Modulation of Isoflavones on Bone-nodule Formation in Rat Calvaria Osteoblasts *in vitro*

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Objective To observe the effects of two main isoflavones, daidzein and genistein on the bone-nodule formation in rat calvaria osteoblasts *in vitro*. **Methods** Osteoblasts obtained from newborn Sprague-dawley rat calvarias were cultured for several generations. The second generation cells were cultured in Minimum Essential Medium supplemented with ascorbic acid and Na-beta-glycerophosphate for several days, in the presence of daidzein and genistein, with or without the estrogen receptor antagonist ICI 182780. Number of nodules was counted at the end of the incubation period (day 20) by staining with Alizarin Red S calcium stain. The release of osteocalcin, as a marker of osteoblast activity, was also determined on day 7 and day 12 during the incubation period. **Results** Compared with the control, the numbers of nodules were both increased by incubation with daidzein and genistein. 17 -estradiol was used as a positive control and proved to be a more effective inducer of the increase in bone-nodules formation than daidzein and genistein. The release of osteocalcin into culture media was also increased in the presence of daidzein and genistein, as well as 17 -estradiol on day 7 and day 12 (day 12 were higher). The estrogen receptor antagonist ICI 182780 completely blocked the genistein- and 17 -estradiol-induced increase of nodule numbers and osteocalcin release in osteoblasts. However, the effects induced by daidzein could not be inhibited by ICI 182780. **Conclusion** These findings suggest that geinistein can stimulate bone-nodule formation and increase the release of osteocalcin in rat osteoblasts. The effects, like those induced by 17 -estradiol, are mediated by the estrogen receptor dependent pathway. Daidzein also can stimulate bone-nodule formation and increase the release of osteocalcin in rat osteoblasts, but it is not, at least not merely, mediated by the estrogen receptor dependent pathway.

Key words: Daidzein; Genistein; ICI 182780; Osteocalcin; Bone-nodules; Osteoblast

INTRODUCTION

Isoflavones are naturally occurring plant compounds that are structurally and functionally similar to 17 -estradiol. The phenolic ring they possess enables them to bind to both types of estrogen receptor, ER- and ER- [1]. Daidzein and genistein, two main isoflavones in soy protein and flaxseed, recently gain much attention after reports that women with diets rich in those compounds had a low incidence of estrogen-related disease, such as cancers, cardiovascular diseases[2], climacteric symptoms[3] and osteoporosis[4]. They are highly

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0895-3988/2003
CN 11-2816

investigated and reported to have the potential to act like estrogen on many tissues, including bone tissue.

In 1998, Potter *et al*. reported soy protein containing high concentrations of isoflavones (90 mg/d for 6 months) could protect against spinal bone loss but not elsewhere in 66 postmenopausal women[5]. Similar results were also observed in other recent studies[6]. There is some evidence that, in addition to slowing bone loss, isoflavones treatment can increase bone mass[7]. However, Kardinaal *et al.* in the Netherlands found that there was no difference in the excretion of isoflavonoids between two groups of women with a high or low rate of bone loss. It was concluded that there did not seem to be a protective effect[8].

Animal models have been used to demonstrate possible effects of isoflavones on bone. A significant decrease in bone loss was shown in ovariectomized rats or mice fed a diet of either genistein or daidzein in placebo-controlled trials[9-11]. In some cell culture studies, daidzein and genistein have been shown to promote cell proliferation and collagen formation of rat osteoblasts *in vitro*[12], inhibit bone resorption by rat osteoclasts[13] and inhibit parathyroid hormone activity[14].

However, to date, few studies have been done to specifically look at phytoestrogens and bone formation in osteoblasts *in vitro*. To determine the effects of isoflavones on the bone formation of osteoblasts, their influences on the osteocalcin release and bone-nodule formation in rat calvaria osteoblasts *in vitro* were investigated in this study.

