β-Ecdysterone Induces Osteogenic Differentiation in Mouse Mesenchymal Stem Cells and Relieves Osteoporosis

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The effect on bone tissue of β-ecdysterone, a type of ecdysteroid found in many plants, has not been previously investigated. In this study, we found that β-ecdysterone treatment significantly induced alkaline phosphatase (ALP) activity in mesenchymal stem cells in a dose-dependent manner. Real-time polymerase chain reaction (PCR) showed that Runx2, osteocalcin, and type I collagen expression also increased. ICI182780, a specific estrogen receptor antagonist, inhibited the upregulation of ALP activity. Moreover, β-ecdysterone promoted estrogen receptor (ER) reporter gene activity; however, ICI182780 reversed its effect, suggesting that β-ecdysterone has stimulatory effects on osteogenic differentiation via the ER. Furthermore, β-ecdysterone alleviated osteoporosis symptoms in a mouse model without obvious side effects. Therefore β-ecdysterone may be a promising candidate drug for the treatment of osteoporosis.

Key words β-ecdysterone; mesenchymal stem cell; osteogenic differentiation; estrogen receptor; osteoporosis

Osteoporosis is the most common bone health problem, affecting millions of people all over the world. Bone marrow mesenchymal stem cells (MSCs) are pluripotent stem cells that can differentiate into osteoblasts, chondrocytes, adipocytes, and other types of mesodermal cells.1–10 It is hypothesized that with increasing age, MSCs are inclined to differentiate into adipocytes rather than osteoblasts, which may be a major cause of osteoporosis.11–13 Chemical agents that could stimulate osteogenic differentiation in MSCs might show promise for osteoporosis treatment.

Estrogen plays an important role in maintaining bone mass through the estrogen receptor (ER). Many menopausal women have severe, rapid bone loss that can be prevented or reversed by estrogen replacement.8,9 Although estrogen replacement has been the main therapy to treat osteoporosis, there are many concerns about its safety. For example, it may increase cardiovascular and breast cancer risk. In the past few years, herbal medicines have received interest as an alternative to estrogen.

β-Ecdysterone [IUPAC: (2S,3R,5R,10R,13R)-2,3,14-trihydroxy-10,13-dimethyl-17-[(2S)-2,3,6-trihydroxy-6-methylheptan-2-yl]-2,3,4,5,9,11,12,15,16,17-decahydroadro-1H-cyclopenta[a]phenanthren-6-one] is a major component of several Chinese herbal medicines, such as Achyranthes bidentata Bl. and Cyanotis arachnoidea C. B. CLARKE. β-Ecdysterone has various activities, including stimulating protein synthesis, promoting carbohydrate and lipid metabolism, alleviating hyperglycemia and hyperlipemia, immunologic modulation, and protecting endothelial cells from apoptosis and inducing their proliferation.10–12 However, little is known about their effects on bone.

β-Ecdysterone has a steroid backbone that is similar to that of estrogen (Fig. 1), and therefore it is possible that β-ecdysterone will have activity similar to that of estrogen. The purpose of this research was to investigate whether β-ecdysterone could induce osteogenic differentiation of MSCs and whether this effect is dependent on the ER. We also performed preliminary research on its role in osteoporosis prevention.

MATERIALS AND METHODS

Chemicals and Reagents Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone. Isobutylmethylxanthine (IBMX), dexamethasone, insulin, ascorbic acid, β-glycerophosphate, and all-trans-retinoic acid (RA) were obtained from Sigma Chemical Co. Alkaline phosphatase (ALP) activity kits and hydroxyproline (HOP) assay kits were purchased from Nanjing Jiancheng Biological Engineering Institute. Protein assay kits were purchased from BioRad. Lipofectamine 2000 and Trizol reagent were purchased from Invitrogen Corporation. First-strand cDNA synthesis kits were obtained from Toyobo Biotech Co. The SYBR green kit for polymerase chain reaction (PCR) reactions was purchased from Takara Biotech Co., Ltd. β-Ecdysterone (supplied by Shanghai Tauto Biotech Co., Ltd.) was dissolved in ethanol and diluted with medium until use. For all experiments, the final concentration of ethanol was 0.1%, and control cultures received the carrier solvent (0.1% ethanol). ICI182780 was obtained from Tocris Bioscience (U.S.A.).

Culture of MSCs and Preosteoblasts MSCs were obtained from 8- to 10-week-old male BABL/c mice (Laboratory Animal Center of Guangzhou University of Chinese Medicine). Briefly, the mice were killed by cervical dislocation, femurs were isolated, and bone marrow was harvested by

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Fig. 1. Structure of β-Ecdysterone
flushing the bone using a syringe needle with DMEM. Then the cells were collected and cultured in DMEM (containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C. After 3 d, the nonadhering cells were discarded by replacing the medium. Subsequently, the medium were changed every 3 d. When the cells reached 80% confluence, they were detached with trypsin-EDTA treatment and subcultured. Our experiments used cells in the third passage.

