Diethylstilbestrol can induce bone marrow stromal cells to differentiate into osteoblasts

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Abstract

BACKGROUND: There are fewer reports about estrogen effects on bone marrow stromal cells (BMSCs).

OBJECTIVE: To study the effect of diethylstilbestrol on the osteogenic differentiation of rabbit BMSCs.

METHODS: Rabbit BMSCs cultured in vitro were intervened with 0, 10^{-7}, 10^{-6}, 10^{-5} mol/L diethylstilbestrol, and BMSCs cultured with dexamethasone 10^{-8} mol/L, β-sodium glycerophosphate 10 mmol/L, and vitamin C 50 mg/L were used as positive controls.

RESULTS AND CONCLUSION: 10^{-6} mol/L diethylstilbestrol significantly improved the proliferative ability of BMSCs at 24, 48, and 72 hours after intervention ($P < 0.01$). 10^{-5} mol/L diethylstilbestrol significantly inhibited the proliferation of BMSCs at 48 hours after intervention as well as 10^{-7} mol/L diethylstilbestrol at 72 hours ($P < 0.01$). Mineralized nodular structures formed at 25 days after intervention with 10^{-7} mol/L diethylstilbestrol. Alkaline phosphatase activities were remarkably increased at 14 and 21 days after intervention with 10^{-7}, 10^{-6} mol/L diethylstilbestrol. It has been proved that diethylstilbestrol has an enhancing effect on the osteogenic differentiation of rabbit BMSCs.

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INTRODUCTION

Studies have demonstrated that Asians who have eaten phytoestrogen-enriched food have a lower incidence of breast cancer, prostate cancer and osteoporosis than Caucasians. However, the effect of estrogen on bone marrow stromal stem cells (BMSCs) has been less reported[1]. To observe the direct effect of estrogen on BMSCs, this experimental study was designed to treat rabbit BMSCs with diethylstilbestrol and to observe the effect of diethylstilbestrol on the osteogenic differentiation of rabbit BMSCs so as to understanding the role and action mechanism of estrogen on the skeletal system.

MATERIALS AND METHODS

Design

A comparative observation.

Time and setting

The experiment was completed at the Central Laboratory of Jining Medical College from October 2010 to January 2011.

Materials

One-week-old New Zealand rabbits were supported by the Animal Center of Jining Medical College. The main reagents are listed as follows.

Main reagent	Source
Low-glucose DMEM (LG-DMEM)	Gibco BRL
Fetal bovine serum (FBS)	Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China
Automatic biochemical analyzer, alizarin red kit	Beijing Chemclin Company
TRIzol	Katara Ltd., USA
Diethylstilbestrol	Wuhan Yuancheng Science Developmental Co., Ltd., China

Methods

BMSCs extraction[2-3]: A 1-week-old New Zealand long-eared white rabbits was sacrificed to obtain bilateral femur and tibia after soaking with 75% ethanol for 10 minutes and povidone-iodine disinfection to remove epiphyseal ends and expose the bone marrow cavity. DMEM medium was used to wash the bone marrow cavity, and bone marrow cells were collected. The nucleated cells were separated by using Percoll method, centrifuged at 1 000 r/min for 5 minutes, and rinsed with DMEM once then to collect BMSCs for primary culture.

Primary culture of BMSCs[4-6]: The harvested BMSCs with high purity were resuspended in DMEM medium. 1×10^9/L cell suspension was incubated into a 50 mL cell culture bottle, and then placed into a humidified incubator at 37 ℃, 5% CO_2. During the primary culture, the cells in the culture bottle were observed at 8, 16, 24 and 48 hours under an inverted microscope for cell adhesion and growth. The medium at 48 hours after primary culture was semi-changed. After that, the medium was exchanged every 3 to 4 days.

Passage culture of BMSCs[7-8]: When 85%−95% cells were confluent, the cells were subcultured. Firstly, the DMEM medium was removed, and the cell suspension were rinsed with PBS twice followed by 1 minute digestion with 1 mL of 2.5 g/L trypsin. When pseudopod retraction appeared under the in inverted microscope, the trypsin was discarded and 3 mL DMEM was added for repeated pipetting and full separation of the adherent cells. The cells were incubated into a new bottle at a density of 3×10^3 cells/cm^2. The medium was changed fully every 3 to 4 days for timely subculture.

BMSCs morphology under the inverted phase contrast microscope: The inverted phase contrast microscope was used to observe the daily changes in BMSCs morphology and growth and to take the picture.

