

Enhancing Effect of Daidzein on the Differentiation and Mineralization in Mouse Osteoblast-like MC3T3–E1 Cells

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The effect of daidzein, an important isoflavone, on the differentiation and mineralization in MC3T3–E1 cells, a mouse calvaria osteoblast-like cell line, was investigated. The MTT assay, the alizarin red S and von Kossa staining, the measurement of calcium (Ca) and phosphorus (P) concentrations by inductively coupled plasma-atomic emission spectrometry and the nitrophenol liberation method were used to determine the cell proliferation, mineralization and intracellular alkaline phosphatase (ALP) activity, respectively. Daidzein enhanced the cell proliferation after the culture for 2 days and the effect reached maximum on day 6. ALP activity and cellular Ca and P contents were increased time- and dose-dependently when the cells were treated with daidzein in the presence of disodium β -glycerophosphate and L-ascorbic acid. Differentiation of the cells to the mature osteoblasts was prompted under incubation in the presence of daidzein for 21 days, by the time the mineralized nodules formed. The calcium depositions of the cells by alizarin red S staining were increased significantly after the culture with daidzein as long as 28 days. It has been demonstrated that daidzein may be able to enhance the bone differentiation and mineralization and prompt the bone formation in the early growing stage and the late growing stage of osteoblasts.

Key words—daidzein; differentiation; mineralization; MC3T3–E1 cells

INTRODUCTION

It is known that bone volume is maintained by two phases of bone remodeling, namely bone formation by osteoblasts and bone resorption by osteoclasts.¹⁾ An imbalance between bone formation and bone resorption leads to metabolic bone diseases.²⁾ Osteoporosis associated with bone loss is a major bone disease in elderly population, particularly in women, which is owing to the decrease in serum estrogen after menopause.³⁾ Although estrogen replacement therapy (ERT) has been considered to be effective in preventing and treating osteoporosis, there are still concerns about its safety including the increase on the risk of breast cancer and other undesirable side-effects, all of which limit the clinical usage of estrogen.^{4,5)} These concerns have prompted to develop new therapeutic and preventive approaches.

In the past few years, soy isoflavones have attracted much more attentions for their decreased incident of osteoporosis in Asian women.^{6,7)} The soy-derived isoflavones, mainly daidzein and genistein, belong to

the family of diphenolic compounds with structural similarities to natural and synthetic estrogen and anti-estrogen (Fig. 1). They are capable of binding to the estrogen receptors ER α and ER β , which may play in estrogen-responsive tissues.^{8,9)} Compared with estradiol, isoflavones have both estrogenic and anti-estrogenic effects.¹⁰⁾

Most of the estrogenic effects of daidzein to prompt bone formation and prevent bone resorption^{11,12)} have been observed in animal models and cultured cells. On the action of daidzein on bone formation, daidzein is shown to stimulate protein synthesis, alkaline phosphatase (ALP) activity and DNA content after the culture in osteoblast-like MC3T3–E1 cells for 2 days.^{13,14)} It has been also demonstrated that daidzein causes a significant increase of ALP activity, DNA and calcium content in bone tissue.¹⁵⁾ On the action of daidzein on osteoblast function, daidzein is shown to act on some cytokine products such as interleukin-6 (IL-6), osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) via estrogen-receptor pathway in hFOB 1.19 cell line¹⁶⁾ and trabecular osteoblasts isolated from young female piglets.¹⁷⁾

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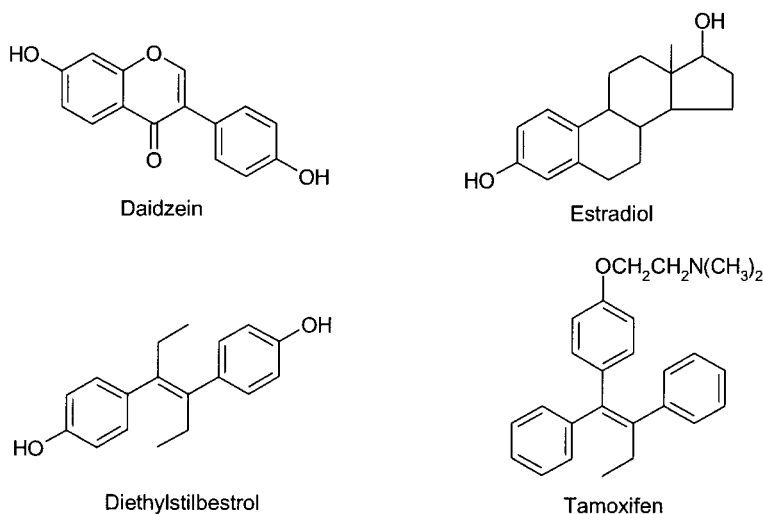


