.3% and 99.2% icariin respectively. They were separately supplemented into the culture media of newborn rat calvarial osteoblasts (ROB) or primary rat bone marrow stroma cells (rMSCs) at 0.1, 1, 10 and 100 μg/ml respectively, in order to observe their effects on the cells. Not any appreciable effect was found on the differentiation of ROB, but an enhancing effect on the osteogenic differentiation of rMSCs was found, and the enhancing degree was icariin-dependent, that is, a higher concentration of icariin in the extract caused more mineralized bone nodules and higher calcium deposition levels. The gene expressions involved in osteogenesis were also improved which was revealed by RT-PCR, including alkaline phosphatase, bone matrix protein (osteocalcin, osteopontin, bone sialoprotein) and cytokines (TGF-β_1 and IGF-I). The effect of icariin on cell proliferation was assayed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Icariin inhibited the proliferation of rMSCs and ROB when its concentration was higher than 10^{-5} μM (6.7 μg/ml), no stimulative effect was found. The above results indicated that icariin may exert bone-strengthening activity by enhancing the osteogenic differentiation of MSCs, which partially explains the anti-osteoporosis action of *Epimedium* herb.

1. Introduction

Icariin is a flavonoid glycoside contained in *Herba Epimedii* and usually used as standard substance in quality evaluation because it is highly related to various pharmacological actions of the herb (Kuroda et al. 2000; Lee et al. 1995; Liang et al. 1997; Xin et al. 2003). Since the total flavonoid extract (TFE) of the herb was found to prevent osteoporosis induced by ovariectomy or retinoid acid in rats (Ji et al. 2001; Ma et al. 2002), we have been attempting to identify the exact compound responsible for the bone-strengthening activity. Our attention naturally focused on icariin and its effects on osteoblasts first, but no positive effect of TFE or further purified extracts containing higher concentration of icariin was found on the differentiation of newborn rat calvarial osteoblasts (ROB) *in vitro* until the serum of rats receiving TFE was used, indicating that metabolites but not icariin itself was effective on osteoblasts (Chen et al. 2004). However, when we latter turned to marrow stromal cells (MSCs), the precursor of osteoblasts in bone marrow, obviously positive results were observed. In this experiment the flavonoid extracts were found to enhance the osteogenic differentiation of rat MSCs (rMSCs) in an icariin-dependent manner, but no such effects were found on ROB.

2. Investigations and results

The total flavonoid extract of *Epimedium sagittatum* (Siebold & Zucc.) Maxim (E_1) was first obtained from the aqueous extract by passing through a macroporous polymer-packed column. It was then analyzed by HPLC. Icariin was found to be the richest and the concentration got to 25.3% (Fig. 1 E_1). E_1 was recrystallized by 60% ethanol for two times and the icariin concentration increased to 51.2% (Fig. 1 E_2). When E_1 was purified by passing through a polyamide resin-packed column, the icariin concentration increased to 82.3% (Fig. 1 E_3). The extract with 99.2% icariin (E_4) was obtained after E_3 was recrystallized three times (Fig. 1 E_4).

The above extracts were separately supplemented into the culture media of ROB at 0.1, 1, 10 and 100 μg/ml respectively, in order to observe their effects on the development of osteoblasts. Neither a stimulative effect on cell proliferation nor an improvement of differentiation and mineralization was found. However, when these extracts were