MTT assay for cell proliferation: After digestion, the cells were seeded onto 96-well plates with a density

of 2.5×10^8/L were seeded in 96 well plates, 200 μL for each well. Forty-eight hours later, the cells were cultured in serum-free medium for 24 hours, and then the cells were incubated with 10^{-7}, 10^{-6}, 10^{-5} mol/L diethylstilbestrol (8 wells for each concentration). BMSCs cultured in DMEM medium containing dexamethasone 10^{-8} mol/L, β-sodium glycerophosphate 10 mmol/L, and vitamin C 50 mg/L were used as controls. After intervention of 24, 48 and 72 hours, a culture plate was selected, 20 μL of 5 g/L MTT was added per well. The cells were cultured at 37 ℃ for 4 hours, then cultured with 150 μL DMSO sulfone per well, and shocked on a micro-oscillator oscillation for 10 minutes. Absorbance (A) values at 490 nm were detected, expressed as A_{490}.

Calcified nodules observation (alizarin red staining): After digestion, the cells at 2.5×10^8/L were inoculated into 50 mL cell culture bottles, and grouped as above. After 25 days intervention, mineralized nodules formed that were rinsed with PBS twice, fixed with 95% ethanol for 10 minutes, washed with distilled water three times, and then dyed with 0.1% alizarin red-Tris-Hcl (pH 8.3) at 37 ℃ for 30 minutes. Following distilled water washing, drying, and cementing, the mineralized nodules were Observed and photographed under a microscope, showing mineralized nodules were orange-red.

Alkaline phosphatase detection: After digestion, the cells at 2.5×10^8/L were inoculated into 50 mL cell culture bottles, and grouped as above (five bottles for each concentration). After intervention of 7, 14 and 21 days, the supernatant was removed, the plates were rinsed with PBS twice and shaken by addition of 0.1% Triton X-100 followed by a centrifugation at 1 000 r/min for 3 minutes. Alkaline phosphatase activity was detected using the automatic biochemical analyzer.

Main outcome measures

BMSCs morphology under inverted phase contrast microscope; different doses of diethylstilbestrol effect on BMSCs proliferation at different time points; different doses of diethylstilbestrol effect on alkaline phosphatase activity of BMSCs at different time points.

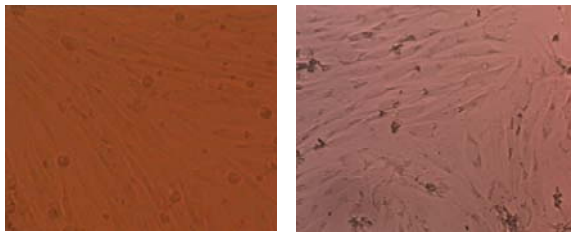
Statistical analysis

The data were analyzed using SPSS 16.0 statistical software and expressed as mean±SD. One-way analysis of variance was done for multiple group comparison and *t* test for intergroup comparison. A value of $P < 0.05$ was considered significant.

RESULTS

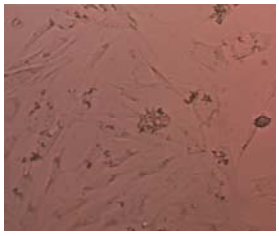
BMSCs morphology under inverted phase contrast microscope

After incubation, BMSCs were in spherical shape, suspended in culture medium and began to adherent growth at 8−10 hours. Early adherent cells were mostly irregular or short spindle followed by faster growth, and the morphology was also changed to long fusiform. After about 8 days, the adherent cells covered 85% to 90% (Figure 1a). Then, the cells began to passage. After passage, the cell growth was accelerated. For the 1st−4th generations, the cells passaged once within 5−7 days and presented with spiral-shaped growth (Figure 1b). After the 5th generation, the cells passaged once within 7−9 days. After induced culture of 14−17 days, the cells were fused into a single layer presenting with round or polygonal shape similar to osteoblasts (Figure 1c). Mineralized nodules were seen at 25−27 days after culture, shown in Figure 1d.