Fig. 1. Structure of Daidzein (Isoflavone) for Comparison with Estradiol (Natural Estrogen), Diethylstilbestrol (Synthetic Estrogen) and Tamoxifen (Synthetic Antiestrogen)

In our previous study, daidzein is shown to enhance the viability and stimulate the late stage of differentiation of osteoblasts isolated from newly born rat calvariae.¹⁸⁾ Moreover, the increased production of bone morphogenetic protein (BMP2) is considered to mediate the effect. In the present study, MC3T3-E1 cells were cultured with different concentrations of daidzein for 30 days by adding some additives to the culture medium. The differentiation and mineralization of the cells were systemically evaluated from the MTT assay, ALP activity, staining assay, Ca and P contents. Although the effect of daidzein on the ALP activity etc. in MC3T3-E1 cells has been studied previously,^{13,14)} the main purpose in our paper was to investigate the effect of daidzein on the growth and differentiation process of the cells after a longer culture time. Thus, it was possible to further clarify the effect of daidzein on the differentiation and mineralization of osteoblast-like MC3T3-E1 cells.

MATERIALS AND METHODS

MC3T3-E1 cells was purchased from Cell Center of Peking Union Medical College (Beijing, China). The content of synthesized daidzein was 98.79% (Huiké Plants Exploiture Co., Shanxi, China). Disodium β -glycerophosphate (β -GP) was from Fluka Chemika (Buchs, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-ascorbic acid (L-AA) and alizarin red S were obtained from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl phosphate (*p*NPP) was purchased from

Amresco Inc. (Solon, Ohio, USA).

Cell Culture MC3T3-E1 cells were cultured at 37°C in 5% CO₂ atmosphere in alpha-modified minimal essential medium (α -MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. β -GP (10 mM) and L-AA (50 μ g/ml) were added to the culture medium. Medium was changed every 3 days. The cells were passaged with 0.25% trypsin and 0.02% ethylenediaminetetraacetic (EDTA) solution. MC3T3-E1 cells were seeded at a density of 2.0×10^4 cells/ml in 96-well tissue culture plates for MTT assay, or at a density of 1.0×10^5 cells/ml in 35 mm dishes for other experiments.

MTT Assay The metabolic activity of MC3T3-E1 cells was determined by MTT assay. After the cells were treated with daidzein for proper time, 20 μ l MTT (5 mg/ml) solution was added and incubation was continued at 37°C for 4 h. The incubation medium was then removed and the resulting formazan crystals were dissolved in 200 μ l DMSO through a shaker for 10 min. The absorbance at 490 nm was measured on a microplate reader (Model 550, Bio-Rad Co., USA).

Mineralization Assay The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution. The alizarin red S solution was freshly made: 1 g of alizarin red S was solved in 100 ml distilled water and 28% ammonia solution was added to make it pH 6.36–6.40. After the cells were cultured

with daidzein for 28 days, the dishes were washed three times with phosphate-buffered saline (PBS, pH 7.4) and fixed by the addition of 95% ethanol solution for 10 min. Then, the cell cultures were washed five times with distilled water and stained by the alizarin red S solution for 5 min. The stained area was photographed using phase-contrast microscope.

The formation of bone nodules was observed by the von Kossa method. After the MC3T3-E1 cells were cultured with daidzein for 21 days, the cells were fixed with 10% neutral formalin. Then, 2 ml of freshly prepared 1% silver nitrate was added to dishes, which were incubated under UV light for 60 min. The dishes were rinsed with distilled water and fixed using 5% sodium thiosulfate for 2 min. Rinse thoroughly with distilled water terminated the reaction. The formed nodules were photographed using a phase-contrast microscope.