MATERIALS AND METHODS

Cell Isolation and Culture Procedure

The procedure for the isolation of osteoblasts was a modification of previously described methods[15-17]. Parietal bones were removed aseptically from newborn Sprague-dawley rats. The periosteum was removed and the bones washed three times with PBS. Bones were then cut into fragments of 3-5 mm diameter and incubated in 0.25% trypsin for 15 min at 37℃ to remove the fibroblast population. Then the cells were isolated twice from the bones using digestion with 0.1% type collagenase solution (1 mg/mL, collagen digestion activity: 380 units/mg, Sigma, USA), each for an hour. Cells obtained from two sequential digestions were mixed up and planted into 80 cm^2 culture flasks (Nunclon, Denmark) in MEM (GIBCO, USA) containing 10% heat-inactivated newborn calf serum (GIBCO, USA) and antibiotics comprising 100 U/mL sodium penicillin and 100 U/mL streptomycin supplemented with 50 g/mL ascorbic acid, 10 mmol/L Na- -glycerophosphate (Merck, German). Growth medium was changed every second day thereafter. The cells reached confluence after 7 d. They were then released with 0.25% trypsin solution and planted in 24-well plates at a density of 2×10^4 cells/well.

Cell Growth

Osteoblasts were grown in 24-well plates *in vitro* and maintained in either standard medium (MEM containing 10% heat-inactivated newborn calf serum and antibiotics comprising 100 U/mL sodium penicillin and 100 U/mL streptomycin supplemented with 50 g/mL ascorbic acid, 10mmol/L Na- -glycerophosphate) or test mediums, which were modified standard medium with daidzein (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}mol/L, Sigma, USA), genistein (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}mol/L , Sigma, USA), 17 -estradiol (10^{-8}, 10^{-7}, 10^{-6}mol/L, Sigma, USA, used as a positive control) in the presence or absence of the estrogen receptor antagonist ICI 182780(10^{-6}mol/L, Tocris, UK). Some cultures were exposed to modified

standard medium only with ICI 182780 (10^{-6}mol/L) to estimate influence of ICI 182780 in this study. The pH of the medium was adjusted to 7.4 before contact with the cells. Plates were maintained at 37℃ in a humidified atmosphere consisting of 95% air and 5% CO_2. Medium was changed every 2 d.

Osteocalcin

Osteocalcin content of the medium was measured using IRMA (China Institute of Atomic Energy, Beijing). Briefly, sample containing osteocalcin was incubated simultaneously with an antibody-coated bead and the ^{125}I-labeled antibody. Osteocalcin contained in the sample was immunologically bound by the immobilized antibody and the radiolabeled antibody to form a complex: bead/Anti-osteocalcin, osteocalcin and ^{125}I-anti-osteocalcin. After incubation for 20 h, the bead was washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead was measured in a gamma counter (GC1200, University of Science & Technology of China, China). The radioactivity of the bound antibody complex is directly proportional to the amount of osteocalcin in the sample[18]. The release of osteocalcin in the medium was determined on day 7 and day 12 during the incubation period.

Bone-nodule Formation and Nodule Quantification

The effects of daidzein and genistein on bone-matrix formation were determined in the nodule assay described by Bellows and Sprague[19-21]. Osteoblasts were maintained for 20 d in standard medium or test mediums supplemented with daidzein, genistein and 17 - estradiol (used as a positive control) in the presence or absence of the estrogen receptor antagonist ICI 182780, as described previously. After incubation, cells were washed three times with PBS, fixed with 90% ethanol, washed with distilled water and stained using 2% Alizarin Red S calcium stain for 30 min. Staining with Alizarin Red S is a standard method for visualization of nodular pattern and calcium deposition of osteoblast cultures *in vitro*. Alizarin Red S stains calcium dark red and the tissue yellow. Bone nodules in each well were counted at 40× magnification using a microscope (Olympus, Japan) by placing the culture plate on a transparent grid ruled in 1mm squares. Data were expressed as the number of bone nodules (nodules with minor diameter < 200 m excluded) per well.

Statistical Analysis

Test for significance of difference, between-group means was performed using analysis of variance. All calculations were performed using the statistical analysis package SPSS. A $P < 0.05$ was considered significant.