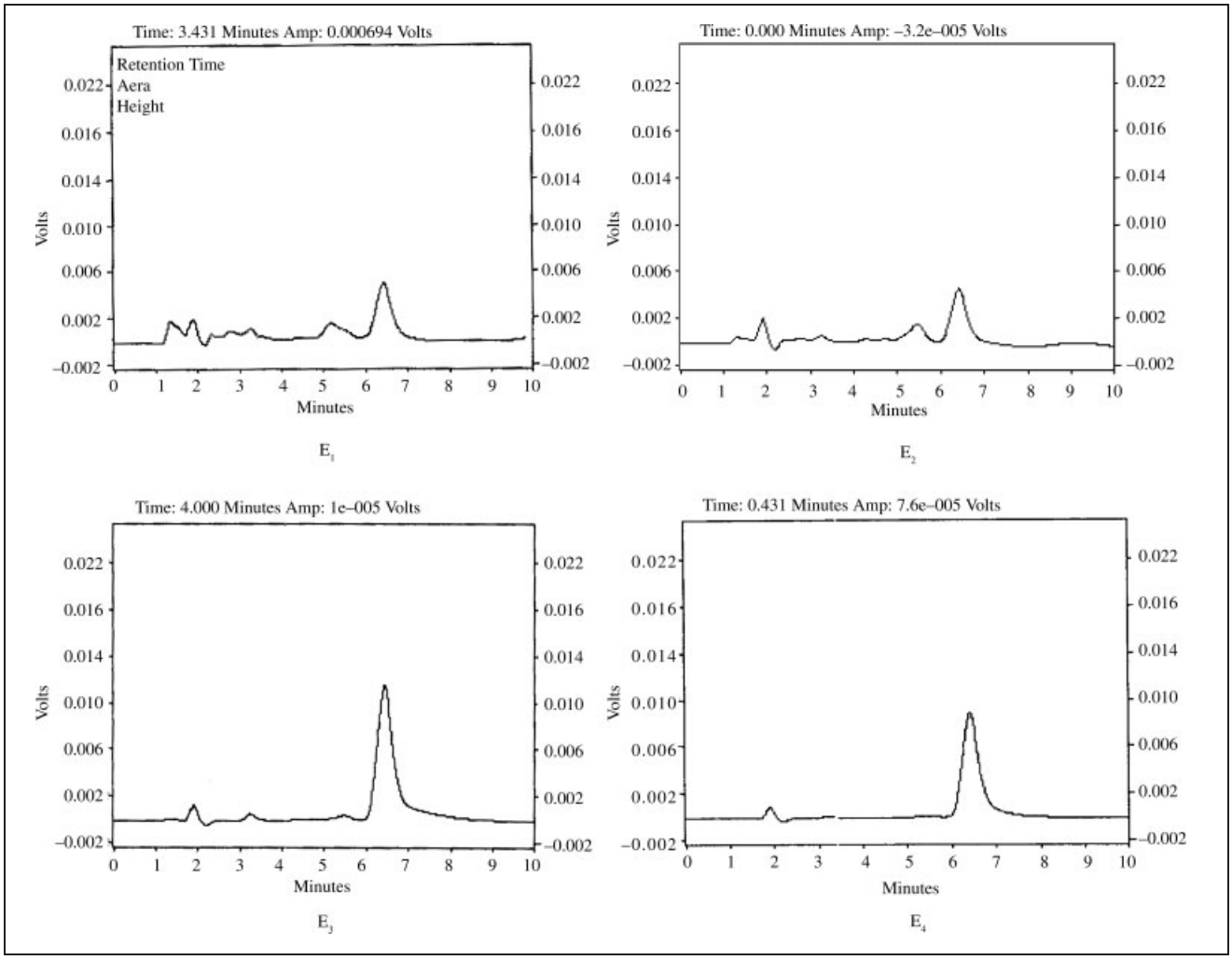

Fig. 1: HPLC analysis results of E_1, E_2, E_3 and E_4, they contained 25.3%, 51.2%, 82.3% and 99.2% icariin respectively

supplemented into the culture media of primary rMSC at the same concentrations, the enhancing effect on osteogenic differentiation was observed in some of them, including 10 μg/ml E_2, 1∼10 μg/ml E_3 and 1∼10 μg/ml E_4, demonstrated by improved cellular ALP activity and osteocalcin secrection as well as the number of colonies stained positive for ALP and the mineralized bone nodules.

To compare their activity, the four extracts were tested in the same culture and in the same concentration. A single-cell bone marrow suspension prepared from one rat was divided into 5 parts and supplemented with 10 μg/ml E_1, E_2, E_3 and E_4 respectively, one part was used as control and no extract was added. They were planted into the wells in triplicate in 6-well plates. After 12 days of osteogenic induction culture, half plates were stained with the von Kossa method. The left plates were used to determine the calcium deposition level after 16 days.

