INTRODUCTION
Osteoporosis is characterized by decrease in bone mass and is widely recognized as a major public health problem. Nutritional and pharmacological strategies are necessary to prevent age-related bone loss. Traditional therapies for postmenopausal osteoporosis have emphasized the use of anti-resorptive agent such as estrogen, calcitonin, and bisphosphonates. Postmenopausal women develop osteoporosis, at least in part, due to estrogen deficiency and estrogen replacement therapy has been shown to reduce postmenopausal osteoporosis (Heikkinen AM et al. 1997). Recent study has shown that overall health risks from the use of combined estrogen plus progesterin therapy exceed benefits among healthy postmenopausal women (Roussouw JE et al. 2002). Furthermore, some agents that stimulate bone formation (e.g. soybean 

Coumestrol is the coumarin-like compound with a close structural relationship to stilbestrol as well as to estradiol. In a recent study, soybean extract containing mainly coumestrol was potent in suppressing urinary excretion of deoxypyridinoline and pyridinium and suppress bone resorption in ovariectomized rats (Ye SF et al. 2003). In the present study, we examined the effects of phytoestrogens: coumestrol and compared with that of the estrogens on osteoblastic differentiation and in vitro bone mineralization using primary osteoblasts cell cultures.

MATERIALS AND METHODS

For the neonatal osteoblasts cell culture, the osteblast-like cells isolated from sequential digestion of calvaria of 4-day newborn ICR mice was performed by using a modification of the methods described by Wong and Cohn (Chang WH et al. 2004). For the mature osteoblast cell culture, 8-Month ICR mice were killed, the femur and tibia bones were used to harvest the osteoblasts derived from mature adult mice. In order to assess the effects of phytoestrogen stimulation on the cellular proliferation throughout the osteoblasts maturation, a series of cultures was stimulated from 1 to 21 days of culture. The effect of coumestrol on osteoblasts differentiation was assayed by analysis of alkaline phosphatase (ALP) in culture medium and the determination of intracellular calcium content. The mRNA was isolated, their cDNA synthesized and then real-time quantitative RT-PCR (QRT-PCR) was performed to analyze the gene expression.

RESULTS

The effect of coumestrol on osteoblasts cell proliferation was examined at coumestrol concentrations of 0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M after 1, 7, 10, 12, and 21 days of culture. Osteoblasts at every concentration proliferated actively with statistically significant differences were observed between cells treated with coumestrol and that of the control (n = 12; P < 0.05). We chose 10^{-3} M coumestrol for the further evaluation because there was maximal beneficial effect on the osteoblasts counts at this concentration at 10 days of culture, which attained 139.5% of the control.

The ALP content of the control samples increased gradually from the 1st day of culture and attained its peak the 14th day of culture; while in the presence of 10^{-3} M coumestrol, the ALP content decreased significantly at the 7th, 14th, and 21st days' samples (P < 0.05), but returned to that of the control level at the 28th day's culture. The intracellular calcium was not detectable until the 14th day of culture, then the intracellular content increased persistently and reached its maximal at the 28th day's culture. The treatment of coumestrol can significantly enhance the osteoblasts differentiation and intracellular calcium precipitation. At the 14th day's culture, the calcium content of coumestrol treated samples was 4 times that of the control (P < 0.05). At the 21st and 28th days' samples, the calcium content of the coumestrol treated osteoblasts was persistent higher than that of the control group, although they did not attain a statistically significant level.

In the presence of 10^{-3} M coumestrol, type I collagen gene expression upregulated 167% at the 1st day's culture; ALP gene expression upregulated 360% at the 7th day's culture and osteocalcin gene expression upregulated 222% at the 14th day's culture.

In the positive control group, adult mice osteoblasts were cultured in the presence of 10^{-3} M estradiol, the effect of estradiol on the osteoblasts attained its maximal effect at the 21st day’s culture with 50% increase in the cell population (P < 0.005). When adult mice osteoblasts were cultured in the presence of 10^{-3} M coumestrol, the osteoblasts population increased significantly since the 7th day’s culture and attained its maximal effect at the 21st day’s culture which was 207.4% of control group (P < 0.0005).

In control samples of neonatal mice osteoblasts, the expression of OPG gene increases as the culture period increased, while the expression of OPG gene attained its maximum at the 7th day’s culture. The ratio of OPG/OPGL reached peak value at the 21st day’s culture. In control samples of 8 month-old mice osteoblasts, both the expression of OPGL and OPG gene were much higher than that of neonatal mice osteoblasts and increased and reached their maximum at the 14th day’s culture. The ratio of OPG/OPGL reached peak value at the 7th day’s culture. In control samples of neonatal mice osteoblasts, the expression of ER-α gene attained its maximal level at the 7th day’s culture, while the expression of ER-β gene attained its maximum at the 21st day’s culture. In presence of 10^{-3} M coumestrol, ER-α gene expression reached its maximum at the 7th day’s culture and ER-β gene expression reached its maximum at the 21st day’s culture. For the neonatal mice osteoblasts cultured in the presence of 10^{-3} M coumestrol, both the ER-α and ER-β expression were lower than that of control at the 21st day’s culture; while these data were increased in the 8 month-old mice osteoblasts culture.

DISCUSSION

Phytoestrogens, which have a structural similarity to 17β-estradiol, are reported to act as agonists/antagonists of estrogen in animals and humans (Kuiper G et al., 1998). In this study, we have employed both neonatal and adult mice osteoblasts cultures to characterize relative gene expression profiles during osteoblasts differentiation. We found that coumestrol can stimulate of neonatal and adult mice osteoblasts proliferation and mineralization. Similar to that described by De Wilde et al., the content of ER-β and OPG secretion by neonatal mouse bone cells gradually increased during osteoblast differentiation, whereas the ER-α and OPG content decreased (De Wilde A et al. 2004). However, the cellular responses to the estradiol and coumestrol were quite different in the neonatal or adult mice origin of osteoblasts.

REFERENCES