RESULTS

Osteocalcin Production

Osteocalcin content of the medium was measured on day 7 and day 12 during the incubation period. The presence of 10^{-6}mol/L daidzein caused a significant increase in osteocalcin content of the medium on both day 7 and day 12, whereas 10^{-12}mol/L, 10^{-10}mol/L and 10^{-8}mol/L daidzein had no effect (except 10^{-8}mol/L daidzein in day 12). When cells were cultured in a medium containing both daidzein and ICI 182780, the daidzein (10^{-6}mol/L)-induced increase in osteocalcin release remained. Genistein at concentrations of 10^{-6}mol/L and 10^{-8}mol/L also increased osteocalcin content of the medium on day 7 and day 12, whereas 10^{-12}mol/L and 10^{-10}mol/L genistein had no obvious effect. The effect of genistein

(10^{-6}mol/L, 10^{-8}mol/L) in increasing osteocalcin release in osteoblasts was not seen in the presence of ICI 182780 (10^{-6}mol/L), although ICI 182780 alone did not have appreciable effect on osteocalcin release in osteoblasts. In the positive control group, the presence of 17 -estradiol at concentrations of 10^{-8}-10^{-6}mol/L also caused a significant increase in osteocalcin release in osteoblasts on both day 7 and day 12. The 17 -estradiol-induced increase in osteocalcin release in rat osteoblasts was prevented completely by the presence of 10^{-6}mol/L ICI 182780. (Figs. 1, 2)

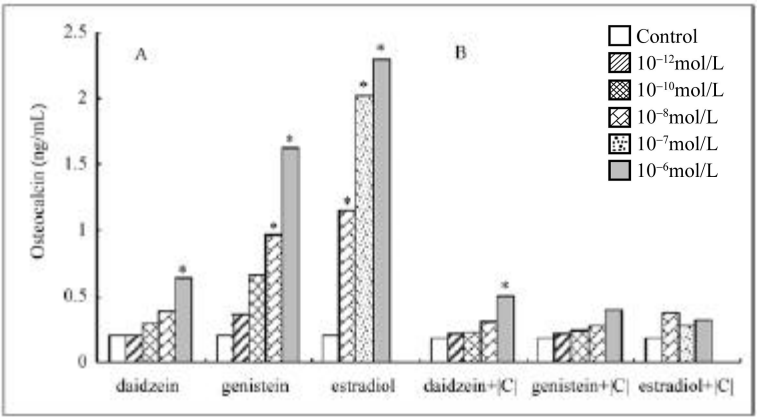

FIG. 1. Effects of isoflavones on the release of osteocalcin in rat osteoblasts. Cells were cultured for 7 d in a standard medium (MEM) or test mediums containing daidzein(10^{-12}-10^{-6}mol/L), genistein(10^{-12}-10^{-6}mol/L) and 17 -estradiol (10^{-8}-10^{-6}mol/L) in the absence or presence of ICI 182780 (10^{-6}mol/L) (A and B respectively). *n*=4 for each group. Key: (*) *P*<0.05, compared with the control value without isoflavones or estrogen. For the concentrations of isoflavones or estrogen; control; 10^{-12}mol/L; 10^{-10}mol/L; 10^{-8}mol/L; 10^{-7}mol/L; and 10^{-6}mol/L.