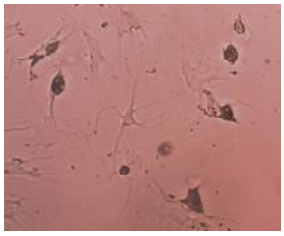


a: Adherent cells with fusiform shape covered 85%−90% (×200)

b: Spiral growth of cells (×200)



c: Cell fusion into a single layer, which was round or polygonal shape similar to osteoblasts (×200)



d: Formation of mineralized nodules after culture (×400)

Figure 1 Morphology of bone marrow stromal stem cells under inverted phase contrast microscope

Effect of different-concentration diethylstilbestrol on BMSCs proliferation at different time points

There was significant difference in BMSCs proliferation after intervention with 10^{-6} mol/L diethylstilbestrol at 24, 48, 72 hours ($P < 0.01$). 10^{-5} mol/L diethylstilbestrol significantly inhibited the proliferation of BMSCs at 48 hours after intervention as well as 10^{-7} mol/L diethylstilbestrol at 72 hours ($P < 0.01$).

Table 1 Effect of different-concentration diethylstilbestrol on proliferation of bone marrow stromal stem cells at different time points ($\bar{x}$±s)

Group	24 h	48 h	72 h
10^{-5} mol/L	0.032 0±0.002 6	0.029 9±0.002 0	0.041 6±0.004 7
10^{-6} mol/L	0.034 8±0.005 3	0.053 4±0.003 0	0.089 4±0.005 0
10^{-7} mol/L	0.032 7±0.005 1	0.030 1±0.002 0	0.024 9±0.002 0
Control	0.030 3±0.002 3	0.033 8±0.003 1	0.041 0±0.004 5

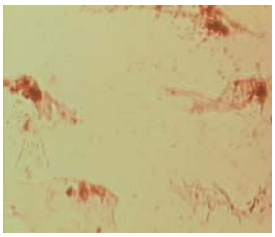
Effect of different-concentration diethylstilbestrol on the osteogenic differentiation of BMSCs

Calcified nodules observation: After 10^{-8} mol/L diethylstilbestrol intervention of 25 days, alizarin red staining was performed. Many orange-red nodules appeared in the site of cell aggregation (Figure 2a); while no orange-red nodules were found without diethylstilbestrol intervention (Figure 2b).

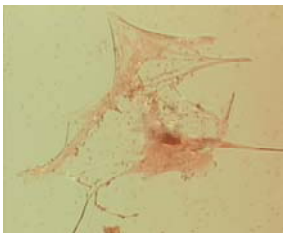
Effect of different-concentration diethylstilbestrol on alkaline phosphatase activity of BMSCs

After 7 days of intervention, there was no difference in the alkaline phosphatase activity. After 14 days of intervention

with 10-6 mol/L and 10^{-7} mol/L diethylstilbestrol, the activity of alkaline phosphatase significantly increased, but inhibited after intervention of 10^{-5} mol/L diethylstilbestrol for 14 days ($P < 0.01$). After intervention of 21 days, 10^{-6} mol/L diethylstilbestrol significantly increased the activity of alkaline phosphatase, and 10^{-5} mol/L diethylstilbestrol obviously inhibited it ($P < 0.01$), seen in Table 2.



a: Many orange-red nodules appeared in the site of cell aggregation after 10^{-8} mol/L diethylstilbestrol intervention (×200)



b: No orange-red nodules were found with no diethylstilbestrol intervention (× 400)

Figure 2 Observation of calcified nodules

Table 2 Effect of different-concentration diethylstilbestrol on alkaline phosphatase activity of bone marrow stromal stem cells ($\bar{x}$±s, nkat/L)

Group (mol/L)	Alkaline phosphatase activity		
	7 d	14 d[a]	21 d[a]
0	255.05±31.17	268.39±17.50	298.39±18.67
10^{-7}	456.76±24.84	636.79±31.01	826.83±26.34
10^{-6}	396.75±28.67	718.48±19.84	1 391.94±23.50
10^{-5}	435.09±22.50	246.72±27.12	226.71±22.67
Negative control	476.76±28.84	718.48±20.67	1 125.22±18.00

