·论著·

Effects of coumestrol on expression of bone markers during primary osteoblastic cells differentiation

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Abstract：**Objective** To observe the effect of phytoestrogen coumestrol on the function of osteoblast including ALP and osteocalcin type I collagen, OPG and RANKL. **Methods** Osteoblasts obtained from fetal mouse calvaria and were incubated with coumestrol (0, 10^{-9}-10^{-5} M) or 17β-Estradiol (10^{-8} M) for 48 hours. Alkaline phosphatase (ALP) activity and type I collagen contents was measured by enzyme digestion. Osteocalcin production was measured by radioimmunity. OPG and RANKL mRNA level were detected by RT-PCR and OPG protein was determined by Western Blot. **Results** Treated with coumestrol for 48 hours, ALP activity and type I collagen contents was dose-dependently increased by all kinds concentration of coumestrol and reached peak at 10^{-6} M ($P < 0.05$) and then decreased slightly when cotreated with 10^{-5} M coumestrol. Coumestrol increased osteocalcin expression slightly, but there were no significant differences among groups. Coumestrol increased OPG mRNA expression and protein production in dose-dependently manner ($P < 0.05$) and slightly decreased RANKL mRNA expression. **Conclusions** Coumestrol has an enhancing effect on the proliferation and differentiation of osteoblasts, at least in part, by stimulating OPG/RANKL expression in osteoblasts.

Key words：Coumestrol；Osteoblasts；Osteoprotegerin；Osteoporosis

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香豆雌酚对成骨细胞分化过程中骨指标表达的影响 吴小涛，韦继南，李永刚，等. 东南大学附属中大医院骨科，江苏 南京 210009

摘要：**目的** 观察植物雌激素香豆雌酚对成骨细胞增殖分化的作用并探讨其作用机制。**方法** 从小鼠颅盖骨获得成骨细胞并用 0，10^{-9}～10^{-5} M 香豆雌酚孵育 48 h，以 17β 雌二醇为阳性对照，用酶消化法测定碱性磷酸酶及Ⅰ型胶原含量，放免法测定骨钙素含量，RT-PCR 法测定 OPG 及 RANKL mRNA 表达情况，Western Blot 测定 OPG 蛋白含量。**结果** 干预 48 h，不同浓度香豆雌酚呈剂量依赖性增加碱性磷酸酶及Ⅰ型胶原含量，10^{-6} M 时达到最大效应($P < 0.05$)，但 10^{-5} M 效应有所降低，香豆雌酚轻度增加成骨细胞骨钙素含量，各组间无统计学差异。香豆雌酚呈剂量依赖性增加 OPG 基因及蛋白的表达($P < 0.05$)，轻度降低 RANKL 基因的表达。**结论** 香豆雌酚能增加成骨细胞增殖及分化，可能其部分通过 OPG/RANKL 发挥作用。

关键词：香豆雌酚；成骨细胞；OPG；骨质疏松

Estrogen can modulate serial cytokines that regulate bone metabolism therefore plays an important role in the postmenopausal osteoporosis. Coumestrol, which was found recently, is a naturally occurring plant estrogen[a]. It is reported that coumestrol can reduce bone loss and bone resorption in ovariectomized rats and stimulate osteoblast proliferation and mineralization[b]. However, the mechanism of action of the coumestrol in osteoblasts has not been elucidated. The purpose of this *in vitro* study was to observe the effect of coumestrol on proliferation and differentiation of fetal mouse osteoblasts and find the mechanism of coumestrol to the osteoporosis.

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1 Materials and Methods

1.1 Materials

DMEM, FBS and TRIzol were purchased from Gibco-BRL. ALP kit was purchased from Nanjing Jiangcheng Bioengineering Institute. Osteocalcin test kit was purchased from Beijing Chemclin Biotech Co. Ltd. RT-PCR kit and β-actin were purchased from Fermentas Inc. USA. Coumestrol, 17β-estradiol and Trypsin were purchased from Sigma. OPG (N-20) goat polycolonal lgG was purchased from Santa Cruz Biotechnology.