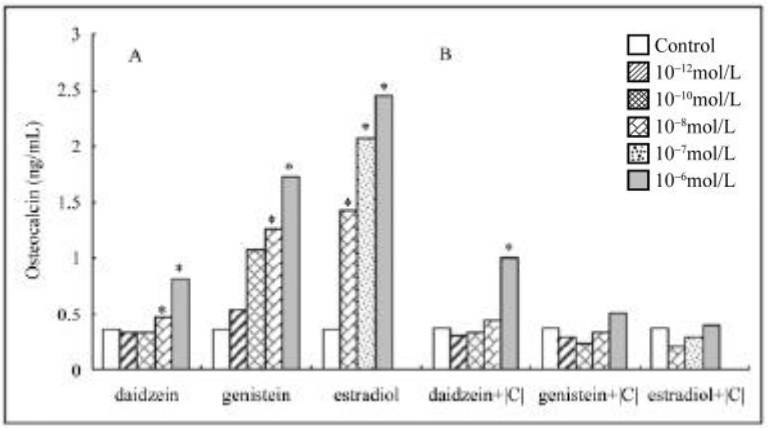

FIG. 2. Effects of isoflavones on the release of osteocalcin in rat osteoblasts. Cells were cultured for 12 days in a standard medium (MEM) or test mediums containing daidzein(10^{-12}-10^{-6}mol/L), genistein(10^{-12}-10^{-6}mol/L) and 17 -estradiol (10^{-8}-10^{-6}mol/L) in the absence or presence of ICI 182780 (10^{-6}mol/L) (A and B respectively). *n*=4 for each group. Key: (*) *P*<0.05, compared with the control value without isoflavones or estrogen. For the concentrations of isoflavones or estrogen; control; 10^{-12} mol/L; 10^{-10} mol/L; 10^{-8} mol/L; 10^{-7} mol/L; and 10^{-6} mol/L.

Nodule Formation

At the end of the experimental period (day 20), culture plates were stained with the Alizarin Red S calcium dye. Cells incubated in control or test medium developed nodules with a typical pattern: larger calcifying foci surrounded by smaller foci in different stages of calcification (Photo 1.). Total number of nodules per well was quantified macroscopically. Compared with the control, cells incubated in 17 -estradiol- supplemented medium, in whatever concentrations, developed more nodules. Daidzein (10^{-6}mol/L) and genistein (10^{-6}mol/L) supplementation also increased the number of nodules. However, the number of nodules of the daidzein (10^{-12}mol/L -10^{-8}mol/L)-treated cultures and genistein(10^{-12}mol/L -10^{-8} mol/L)-treated cultures were similar to that of the control. In the 17 -estradiol-treated and genistein-treated cells, the number of nodules per well formed in the presence of ICI 182780 (10^{-6} mol/L) was not different from that of control, although ICI 182780 (10^{-6} mol/L) alone did not have appreciable effect on the bone-nodules formation in rat osteoblasts. However, the number of nodules formed in daidzein (10^{-6}mol/L)-treated cells in the presence of ICI 182780 (10^{-6}mol/L) remained higher than control (Fig. 3) .

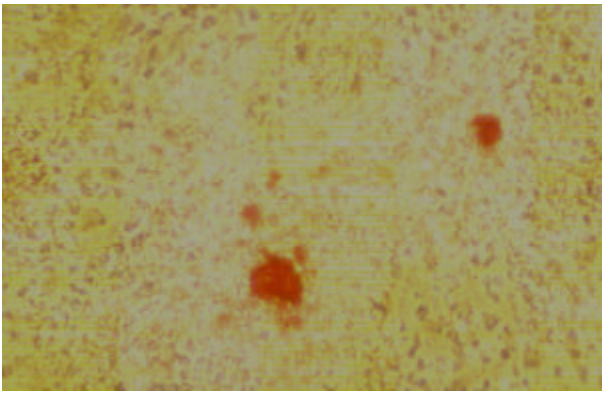

Photo1. Light micrograph of mineralized nodules stained by ARS showing the red calcificated extracellular matrix. (×100)