The von Kossa staining results are shown in Fig. 2. The amount of mineralized bone nodules (von Kossa staining-positive spot) in each group was $E_4 > E_3 > E_2 > E_1 \approx$ control, indicating an icariin-dependent manner. That is, the higher the concentration of icariin, the more mineralized bone nodules were formed. The calcium deposition level was consistent with the amount of mineralized bone nodules (Fig. 3).

To further identify the role that icariin played in enhancing osteogenic differentiation of primary rMSCs, the expression of bone-related genes was compared between the 10 μg/ml-E_4-supplemented group and the control by RT-PCR. As a result, the gene expression of ALP was found to be obviously stronger in the E_4 group than in the control after 4 day and 8 day culture (Fig. 4). The genes of bone matrix proteins, usually used as marker proteins for bone formation including osteocalcin, osteopontin and bone sialoprotein, were all improved to different degrees. The genes for TGF-β_1 and IGF-I, which are active and important cytokines in bone formation, were also found to be expressed at a higher level in E_4 group than in the control.

Basing on the above results, it can be concluded that icariin might be responsible for enhancing the osteogenic differentiation of rMSCs. The effect of icariin on cell proliferation was then investigated. E_4 was supplemented into the culture media of primary rMSCs at 0.01, 0.1, 1, 10 and 100 μM respectively. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method was used to analyze cell proliferation. No stimulative effect was found on the proliferation of rMSCs (Fig. 5). When the concentration was higher than 10 μM (6.7 μg/ml), icariin inhibited cell proliferation in a dose-dependent manner. The effect of icariin on ROB had the same tendency with rMSCs.

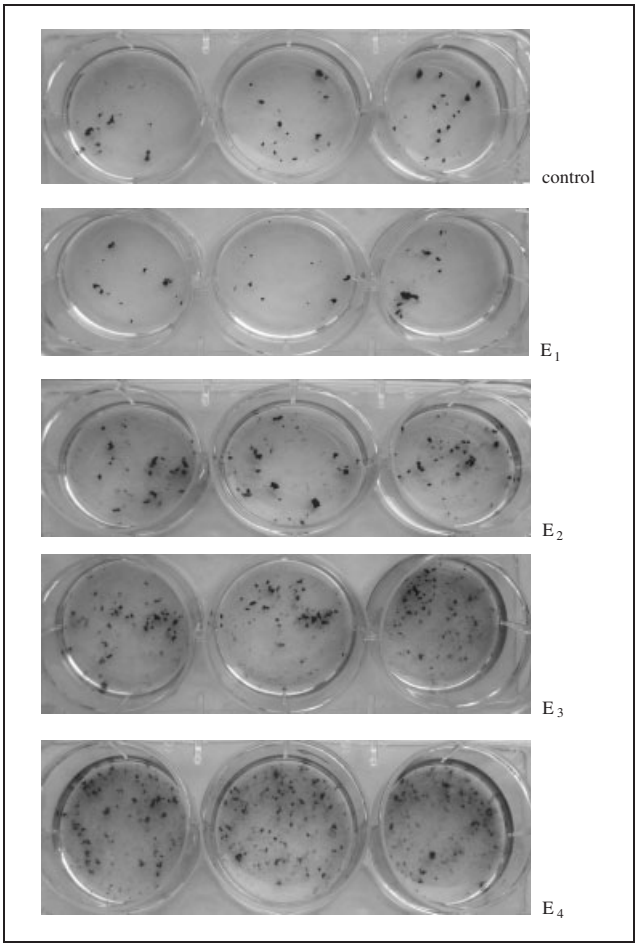

Fig. 2: The mineralized bone nodules stained by von Kossa method. The primary rMSCs planted in 6-well plates was cultured for 12 days under osteogenic induction. The extracts (E_1, E_2, E_3 and E_4) were supplemented into the culture media at 10 μg/ml from the beginning of culture