Preosteoblast MC3T3-E1 cells obtained from the American Type Culture Collection (ATCC) were cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was changed twice weekly.

**ALP Activity Measurement** MSCs and MC3T3-E1 were plated in 24-well plates, and differentiation was induced by the addition of β-ecdysterone at various concentrations (0.01—10 μmol/l) for 5 or 8 d. On the indicated days, the cells were washed with ice-cold PBS three times and lysed. The ALP activity was measured using the ALP activity kit, and the protein concentration in cell lysates was determined with the protein assay kit.

**Oil Red O Staining** MSCs were treated with β-ecdysterone (1 μmol/l) with or without adipogenic supplement (0.5 mmol/l IBMX, 1 μmol/l dexamethasone, 10 mg/l insulin; DMIX) for 12 d. At the end of culture, fat in differentiated adipocytes from MSCs was observed using the oil red O staining method. Briefly, cells were washed twice with PBS, fixed with 10% formalin for 20 min, then stained with a 0.5% oil red O solution for 45 min and photographed.

**Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** MSCs were cultured in DMEM (containing 10% FBS) and 1 μmol/l β-ecdysterone was added to the culture medium. After 4 or 8 d, total cellular RNA was isolated with Trizol, and reverse transcription was carried out using the first-strand cDNA synthesis kit. Real-time quantitative RT-PCR was performed using the SYBR green kit for PCR reactions. The reaction conditions were an initial step of 95 °C for 4 min; followed by 40 cycles of 95 °C for 20 s; 60 °C for 30 s; and 72 °C for 30 s. The tested gene expression levels were normalized to GADPH levels. The primer sequences are shown in Table 1.

**DNA Transfection and Reporter Gene Assays** Three ER-responsive elements were cloned into the plasmid GL3 (pGL3) vector and named pGL-ER. 293T cells were transfected with pGL-ER and the expression vector for ER-β or empty vector using Lipofectamine 2000. After 48 h, cells were treated with ecdysterone (1 μmol/l). Twenty-four hours later, cells were lysed and luciferase activity was measured using firefly luciferase reagents.

**MTT Assay** MSCs were plated in 96-well culture plates. IC1182780 was added at final concentrations of 1 μmol/l or 0.1 μmol/l. The cells were cultured for another 6 d, and then 20 μl MTT solution (5 g/l) was added to each well. After 4 h, the supernatant was removed carefully. One hundred and fifty microliters DMSO was added and the plate was shaken for 10 min. The optical density was measured at a wavelength of 492 nm.

**Animal Model of Osteoporosis** RA is widely used to create animal models of osteoporosis. In brief, 4-week-old male mice were divided into three groups of 8 mice each. Mice in the control group received water orally, and those in the RA group and β-ecdysterone (Ec) group received RA orally (70 mg/kg body weight) daily for 1 week. After that, only the mice in the Ec group were treated with β-ecdysterone (1 mg/kg body weight) through intravenous injection every day for 3 weeks.

**Bone Mineral Density and Radiographic Analysis** The bone mineral density (BMD) of the entire body and spine was determined using a PIXImus densitometer (GE Healthcare, Fairfield, CT, U.S.A.). The coefficient of variation of BMD of the entire body and spine was 0.9% and 0.6%, respectively. Results are given in milligrams per square centimeter. Radiographic analysis of the whole body was also performed using a soft X-ray system.

**Drying and Ashing of Bones** Bone ash weighting was done based on Wade’s method. Briefly, mice femurs were collected and dried at 110 °C for 48 h. The dry weights were measured, the samples were kept at 600 °C for 24 h, and then weighed to determine the weight of bone ash.

**HOP Content in Bone Tissue** Mice femurs were decalcified in trichloroacetic acid for 48 h and resolved in 6 mol/l HCl. The content of HOP was measured at a wavelength of 550 nm, according to the protocol.

**Statistical Analysis** Data represent mean±S.D. of several experiments (for experiments on cells, n=3; for experiments on animals, n=8). The data were statistically analyzed using ANOVA.

**RESULTS**

**Effects of β-Ecdysonerone on Osteogenic and Adiopogenic Differentiation** We first examined the effects of β-ecdysterone on ALP activity, which is an early marker of osteogenic differentiation. MSCs were treated with various concentrations of β-ecdysterone (0.01—10 μmol/l) for 8 d, and then ALP activity was measured. Our results showed that β-ecdysterone markedly stimulated ALP activity and its effect was dose dependent (Fig. 2A). ALP activity in control cells was 2.71 U/g protein (when 1 U is 1 mg of phenol produced by 1 g of protein collected in the cell lysate at 37 °C in 15 min).