Preparation of Cell Lysates The alkaline phosphatase (ALP) activity, calcium and phosphorus contents of the cells cultured with daidzein for 10, 20 or 30 days were measured using the cell lysates. The cells were washed three times with ice-cold PBS, scraped into 0.5 ml of ice-cold 1% Triton X-100 solution and broken up by ultrasonic in an ice bath for 2 min. The supernatant, centrifuged at 14000 g and 4 °C for 5 min, was stored frozen at -20°C until measurement.

ALP Activity Intracellular ALP activity was determined using a method described previously.¹⁸⁾ Briefly, twenty microliters of the diluted cell lysates was incubated in a 96-well plate with 180 μ l of 0.1 M NaHCO₃-Na₂CO₃ buffer (pH 10.0) containing 1.0 % Triton X-100, 2 mM MgCl₂ and 8 mM *p*NP for 30 min at 37°C. The absorbance of *p*-nitrophenol liberated in the reactive solution was read at 405 nm. Twenty-five microliters of the diluted cell lysates was measured at 550 nm for total protein content by BCA method (BCA protein assay kit, Pierce, Rockford, IL, USA). ALP activity in the cells was normalized for total protein content of the cell lysate.

Ca and P Contents The cell lysates were diluted with deionized water. Concentrations of Ca and P were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Vista-MPX model, Varian Co., USA). The total protein content in the diluted cell lysates was also measured by BCA method. Ca and P contents in the cells were normalized for total protein content of the cell lysate.

Statistical Analysis All of the experiments were performed at least three times. Data are presented as means \pm standard deviation of means. Statistical analysis was performed using Student's paired *t*-test. $p < 0.05$ was considered to be significant.

RESULTS

The influence of daidzein on metabolic activity in MC3T3E-1 cells was assessed using the MTT assay. Cell metabolic activity was greater in daidzein treatment group than in the control group. The formation of MTT formazan was significantly increased dose-dependently when cells were treated with daidzein (10^{-9} M to 10^{-5} M) for 2 days and the effect was up to 132.3% of the basal value at the concentration of 10^{-5} M (Fig. 2(a)). In the time-related profile (Fig. 2(b)), the effect of daidzein (10^{-7} M) reached maximum on day 6 but decreased on the following day 8 and day 10.

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution. Because alizarin red S combines with the calcium and forms the lac of poor solubility, the calcification parts are stained dark-red. Figure 3 shows the calcification parts clearly increased when cells were treated with daidzein (10^{-6} M). As illustrated in Fig. 4, daidzein induced cell mineralization nodules after 21 days of exposure in the presence of β -GP and L-AA. The cells in the control group showed no mineralization nodule. On the other hand, by day 21 the cells treated with daidzein at the concentration of 10^{-6} M formed nodules that could be visualized with the von Kossa stain. The black plot in Fig. 4 showed the mineralization nodules. The results showed that daidzein further stimulated the cells at the late stage of differentiation and prompted mineralization.

ALP activity, as an indicator of osteoblastic differentiation, is a representative marker for the mature osteoblast. Figure 5 shows the effect of daidzein on the differentiation of MC3T3E-1 cells by determining ALP activity in the cells. The result suggested that the effect of daidzein on the ALP activity was dose-dependent and time-dependent in the groups. Daidzein at the higher concentration of 10^{-5} M significantly increased ALP activity in the MC3T3E-1 cells cultured for 10, 20 and 30 days ($p < 0.05$, $p < 0.01$ and $p < 0.01$ respectively). When cultured with the cells for 30 days, daidzein at the concentrations of 10^{-6} M and 10^{-7} M also increased ALP activity ($p <$