[a]$P < 0.01$, comparison among groups

DISCUSSION

The current studies have confirmed that estrogen can promote osteoblasts and inhibit osteoclasts[9], but its direct role in BMSCs is reported rarely. BMSCs are a class of multi-differentiation potential stem cells, and in certain conditions can be induced to differentiate into osteoblasts[10]. Thus, we studied the effect of direct intervention of diethylstilbestrol on osteogenic differentiation of BMSCs. Wang *et al*[11] performed experimental studies regarding the effect of coumestrol on BMSCs and the results showed that 10^{-8},10^{-7} mol/L coumestrol could significantly promote the proliferation of BMSCs in rats; 10^{-5} mol/L coumaric phenol played an inhibitory effect on cell proliferation after intervention for 24 and 72 hours; after intervention with 10^{-8} mol/L coumestrol for 25 days, calcified nodules formed. In BMSCs treated with 10^{-8},10^{-7} mol/L coumestrol, alkaline phosphatase activity was increased significantly after 14 and 21 days, and Hyp increased remarkably after 28 days. After intervention with 10-8 coumestrol for 28 days, OC expression had a significant increase; however, OC expression was inhibited after intervention with 10^{-5} mol/L coumestrol. 10^{-7} mol/L coumestrol could up-regulate the expression of OPG mRNA and protein as well as increase OPG/RANKL relative gene expression in BMSCs after 7 and 14 days intervention. Taken together, coumestrol can promote the proliferation and osteogenic differentiation of BMSCs and exert a protective effect on the the skeletal system through regulation of OPG/RANKL expression.

In the present study, 10^{-6} mol/L diethylstilbestrol intervention for 24, 48, 72 hours significantly promoted the proliferation of BMSCs ($P < 0.01$). 10^{-5} mol/L diethylstilbestrol intervention for 48 hours significantly inhibited cell proliferation as well as 10^{-7} mol/L intervention for 72 hours ($P < 0.01$). After intervention with 10^{-7} mol/L diethylstilbestrol for 25 days, calcified nodules appeared; and after intervention with 10^{-7}, 10^{-6} mol/L diethylstilbestrol for 14 and 21 days, alkaline phosphatase activity increased significantly. It has confirmed that diethylstilbestrol can promote differentiation of BMSCs into osteoblasts.

Another experimental studies have shown that estrogen can reduce apoptosis of osteoblasts so as to enhance the function of osteoblasts[12-15]. Tang *et al*[16] found that lipopolysaccharide (LPS) strengthened the expression of Bax and Fas in osteoblasts, and had no influence on the expression of Bcl-2; estrogen could inhibit the LPS-enhanced Bax and Fas expression, and while enhanced the expression of Bcl-2 to significantly increase Bcl-2/Bax ratio so as to inhibit the LPS-induced osteoblast apoptosis. In addition, estrogen may promote osteoblast OPG gene expression to induce the proliferation and osteogenic differentiation of BMSCs[17-21]. In the study of Ouyang and his colleagues[22], 17β-estradiol was found to promote the expression of human osteoblast OPG gene. OPG can promote the proliferation and differentiation of BMSCs into osteoblasts[23-25]. Wang *et al*[26] found that estrogen-induced cytokines could promote the proliferation and osteogenic differentiation of BMSCs. It can be seen through a variety of ways estrogen can facilitate the proliferation and osteogenic differentiation of BMSCs.

In summary, diethylstilbestrol can promote the proliferation and osteogenic differentiation of BMSCs through increasing the OPG/RANKL relative gene expression, inhibiting osteoblast apoptosis and promoting the production of multiple cytokines, providing a theoretical basis for BMSCs as seed cells to repair bone defects. But the in vivo effect of estrogen on BMSCs and how to reduce estrogen-induced adverse effects on the human body needs further studies.

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乙烯雌酚可诱导骨髓基质干细胞向成骨细胞分化

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摘要

背景：关于雌激素对骨髓基质干细胞作用的报道尚不多。

目的：观察乙烯雌酚对兔骨髓基质干细胞成骨分化的影响。

方法：体外培养骨髓基质干细胞，用0，10^{-7}，10^{-6}，10^{-5} mol/L浓度乙烯雌酚干预，并设地塞米松10^{-8} mol/L、β-甘油磷酸钠10 mmol/L、维生素C 50 mg/L为阳性对照。

结果与结论：乙烯雌酚干预培养24，48，72 h，10^{-6} mol/L组显著促进了骨髓基质干细胞增殖($P < 0.01$)。干预48 h 10^{-5} mol/L组显著抑制细胞增殖，干预72 h 10^{-7} mol/L组显著抑制细胞增殖($P < 0.01$)。10^{-7} mol/L组乙烯雌酚干预25 d后开始出现钙化结节；10^{-7}，10^{-6} mol/L组干预14，21 d碱性磷酸酶活性显著增加。证实乙烯雌酚能促进兔骨髓基质干细胞的成骨分化。

关键词：乙烯雌酚；骨髓基质干细胞；成骨分化；增殖；碱性磷酸酶
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