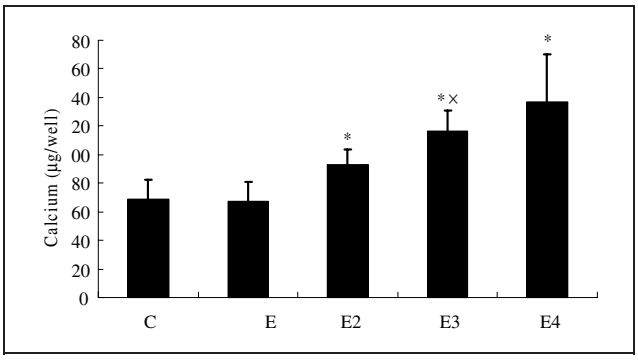

Fig. 3: The calcium deposition in primary culture of rMSCs after 16 days of osteogenic induction. The extracts (E_1, E_2, E_3 and E_4) were supplemented into the culture media at 10 μg/ml from the beginning of culture. Results are the mean of triplicate culture. Standard deviation of mean is shown by vertical bars. $^{*}P < 0.05$ vs. control, $\times P < 0.05$ vs. E_2.

3. Discussion

There are several research groups in China who have been engaged in the study of icariin for its enhancing or improving activity on osteoblasts (Liu et al. 2001; Wang et al. 2005; Xue et al. 2005; Yin et al. 2005), however, no strong persuasive experimental results can be provided until now. We have established an *in vitro* culture of newborn rat calvarial osteoblasts with good repeat of differentiation and mineralization (Fig. 6), with which we have found several compounds with stimulating activity, but none of them was from *Epimedium* herbs.

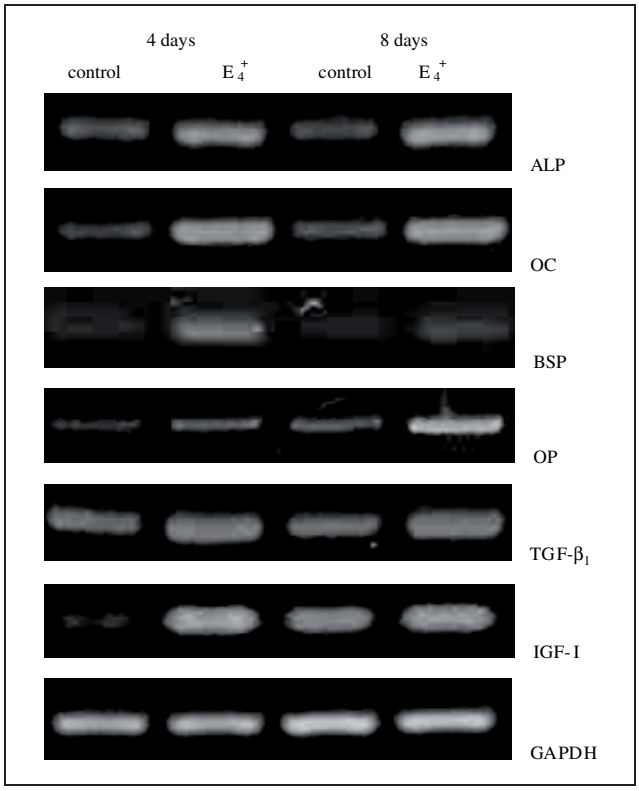

Fig. 4: Comparison of the bone-related gene expression of rMSCs after 4 day and 8 day osteogenic induction. The expression of genes for alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OP), bone sialoprotein (BSP), TGF-β_1 and IGF-I were revealed by RT-PCR

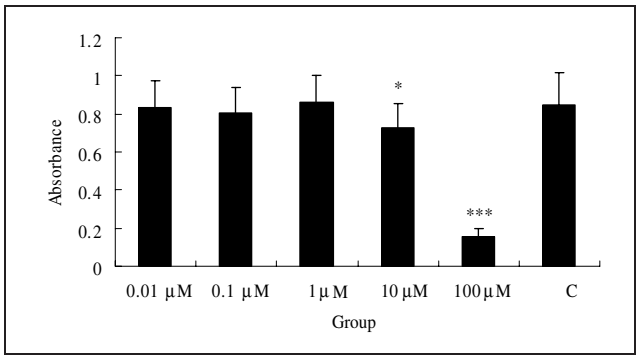

Fig. 5: Effect of icariin on the proliferation of rMSCs at different concentration. The proliferation was assayed by MTT mrthod and expressed by the absorbance value at 570 nm. The results are the mean value of six parallel cultures. Standard deviation of mean is shown by vertical bars. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control