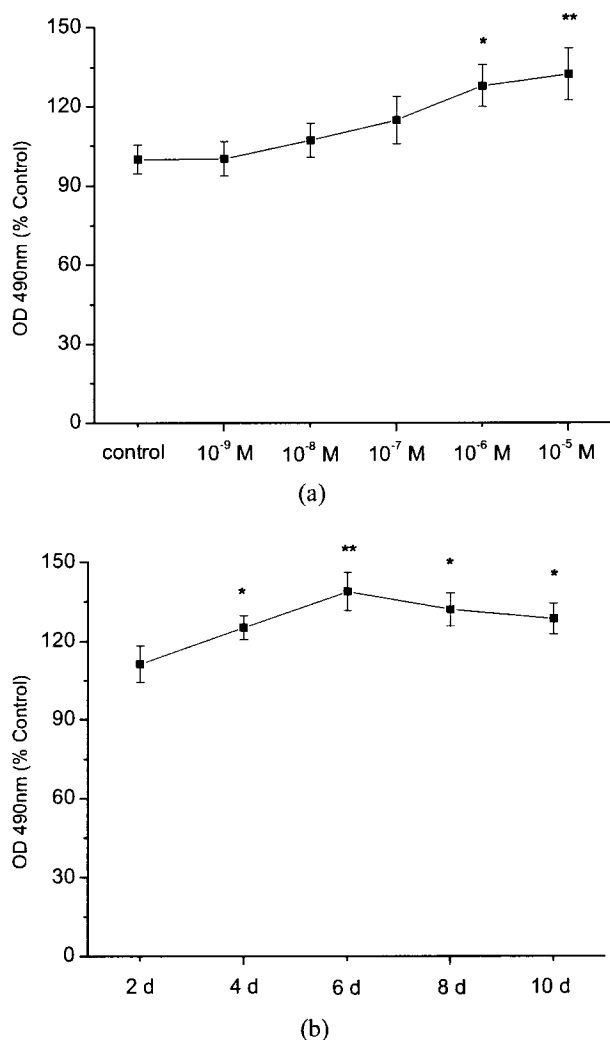


Fig. 2. Dose-related (a) and Time-related (b) Effects of Daidzein on Metabolic Activity in MC3T3-E1 Cells

The cells were incubated with daidzein (10^{-9} M to 10^{-5} M) for 2 days or daidzein (10^{-7} M) for serial time intervals. An MTT assay was performed according to the procedure described in the Material and Methods section. Each value is the mean \pm S.D. of six experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared with the control value without daidzein.

0.05) significantly. With the culture time prolonging, ALP activity in the daidzein-treated groups on day 20 was higher at least twice than the values on day 10, but the increased extent of ALP activity from day 20 to day 30 was not similarly visible.

The cells were cultured with daidzein (10^{-9} to 10^{-5} M) for 10, 20 or 30 days, and the contents of cellular Ca and P were measured by ICP-AES. Figures 6(a) and 6(b) show dose-related and time-related changes in cellular Ca and P contents, respectively. Daidzein significantly increased Ca content at higher concentration than 10^{-7} M in the cells cultured for 10, 20 and 30 days ($p < 0.01$). When the cells were cultured in the presence of β -GP and L-AA from day 20 to day 30, the increased extent of Ca content in the daidzein-treated groups was higher than that from day 10 to day 20. But the dose-related and time-related profiles in Fig. 6(b) presented that the cellular P content had no similar change to the Ca content in the cells. Only daidzein at the concentration of 10^{-7} M increased the cellular P content on day 20 and day 30.

DISCUSSION

Recently, many studies in humans, animals and cell culture systems have suggested that isoflavones play an important role in the prevention of cancer, heart diseases, menopausal symptoms and osteoporosis. Daidzein is a natural isoflavone found in *Leguminosae*. Studies about the effect of daidzein on the bone formation and osteoblast function have been reported.^{13-15,17} These studies demonstrated that daidzein can stimulate the early differentiation of osteoblasts and act on osteoblasts via estrogen receptor

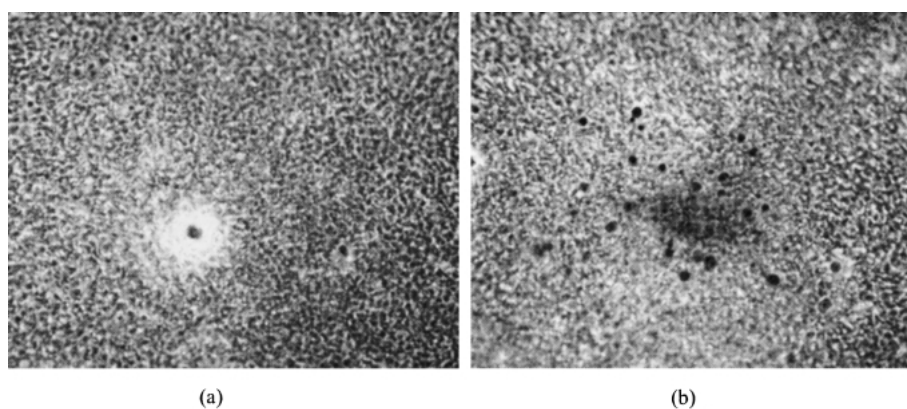


Fig. 3. Phase-contrast Micrographs ($\times 100$) of (a) Control and (b) Daidzein (10^{-6} M)-treated MC3T3-E1 Cells by Alizarin Red S Staining

Cells were treated for 28 days with daidzein. The alizarin red S solution was used to stain calcium deposition.