We also investigated whether β-ecdysterone affected the differentiation of MC3T3-E1 cells. Cells were treated with β-ecdysterone in a similar manner for 5 d, and then

<table>
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<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
<th>Gene</th>
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<tr>
<td>CTGCCAAATATGATGACA</td>
<td>CCCAGGATGCCCCCTGA</td>
<td>GADPH</td>
</tr>
<tr>
<td>AATGCTCTCCGTGTATGAA</td>
<td>GCTCCGGCCCACAATAC</td>
<td>Runx2</td>
</tr>
<tr>
<td>ACGTCTCGTGAGTGTGTC</td>
<td>CAAGGAAGCTTTCCTTCTCT</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>AAGCAGGAGGGCAATAAGGG</td>
<td>AGCTGCTGTGACATCCATA</td>
<td>OCN</td>
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ALP activity was measured. The results were similar to those of MSCs (Fig. 2B). ALP activity in control cells was \(81.26 \pm 8.25\) U/g protein. However, 12-d treatment with \(\beta\)-ecdysterone (1 \(\mu\)mol/l) did not induce adipogenic differentiation, as shown in Fig. 2C. Our results also showed that \(\beta\)-ecdysterone did not influence adipogenic differentiation induced by DMIX (Fig. 2C).

**mRNA Expression**  To confirm the induction effect of \(\beta\)-ecdysterone on osteogenic differentiation, real-time PCR was performed to examine gene expression. Runx2 is a critical transcriptional factor that controls many subsequent osteogenic differentiation-related gene expression events, and mice with Runx2 knockout have severe defects in bone.\(^{17-19}\) Type I collagen is an important extracellular matrix of osteoblasts which is deeply involved in differentiation and proliferation. Osteocalcin (OCN) is another marker expressed much later during differentiation. Therefore we examined whether \(\beta\)-ecdysterone treatment could upregulate these three genes.

![Fig. 2. Effects of \(\beta\)-Ecdysterone on the Differentiation of MSCs and Preosteoblasts](image)

(A) Alkaline phosphatase activity of MSCs. *\(p<0.01\) vs. control group. (B) Alkaline phosphatase activity of preosteoblasts. *\(p<0.01\), **\(p<0.05\) vs. control group (ALP activity of control cells was defined as 100%). The ALP activity of \(\beta\)-ecdysterone-treated cells was calculated as the percentage of control). (C) MSCs were treated with \(\beta\)-ecdysterone (1 \(\mu\)mol/l) with or without DMIX. After 12 d, cells were fixed, stained with oil red O, and photographed (\(\times100\)).

![Fig. 3. MSCs Were Treated with 1 \(\mu\)mol/l \(\beta\)-Ecdysterone for 4 d (for Runx2 and Type I Collagen) or 8 d (for OCN), Then RNA Was Collected and Real-Time PCR Was Performed](image)

The gene expression levels were normalized by GADPH. *\(p<0.01\), **\(p<0.05\) vs. control group.

Our results showed that \(\beta\)-ecdysterone induced Runx2, type I collagen, and OCN expression compared with the untreated controls (Fig. 3). Collectively, these results indicate that \(\beta\)-ecdysterone has stimulatory effects on MSC os-
teogenic differentiation during the early, middle, and late phases.

**B-Ecdysterone Induces Osteogenic Differentiation through the ER** To investigate whether the induction effect of β-ecdysterone on osteogenic differentiation is specifically mediated by the ER, we applied ICI182780, a specific ER antagonist that inhibits estrogen action.20) We first performed the MTT assay to evaluate the effects of ICI182780 on cell growth. In the presence of 1 μmol/l ICI182780, cell viability decreased; however, 0.1 μmol/l ICI182780 did not have obvious effects on viability (Fig. 4A). 0.1 μmol/l ICI182780 was therefore used in the following experiments.

MSCs were treated with β-ecdysterone with or without the presence of ICI182780. ICI182780 reduced the ALP activity induced by β-ecdysterone (Fig. 4B). These data indicate that β-ecdysterone stimulates osteogenic differentiation at least partly through the ER. To confirm ER activation by β-ecdysterone, the luciferase reporter gene assay was performed. 293T cells transfected with pGL-ER, pRL-TK, and ER-β or empty vector. Then cells were treated with β-ecdysterone (Ec) with or without ICI182780 (ICI). Cells lysates were prepared and subjected to luciferase activity assays. The left bar of each group represents cells transfected with empty vector and the right bar represents cells transfected with ER-β expression vector. The relative reporter luciferase activity was normalized with pRL-TK. ∗p<0.01 vs. a and b.