1.2 Cell culture

Primary osteoblast cell cultures were prepared from neonatal rat calvarias through the sequential trypsin/collagenase digestion method[1]. P_3 cells were used in our experiment. Experiments were initiated by seeding 2×10^5 cells per well in flat-bottom 6-well plates. All experiments were replicated with 8 wells for each treatment. The cells were treated with coumestrol (0, 10^{-9} M-10^{-5} M) or 17β-Estradiol (10^{-8} M) for 24 h, 48 h or 72 h according to the schedule.

1.3 Assay of ALP activity and type I collagen content

After treatment with coumestrol, the supernatant was discarded and the cell layer was washed twice with PBS. The cells were collected by a tuber scraper. Then, cells were disrupted (3×7 s) with ultrasonic disrupter in water bath at 0℃. The specimen was collected and centrifuged at 12000 g at 4℃ for 10 min. The ALP activity was determined in the supernatant using ALP colorimetric assay kit. Absorbance was obtained at 520 nm against a blank prepared with distilled water to determine the concentration of der quinine. Total protein concentrations were determined by Bradford protein assay method. The ALP activity was normalized to the protein content.

Type I collagen content was detected by enzyme digestion. Absorbance was measured spectrophotometrically at 550 nm and the collagen content was calculated from a standard curve and expressed as μg/ml, normalized by the number of cells counted at 48 h.

1.4 Assay of osteocalcin production

At the end of the treatment period, the culture medium was collected for osteocalcin assaying. Osteocalcin released into the culture media was measured using a specific radioimmunoassay kit according to the manufacturer's instructions. To normalize the protein expression of total cellular protein, protein from the lysate solution was quantified by a Bradford assay.

1.5 Reverse transcription polymerase chain reaction (RT-PCR).

Total cellular RNA was extracted by Tri-Reagent and 2 μg RNA was reverse transcribed. Primers: OPG: forward, 5′-TCC TGG CAC CTA CCT AAA ACA GCA-3′; reverse, 5′-CTA CAC TCT CGG CAT TCA CTT TGG-3′; RANKL: forward, 5′-ATG ATG GAA GGC TCA TGG TTG-3′; reverse, 5′-TGT TGG CGT ACA GGT AAT AGA A-3′. Amplifications were performed in a GeneAmp 9600 thermal cycler for 30-33 cycles (typically 94℃ for 1 min, 55℃ for 2 min, 72℃ for 1 min) after an initial denaturation at 94℃ for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. All signals were quantified by scanning densitometry, and the OPG/β-actin and RANKL/β-actin ratios were calculated.

1.6 Western blot analysis

At the completion of the incubation period, cells were harvested after removing the media, washing twice with PBS, and then scraped into appropriate volumes of ice-cold Triton buffer, sonicated and centrifuged at 12000 g at 4℃ for 15 min. Protein concentrations were determined by Bradford protein assay method. Fifty micrograms of protein from each group was loaded onto a 10% polyacrylamide gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to PVDF membranes. The membrane was blocked with 5% nonfat dry milk in PBS plus 0.1% Tween-20 and incubated with goat polycolonal antibody OPG(N-20) in PBS overnight at 4℃. Then, the membrane was incubated with rabbit anti-goat IgG conjugated with horseradish peroxidase at 1:1000 in PBS for 1 h. Blots were detected through the ECL system(Pierce, Rockford, IL, USA), according with the manufacturer's instructions and the signals were quantified by scanning densitometry.

1.7 Statistical analysis

The data were expressed as the means ± SD. Differences between each treated group and controls were analyzed by one-way ANOVA, and $P < 0.05$ was considered statistically significant.

2 Results

The neonatal rat calvarias osteoblastic cells display a characteristic pattern of gene expression and protein production of various osteoblastic differentiation markers (type Ⅰ collagen, alkaline phosphatase, and osteocalcin) that were developmentally regulated over time in culture and were stimulated by differentiating agents such as dexamethasone and vitamin D, indicating that they represent the mature osteoblastic phenotype[2]. In this experiment, dose-response and time-course experiments were performed to determine the effects of coumestrol on neonatal rat calvarias osteoblastic cells with negative control (zero concentration) and positive control (17β-estradiol 10^{-8} M).