Marrow stromal cells, also named mysenchymal stem cells (MSCs), are the precursor cells of osteoblasts in bone marrow as well as of adipocytes and other several cell lines. It is reported that a reciprocal relationship exists between the differentiation of MSCs into osteoblasts and adipocytes, the bone volume loss associated with osteoporosis is accompanied by reduced osteoblastic bone formation and increased marrow adipose tissue. Therefore MSCs can be taken as the target for prevention and treatment of osteoporosis (D'Ippolito et al. 1999; Rodriguez et al. 1999; Rodriguez et al. 2000, 2004; Hess et al. 2005; Liu et al. 2005). Our results indicated that icariin may im-

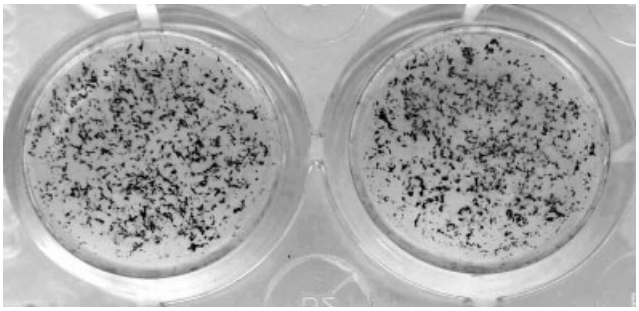

Fig. 6: Bone mineralized nodules formed by ROB. The cells were plated in 24-well plates in duplicate. When they grew to near confluence, the media with osteogenic supplements were changed. The nodules were stained by von Kossa method after 12 days

prove bone formation by enhancing the differentiation of MSCs into osteoblasts and thus exert a preventive effect on osteoporosis.

In general, our findings about the effects of icariin on MSCs provide a possible mechanism for the bone-strengthening action of *Epimedium* herb, and demonstrate that the original form of icariin is active, whereas its metabolites have been found to be active as well.

4. Experimental

4.1. Plant material

The dried aerial parts of *Epimedium sagittatum* (Siebold & Zucc.) Maxim was purchased from Lanzhou Pharmaceutical Company Ltd., Gansu, China, which was collected in Bozhou, Anhui, China. The same herb was authenticated by Associate Professor Xun Pu of Botany Department, School of Life Sciences, Lanzhou University.

4.2. Preparation of flavonoid extracts

The dried aerial parts of *Epimedium saggitattum* (Siebold & Zucc.) Maxim were crushed and soaked in ten times their weight of distilled water, boiled 1.5 h for two times. The aqueous extracts were filtered and then loaded onto a macroporous polymer-packed column for hydrophobic chromatography. Ethanol (60%) was used to elute the column. E_1 was obtained from the elute after it was evaporated. E_2 was the purified product of E_1 crystallized twice with 60% ethanol. When E_1 was processed by a second chromatography in a polyamide resin column, E_3 was obtained. E4 was received from E3 after three times crystallization. All products were analyzed by HPLC (LC-6A, Shimadzu, Japan). The standard preparation of icariin was bought from Nation Institute for the Control of Pharmaceutical and Biological Products (NICPBP).

4.3. Cell culture

4.3.1. Isolatin and culture of newborn rat calvarial osteoplasts (ROB)

ROB was isolated enzymatically from calvariae of newborn Wistar rats (within 48 h) basically as previously reported (Chen et al. 2004). Briefly, 6∼7 calvariae without adhering soft tissue were cut into pieces and subjected to 20 min digestions with 5 ml enzyme mixture containing 1 mg/ml collagen II and 0.5 mg/ml trypsin. The pieces were then digested two times with 1 mg/ml collagen II for 60 min. The released cells were collected and suspended in α-MEM (GIBCO) supplemented with 10% fetal bovine serum (National Hyclone, Lanzhou, China), 100 units/ml penicillin and 100 μg/ml streptomycin, and plated in 100-mm plastic culture dish (Corning, USA) at 37 °C in 5% CO_2 with humidification. When reaching confluence, the cells were detached by treatment with 0.25% trypsin and 1 mM EDTA, and planted into 24-well plates for differentiation or 96-well plates for proliferation assay.