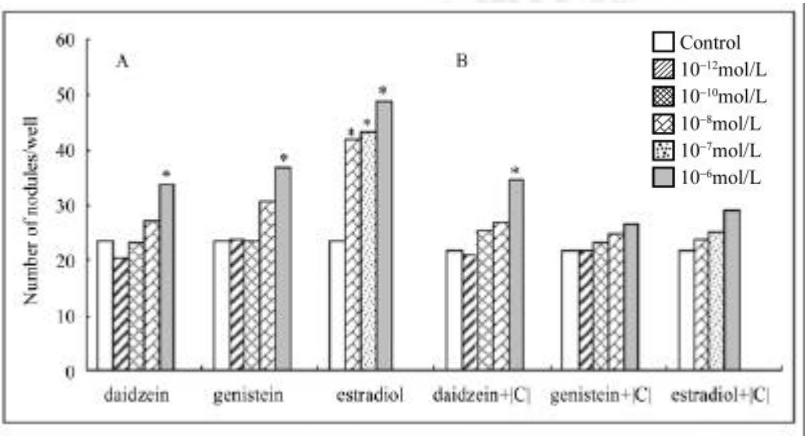

FIG. 3. Mean number of bone nodules per culture well produced by cells exposed to various dilutions of isoflavones in the presence or absence of ICI 182780 (10^{-6}mol/L). Cells were cultured for 20 days in a standard medium (MEM) or test mediums containing daidzein (10^{-12}-10^{-6}mol/L), genistein (10^{-12}-10^{-6}mol/L) and 17 -estradiol (10^{-8}-10^{-6}mol/L) in the absence or presence of ICI 182780 (10^{-6}mol/L) (A and B respectively). *n*=4 for each group. Key: (*) *P*<0.05, compared with the control value without isoflavones or estrogen. For the

concentrations of isoflavones or estrogen; control; 10^{-12}mol/L; 10^{-10}mol/L; 10^{-8} mol/L; 10^{-7}mol/L; and 10^{-6}mol/L.

DISCUSSION

Osteocalcin, a marker of late osteoblast differentiation, is expressed by highly differentiated osteoblasts during the phase of mineralization[22]. Both osteocalcin and alkaline phosphatase are generally considered as markers of mature osteoblast phenotype; however, during osteoblasts development, gene expression of alkaline phosphatase reaches peak values earlier than that of osteocalcin. In the present study, osteocalcin released into the culture medium was higher on day 12 than on day 7, using either control, daidzein, genistein and 17 -estradiol treatment. It is possible that the peak of osteocalcin gene expression was missed in our study, but the tendency showed in the results is in agreement with the findings that osteocalcin gene expression increases during osteoclasts development and nodules formation.

Although both daidzein and genistein increased the osteocalcin content of the medium at some concentrations, the dose responses for these two different isoflavones are not identical: At a concentration of 10^{-6}mol/L, the osteocalcin concentration in the medium after daidzein-treatment for 7 days was 3-fold higher in comparison to control levels, while genistein-treatment was 8-fold higher. Genistein at a concentration of 10^{-8}mol/L increased the osteocalcin content of the medium, while daidzein at the same concentration had no effect. A complete comparison between the effect of daidzein and genistein on osteocalcin release in rat osteoblasts showed that genistein is a more effective inducer of an increased osteocalcin release. Some differences between daidzein-treated and genistein-treated cells were significant (10^{-10}-10^{-6}mol/L in day 7, 10^{-8}mol/L in day 12). It is partly due to the fact that daidzein binds with a lower affinity to the estrogen receptor than genistein.

It was found in this study that 1 mol/L of both daidzein and genistein increased bone-nodule formation. A comparison of these two isoflavones with 17 -estradiol showed that 17 -estradiol is more effective in promoting bone-nodules formation than daidzein and genistein. The fact that the relative binding affinity of phytoestrogen to the estrogen receptor is weaker than that of 17 -estradiol can explain the difference between 17 -estradiol and two isoflavones on bone-nodules formation, but it does not provide any evidence to explain the similar effect between daidzein and genistein.

The 17 -estradiol- and genistein-induced increase in osteocalcin release and bone-nodules formation in rat osteoblasts were prevented completely by the presence of the estrogen receptor antagonist ICI 182780 (10^{-6}mol/L), which indicates that these types of response are estrogen receptor mediated. However, daidzein-induced increase in osteocalcin release and bone-nodules formation in rat osteoblasts was not inhibited by the presence of ICI 182780 (10^{-6}mol/L). It is assumed that daidzein increases osteocalcin release and bone-nodules formation in rat osteoblasts not merely by activation of the conventional gene-stimulating nuclear estrogen receptor pathway.

In summary, this study has shown that genistein, like 17 -estradiol, can induce increase in osteocalcin release and bone-nodules formation in rat osteoblasts. This estrogen receptor dependent effect can be blocked by an ICI 182780 treatment. Daidzein also can increase osteocalcin release and bone-nodules formation in rat osteoblasts. Although daidzein is weaker than genistein in promoting osteocalcin release, its effect is non-ER dependent and cannot be blocked by an ICI 182780 treatment.

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(*Received June* 23, 2002 *Accepted December* 21, 2002)