As show in Fig.1, ALPase activity was elevated in a dose-dependent manner after being treated with coumestrol (0, 10^{-9} M-10^{-5} M) for 48 h. It reached peak at 10^{-6} M of coumestrol and then decreased slightly. The increment in ALPase activity of all groups was significant and the max effect attained 155.2% compared to the negative control ($P < 0.05$). Also, coumestrol increased type I collagen synthesis in a dose-dependent manner. At the most effective dose of coumestrol (10^{-6} M), the synthesis of type I collagen was 128.9% compared to the negative control (Fig.2).

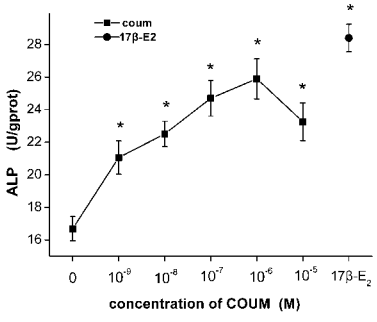

Fig.1 Effects of coumestrol on the ALPase activity in osteoblasts

However, coumestrol dose-dependent slightly increased osteocalcin production in various concentrations of coumestrol. But there were no significant differences among all groups (Fig.3).

As shown in Fig.4, treatment of osteoblasts with various concentrations of coumestrol for 48 h resulted in a dose-dependent increase in the steady-state levels of OPG

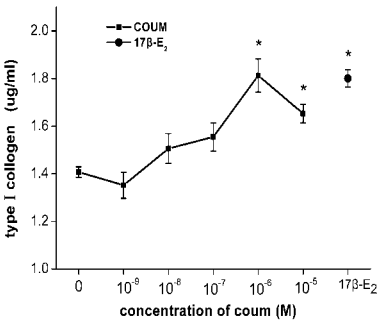

Fig.2 Effects of coumestrol on the type I collagen content in osteoblasts

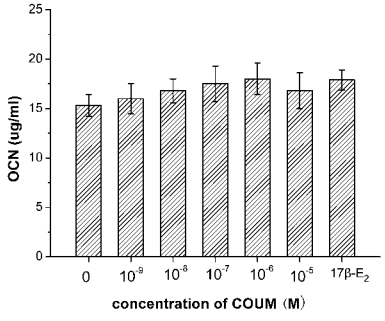

Fig.3 Effects of coumestrol on the osteocalcin production in osteoblasts

mRNA and protein production, expressed as a relative ratio to β-actin as an internal control. B-actin expression did not change significantly with coumestrol treatment in present experiment. At the most effective dose of coumestrol (10^{-6} M), the induction of OPG mRNA level and protein concentration was 279.5% and 235.3% compared to the negative control ($P < 0.05$; $P < 0.01$), respectively. However, the OPG protein production became lower at the highest concentration of coumestrol (10^{-5} M).

RANKL mRNA lever were also detected in our experiment. Nevertheless, treatment of osteoblasts with various concentrations of coumestrol for 48 h, RANKL mRNA level only increased slightly and then decreased. There were no significant differences of all groups compared to the negative control.

3 Discussion

Estrogens have major beneficial effects on bone[3]. The primary mechanism of estrogens is to decrease bone resorption, which has been attributed to direct inhibitory effect on osteoclasts, the suppression of bone-resorbing