**Attenuation of the RA-Induced Osteoporosis Phenomenon by B-Ecdysterone** First, we examined whether β-ecdysterone had any effect on the growth of mice. β-Ecdysterone (1 mg/kg body weight) was injected into the vein every day for 3 weeks. No obvious difference in body weight was observed between control and β-ecdysterone-treated mice. The initial body weight of control and β-ecdysterone-treated mice were 19.06±0.71 g and 18.83±0.64 g, respectively. After 3 weeks, the body weight of control mice increased to 24.13±0.78 g, and that of β-ecdysterone-treated mice to 23.86±0.69 g. Therefore we used this dosage of β-ecdysterone in the following experiments.

Mice treated with RA for one week showed a remarkable decrease in bone ash weight, HOP content in bone tissue, and BMD (especially in the spine) (Figs. 5, 6). Three-week treatment with β-ecdysterone reversed the osteoporosis symptoms. In dual-energy absorptiometry X-ray analysis (DEXA), the decrease in BMD caused by RA was inhibited by β-ecdysterone treatment (Fig. 5A). Radiography of the whole body also revealed that marked bone loss occurred in RA-treated mice and treatment with β-ecdysterone markedly inhibited bone loss (Fig. 5B). Detectable increases in bone ash weight, HOP content in bone tissue, and BMD were observed compared with the RA-treated group (Fig. 6), which
implies that \(\beta\)-ecdysterone hinders the process of osteoporosis.

**DISCUSSION**

In the present study, we demonstrated that \(\beta\)-ecdysterone stimulates osteogenic differentiation of MSCs \textit{in vitro} and its effect was dependent on the ER. We also showed that it prevented the process of osteoporosis. Estrogen has beneficial effects on bone, partly because it induces bone formation through osteogenic differentiation. However, because of its side effects, alternative therapies are desirable. Increasing data show that many chemical components isolated from herbal medicines with structures similar to that of estrogen have functions similar to those of estrogen.\(^{21–23}\) Our findings provided the first evidence that \(\beta\)-ecdysterone isolated from traditional Chinese medicine could induce the osteogenic differentiation of MSCs and did not have obvious effects on adipogenic differentiation.

During differentiation \textit{in vitro}, osteoblast phenotypic markers appear in the following order: formation of type I collagen matrix, expression first of ALP and then of Runx2, secretion of osteocalcin, and finally mineralization of bone nodules.\(^{24–26}\) Our results showed that \(\beta\)-ecdysterone stimulates ALP activity and induces the expression of type I collagen, Runx2, and OCN. \(\beta\)-Ecdysterone not only determines the osteogenic lineage determination of MSCs but also directs this differentiation to the terminal stage.

Considering its induction effects on osteogenic differentiation and its structural characteristics, \(\beta\)-ecdysterone can be categorized as a phytoestrogen. Many phytoestrogens exert their effects through the ER-signaling pathway,\(^{27}\) and therefore, we hypothesized that \(\beta\)-ecdysterone might interact with and activate steroid receptors, such as the ER. Our data showed that ALP activity induced by \(\beta\)-ecdysterone was obviously blocked by the ER antagonist ICI182780. Moreover, the ER-responsive reporter gene was activated by \(\beta\)-ecdysterone. These data suggest that the ER mediates, at least in part, the induction effect of \(\beta\)-ecdysterone.

\(\beta\)-Ecdysterone is a major component of some traditional herbal medicines. Among them, \textit{Achyranthes bidentata} Bl. has been used in China for the treatment of osteoporosis for several hundred years. But the molecular and cellular bases of its effect remain largely unknown. The stimulation of osteogenic differentiation of MSCs as well as osteoblasts by \(\beta\)-ecdysterone is substantial and dose dependent, and \textit{in vivo} study showed that \(\beta\)-ecdysterone attenuates the progress of osteoporosis. Therefore it is likely that the preventive effect of \textit{Achyranthes bidentata} Bl. on osteoporosis is mediated by \(\beta\)-ecdysterone. As a traditional medicine, \textit{Achyranthes bidentata} Bl. has been extensively used in China and no side effects have been reported. Meanwhile, \(\beta\)-ecdysterone treatment did not cause obvious differences in the body weight of mice. \(\beta\)-Ecdysterone may thus be developed into a promising medicine for osteoporosis.

In conclusion, \(\beta\)-ecdysterone induced \textit{in vitro} osteogenic differentiation of MSCs in an ER-dependent manner and alleviated the osteoporotic process in a mouse model.

**REFERENCES**