4.3.2. Primary culture of rat bone marrow stroma cells (rMSCs)

The primary culture of rMSCs was established as described previously (Chen et al. 2005). A single-cell suspension was obtained from a two-month-old Wistar rat, by flushing the bone tubes of tibias and femurs with MEM medium (GIBCO) supplemented with 10% fetal bovine serum, and finally filtered through a 76 μm stainless steel cellcribble. The suspension was adjusted to 10^7 cells/ml and planted into 6-well plates. After 72 h incubation, the medium was removed and all wells were washed two times with phosphate buffered saline (PBS). Thereafter, the osteogenic differentiation and cell proliferation were analyzed. All extracts were dissolved in DMSO with final concentrations in culture media less than 0.05%.

4.4. Cell proliferation and osteogenic differentiation assay

ROB proliferation was analyzed as reported previously (Chen et al. 2004). When rMSCs were assayed, the single-cell suspension was adjusted to 10^6 cells/ml and plated in 96-well plates. After 72 h, the medium was removed and the wells were gently washed twice with PBS. Fresh medium supplemented with E_1, E_2, E_3 or E_4 at 0.01, 0.1, 1, 10 and 100 μM was added respectively, each six time in parallel. After 48 h, the proliferation was analyzed by the reduction of MTT.

When ROB in 24-well plates or rMSCs in 6-well plates grew to 70∼80% confluence, the osteogenic supplements (10^{-8} M dexamethasone, 100 mM AsAP and 10 mM β-glycerophosphate) were added and the osteogenic differentiation was evaluated every third or fourth day by the assay of cellular alkaline phosphatase (ALP), osteocalcin secretion, and calcium deposition as well as colonies stained positive for ALP and bone mineralized nodules. The methods were previously reported (Chen et al. 2004, 2005).

4.5. RT-PCR

Total cellular RNA was extracted from primary rMSCs cultured in 100 mm plastic culture dishes after 4 and 8 day osteogenic induction. TaKaRa RNAiso Regent (TaKaRa Biotechnology Co., Ltd China) was used. 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 1U Taq DNA polymerase (all provided by TaKaRa). Primers for ALP (sense: 5′-TCCATGGTGGATTATGCTCA-3′, antisense: 5′-TTCTGTTCCTGCTCGAGG TT-3′), osteocalcin (sense: 5′-AGGACCCTCTCTCTGCTCAC-3′, antisense: 5′-AA CGGTGGTGCCA-TAGATGC-3′), osteopontin (sense: 5′-AAGGACCAACTACAA CCA-3′, antisen: 5′-GATTCATCC GAGTTCACA-3′), bone sialoprotein (sense: 5′-GCGAATTCTGAACGGGTTTCAGCAGAC-3′, antisense: 5′-GCGAATT-CTGG TGGTAGTAATAATCCT-3′), TGF-β_1 (sense: 5′-GGCTTTCGCTT-CAGTGCTCA-3′, antisense: 5′-CGGGTTGTGTTGGTTGTAGA-3′), IGF-I (Sense: 5′-AACTTTC TTTCCGTGCTG-3′, antisense: 5′-CATTGTATGG-CTATCTGTCTT-3′), GAPDH (sense: 5′-TGAACGGGAAGCTCACTGG-3′, antisense: 5′-TCCACCACCCTGTT GCTGTA-3′) were prepared based on published DNA sequences (Gene bank accession numbers J03572, X04141, M14656, J04215, X52498, NM178866 and X02231 respectively). GAPDH was used as internal control. DNA amplification included an initial denaturation at 95 °C for 5 min, followed by 30 (ALP, Osteocalcin, Osteopontin, TGF-β_1 and GAPDH) or 35 (bone sialoprotein and IGF-I) cycles of denaturation at 94 °C for 30S, annealing at 52 °C (Osteopontin and bone sialoprotein), 53 °C (IGF-I) or 60 °C (osteocalcin, TGF-β_1 and GAPDH) for 30S, and extension at 72 °C for 1 min. 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.

4.6. Statistics

Values were expressed as means ± S.D. Statistical analysis was carried out by SPSS10.0. An analysis of variance was first performed to test for any significant difference among groups. The Dunnett's multiple test (for comparison with control in cell proliferation) or Student's t-test (E_3 group vs. E_2 group, E_4 group vs. E_3 group in calcium deposition assay) was used to determine the specific differences between means.

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