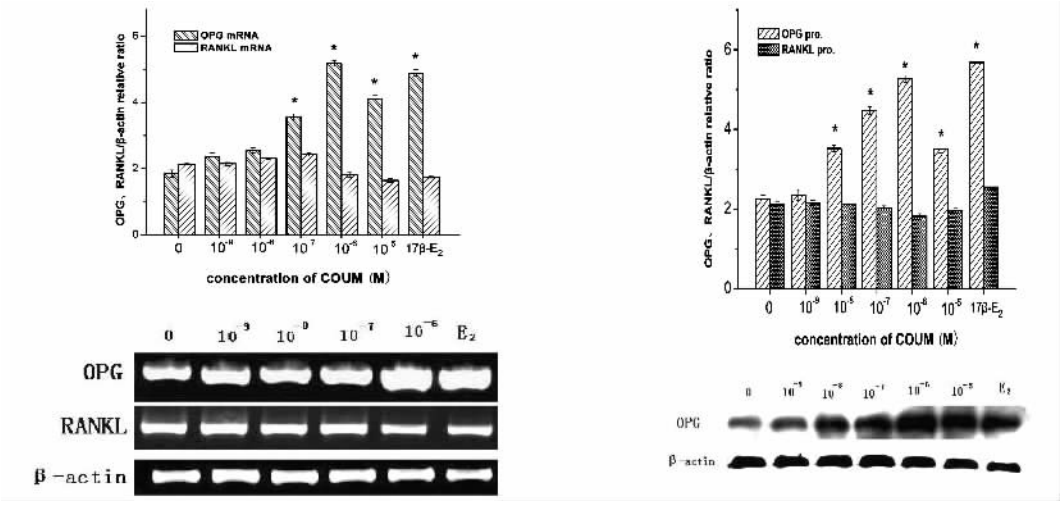

Fig.4 Effects of coumestrol on RANKL and OPG mRNA level and protein expression on osteoblasts
The OPG content increased gradually from zero to 10^{-6} M and attained peak at 10^{-6} M both in mRNA and protein; while decrease at 10^{-5} M

cytokines, and the stimulation of anti-resorptive factors[4]. However, because of the concomitant side-effect of exogenous administration, alternative to the conventional estrogen replacement therapy are desirable[5]. Phytoestrogen such as daidzein, genistein, and coumestrol are natural compounds that structurally and functionally mimic mammalian estrogens and therefore are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis[6]. Several studies have shown that phytoestrogens increase bone mass density and prevent bone loss both *in vivo* and *in vitro*[7]. However, the mechanisms of phytoestrogen action on bone cells are only partially understood. In addition, RANKL and OPG are the essential regulators of osteoclast biology and bone resorption[8] and are modulated by various cytokines and hormone known to affect bone metabolism[9]. The purpose of this *in vitro* study was to characterize the actions of coumestrol on bone marker synthesis during cell differentiation of neonatal rat calvarias osteoblastic cells. Based on the changes of osteoblastic phenotype during neonatal rat calvarias osteoblastic cells differentiation, the possible roles of ERs and OPG in osteoblastic cells following coumestrol treatments will be examined.

Coumestrol, a common phytoestrogen derived from clover and cabbage, exerts its effects on osteoblast-like cells at dietarily achievable concentrations, and the beneficial effects of coumestrol may be partly related to inhibition of osteoclastogenesis as mediated by cytokine production in osteoblasts. which is supported by the finding that estrogen loss increases the activation of osteoclasts[10].

In the present study, coumestrol acts on osteoblasts to promote cell proliferation in time- and dose-dependent manner by MTT (data not showed). The effective dose range for the actions of coumestrol in this assay system was from 10^{-9} M to 10^{-5} M. The most effective dose and time for the action of coumestrol was 10^{-6} M at 48 h, which was similar to that observed with 17β-E_2. But at the highest doses (10^{-5} M) the efficiency became lower, maybe it is because high concentration of coumestrol was cytotoxic.[c] It is likely that coumestrol increase cell proliferation and this may be due to estrogenic activity of coumestrol on the osteoblast cells. Our results are in accordance with the study of Kanno[11].