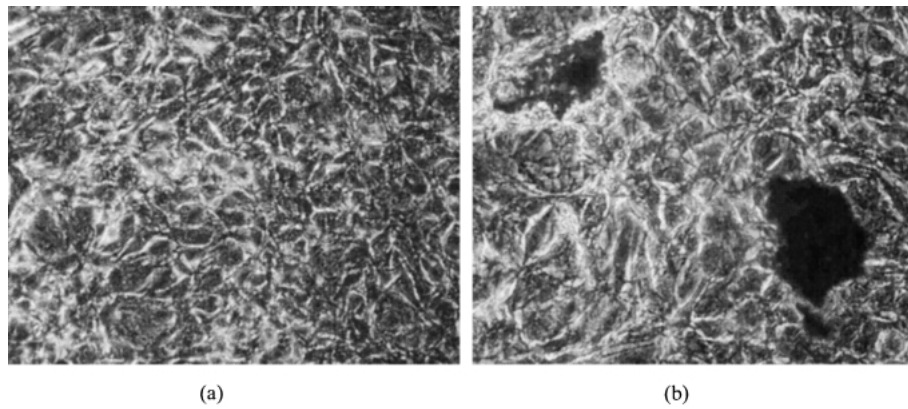


Fig. 4. Phase-contrast Micrographs ($\times 200$) of (a) Control and (b) Daidzein (10^{-6} M)-treated MC3T3-E1 Cells by Von Kossa Staining

Cells were treated for 21 days with daidzein. Von Kossa stain was used to visualize mineralized nodules

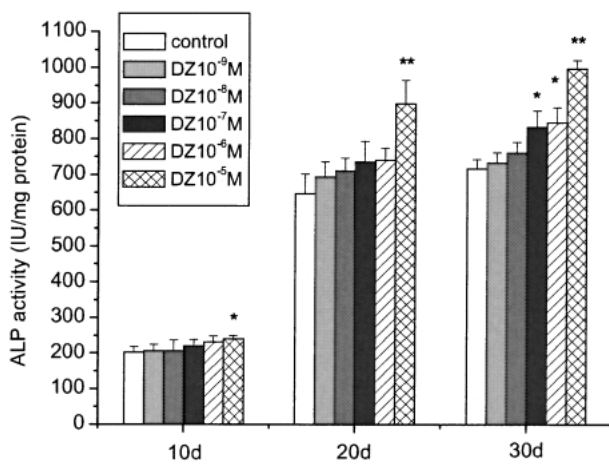
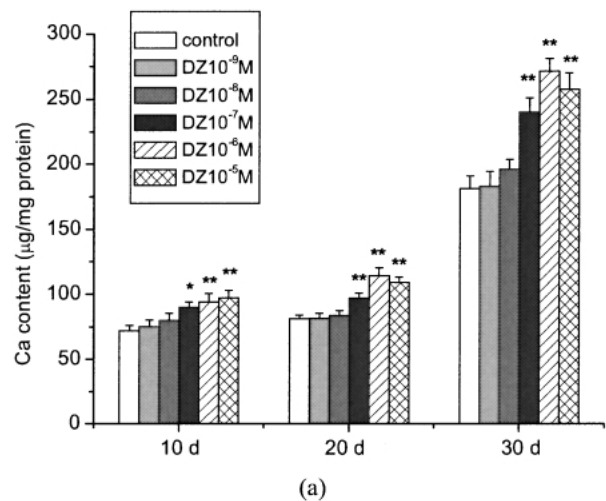


Fig. 5. Effect of Daidzein on ALP Activity in MC3T3-E1 Cells

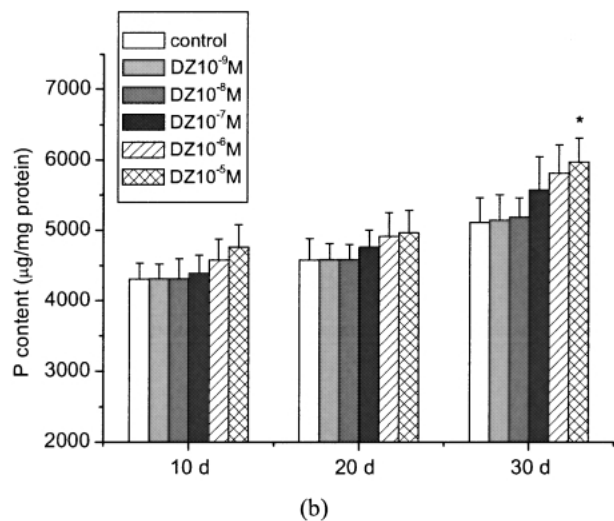
The cells were cultured in α -MEM containing β -GP (10 mM) and L-AA (50 μ g/ml) and incubated with daidzein (10^{-9} M to 10^{-5} M) for 10, 20 or 30 days. The ALP activity and protein concentration were measured according to the procedure described in the Material and Methods section. Each value is the mean \pm S.D. of quadruplicate experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared with the control value without daidzein.

pathway, but the effect of daidzein on the differentiation and mineralization of osteoblasts through long time action has not been investigated. The present study utilized the mouse osteoblastic-like MC3T3-E1 cell to analyze the effect of daidzein on osteoblastic cells *in vitro*.

MC3T3-E1 cells are pre-osteoblastic cells that undergo osteoblastic differentiation and mineralization when grown in the presence of β -GP and L-AA. It is known that proliferation, matrix maturation and mineralization are three sequential processes in the differentiation of osteoblasts. The osteoblast phenotypes are acquired in two stages. There is an initial period of proliferation and biosynthesis of the ex-



(a)



(b)

Fig. 6. Effect of Daidzein on Ca (a) and P (b) Contents in MC3T3-E1 Cells

The cells were cultured in α -MEM containing β -GP (10 mM) and L-AA (50 μ g/ml) and incubated with daidzein (10^{-9} M to 10^{-5} M) for 10, 20 or 30 days. The cellular concentration of Ca was measured by ICP-AES. The protein concentration was determined by BCA method. Each value is the mean \pm S.D. of quadruplicate experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared with the control value without daidzein.

tracellular matrix (type I collagen), followed by a period of cell differentiation in two phases.¹⁹⁾ In the first stage, the matrix matures and specific proteins associated with the bone cell phenotype such as ALP are detected. In the second stage, matrix becomes mineralized, and late markers such as osteocalcin are produced. Osteoblasts produce ALP, type I collagen, osteopontin, which are associated with matrix maturation and mineralization. Thus we examined cell proliferation, mineralization assay, ALP activity and cellular Ca and P contents to investigate the effect of daidzein on the differentiation and mineralization of osteoblast-like cells.

MC3T3-E1 cells experienced a significant increase in the cell proliferation when they were cultured with daidzein at higher concentrations for 2 days. But the effect decreased on the following day 8 and day 10 at the concentration of 10^{-7} M. Maybe it indicated that differentiation began in MC3T3-E1 cells owing to the long culture time. ALP activity and cellular Ca and P contents increased when the cells were treated with daidzein in the presence of β -GP and L-AA. In the culture time, ALP activity in all groups rapidly rose from day 10 to day 20, compared with those from day 20 to day 30. But the change of cellular Ca and P contents from day 20 to day 30 was more significant than those from day 10 to day 20. Differentiation of the cells to mature osteoblasts was prompted under incubation in the presence of daidzein (10^{-6} M) for 21 days, by the time the mineralized nodules formed. The calcification of the cells by alizarin red S staining was increased significantly after the culture with daidzein (10^{-6} M) as long as 28 days. These results were consistent with the proliferation, matrix maturation and mineralization stages in the growth of the osteoblastic-like MC3T3-E1 cells. It also presented that daidzein was able to prompt the bone formation in the early growing stage and the late growing stage of osteoblasts.

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