Proliferation, matrix maturation, and mineralization are three sequential processes in the mature of osteoblasts[12]. It is known that osteoblasts produce ALP, type I collagen, and osteocalcin, which are associated with matrix maturation and mineralization[13]. ALP is the most widely accepted marker of osteoblast differentiation[14]. Although the exact role of the membrane-bound alkaline ALP (bone-specific ALP) is unknown, the enzyme attached to the glycosyl-phosphatidylinositol moieties located on the outer cell surface may play a role in osteoid mineralization[15]. Therefore,

we examined the effect of coumestrol on the ALP activity of freshly isolated osteoblastic cells from neonatal rat calvaria. Our results in the present study suggested that ALP activity significantly increase in dose-dependent manner co-treated with various concentrations of coumestrol (Fig.1). Recently, Kanno[11] also reported that phytoestrogen coumestrol increased cell proliferation and ALP activity and enhanced bone mineralization in MC3T3-E1 cells. It is reported that soy extract or resveratrol, one of the phytoestrogens, increased cell proliferation and ALP activity in MC3T3-E1 cells and these effects were mediated through an estrogenic action, since the effects were blocked by tamoxifen, an estrogen receptor antagonist[16].

During the osteoblast maturation stages, osteoblasts synthesize two types of extracellular matrix proteins: the collagens, mostly type Ⅰ collagens which resemble 90% of the bone matrix proteins, and the noncollagenous proteins, including osteocalcin. In current study, coumestrol increased the activity of type I collagen content in a dose-dependent manner. At most effective dose of coumestrol (10^{-6} M for 48 h), the induction of type I collagen was 1.28-fold compared to the negative control. Therefore, it is likely that the coumestrol play an important role in the maturation of osteoblasts.

Osteocalcin, which also know as bone gla-protein, is a hydroxy-apatite-binding protein that is exclusively synthesized by the osteoblasts, odontoblasts and hypertrophic chondrocytes, and is expressed mainly during the osteoid mineralisation phase of bone formation[17]. Osteocalcin is currently used in clinical practice and experiment as a specific biomarker of bone formation. In our current study, 10^{-9} M to 10^{-5} M coumestrol co-treated with osteoblasts slightly increased osteocalcin content of culture media in dose-dependent fashion. But there were no significant differences among groups. Our result was supported by a previous report by Luo[18], where estradiol had no influence on osteocalcin production in MG-63 cells. However, our experiment period was very short, maybe it shows anther results while prolong experiment period.

The expression of RANKL and OPG by osteoblastic cells is modulated by various osteotropic factors, including commonly employed drugs such as estrogens, glucocorticoid and bisphosphonate[19]. We also observed a stimulation of OPG production by 17β-E_2, bisphosphonate, dexamethasone, PE_1, PE_2 and so on in our previous studies. In this study, our findings provide the first evidence that coumestrol acted on osteoblastic cells to increase the production of OPG. Both coumestrol stimulated OPG mRNA steady state levels and protein production in a dose-dependent fashion by 2.79- to 2.35-fold respectively. However, the OPG content reduced when co-treated with 10^{-5} M coumestrol, which in part maybe due to the cytotoxic effect. Furthermore, RANKL mRNA level was not change obviously between all concentration groups. Together, our data indicated that the increase of OPG production following coumestrol treatment meet the criteria for a specific and physiologically relevant response. Nevertheless, RANKL expression has been shown to decrease during human osteoblastic differentiation[20]. Thus, similar to 17β-estradiol[21], the primary effect of coumestrol on decreasing the RANKL-to-OPG ratio in the bone microenvironment is to enhance OPG production. A local increase of OPG levels in the bone microenvironment may be an important component of the paracrine mechanisms by which coumestrol increase bone formation and reduce bone resorption. Since OPG production is a function of osteoblastic cell maturation, enhancement of OPG expression by coumestrol may, at least in part, be related to the stimulate effect of coumestrol on osteoblastic differentiation. Therefore, it is likely that coumestrol can up-regulate the gene expression and protein production of OPG and these effects are due to the estrogenic activities of coumestrol on the osteoblast cells.

However, OPG production was measured only in osteoblastic cells and it is possible that levels of secreted OPG in the culture medium may differ between treated and untreated cultures. Further work is required to determine this fact. Of course our data do not exclude the contribution of other unknown bone-resorbing and pro-inflammatory osteoblastic cytokines and growth factors, which may be modulated by coumestrol.

In summary, the data described in this report suggested that coumestrol stimulate the osteoblastic mature and enhance OPG mRNA and protein expression by osteoblasts, therefore elevate the OPG/RANKL radio.

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