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Effect of different concentrations of medroxyprogesterone acetate combined with 17β-estradiol on endothelial progenitor cells

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Abstract. – OBJECTIVE: Endothelial progenitor cells (EPCs) have the ability to differentiate into mature endothelial cells. Inhibition of EPC proliferation and migration may be a new method for anti-tumor therapy. Medroxyprogesterone acetate (MPA) may act on tumor angiogenesis by impacting biological functions of EPCs. The aim of this work was to study the effect of different concentrations of MPA combined with 17β-estradiol (17β-E2) on proliferation, migration, and apoptosis of EPCs *in vitro*.

MATERIALS AND METHODS: Proliferation tests (MTT analysis) and migration assay of EPCs, isolated from bone marrow of canine, were performed to detect their response to different concentrations of MPA combined with 17β-E2 (1×10^{-8} mol/L). The growth curves were drawn every 24 h for 7 consecutive days. The cell cycle and apoptosis of EPCs were analyzed by flow cytometry.

RESULTS: 17β-E2 (1×10^{-8} mol/L) increased EPC proliferation, while lower concentration of MPA (≤ 10^{-5} mol/L) partially inhibited it. The higher concentration of MPA (≥ 10^{-4} mol/L) combined with 17β-E2 had a significant inhibitory effect on EPC growth, arresting it in the S phase. It also increased the apoptosis rate and damaged the migration ability of EPCs.

CONCLUSIONS: Low concentration of MPA partially inhibited the function of 17β-E2 that promotes the proliferation of EPCs. However, high concentration of MPA combined with 17β-E2 inhibited a variety of biological functions of EPCs. So, the MPA has a bidirectional effect combined with 17β-E2 on the cell biology of EPCs.

Key Word:
Endothelial progenital cells (EPCs), Medroxyprogesterone acetate (MPA), Progesterone receptor (PR), Angiogenesis.

Introduction

Endothelial progenitor cells (EPCs) are defined as precursor cells that have the ability to differentiate into mature endothelial cells (characterized by their spindle shape), incorporate Dil-labeled acetylated low-density lipoprotein, express endothelial cell surface molecules such as CD31, Flk-1 (vascular endothelial growth factor receptor-2, VEGFR-2), and Tie-2, and release nitric oxide[1-2]. There are evidences showing that EPCs take part in tumor angiogenesis and metastasis and prompt the development of tumor and metastasis. Inhibition of EPC proliferation and migration may be a new method for anti-tumor therapy[3].

In our previous study[4], the high concentration of MPA could suppress EPC proliferation and increase the apoptosis rate by blocking the cell cycle in the S phase. MPA also inhibited the migration and tube formation capacity of EPCs, indicating that MPA could act on tumor angiogenesis by impacting the biological function of EPCs.

Estrogen may affect tumor neovascularization by inducing the mobilization of bone marrow-derived EPCs[5]. In the recent study[6], 17β-estradiol (17β-E2), in particular, and ER-α agonists, in general, may promote the healing of injured vascular beds by promoting EPC activity, which leads to more rapid endothelial recovery and capillary formation after injury. EPC proliferation is induced during the menstrual phase, and proliferation can be affected by estrogen through ER alpha activation[7].

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The main purpose of the present study was to investigate the influence of 17β-E2 combined with MPA on EPCs.

Materials and Methods

Experimental Animal

Canine (Beagle's dog, female, Level I, 12 months old, 8 kg) was supplied by Animal Center of Sichuan University, and it was fed in a single cage. The treatment of the animal was in accordance with the regulation of ethics.

Isolation and Culture of EPCs

The bone marrow mononuclear cells of the canine was isolated by Histopaque®-1077 (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation (500 g, 25 min) and suspended into endothelial cell basal medium 2 (Clonetics, Walkersville, MD, USA) supplemented with EGM-2 MV (Clonetics). They were then inoculated into the culture flasks precoated with fibronectin (Roche, Basel, Switzerland) under 37°C in a humidified environment with 5% CO_2. The culture medium was replaced in every 4 days. Cells were passaged when they reached to 90% confluency. Cells from the third generation to the fifth generation were used for the following experiment.

MTT Assay

EPCs were seeded on a 96-well plate (2.5×10^3/well) for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The concentrations of MPA used were 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 0 mol/L combined with 17β-E2 (1×10^{-8} mol/L). The growth curves were drawn using the absorbance values (optical density, OD) for 7 days continuously.

Cell Cycle and Apoptosis Assay

EPCs were cultured in 6-well plates (5×10^5/well). The concentrations of MPA used were 5×10^{-4}, 2.5×10^{-4}, 1×10^{-4}, and 0 mol/L combined with 17β-E2 (10^{-8} mol/L). The cells were collected after 24 h, fixed in 70% ethanol, and stained with propanol iodide. The cell cycle and apoptosis ratio of EPCs were analyzed by flow cytometry.

Transwell Assay

EPCs (4×10^4 cells/well) were seeded in the upper chamber of Transwell (8 μm pore size, 24 wells, Corning). EBM-2 culture medium was added in the lower chamber with various concentrations of MPA (5×10^{-4}, 2.5×10^{-4}, 1×10^{-4}, and 0 mol/L) combined with 17β-E2 (1×10^{-8} mol/L). The upper chamber was taken out after 24 h. Cells from the top of the upper chamber film were carefully removed using a cotton swab; the film was then fixed in 4% paraformaldehyde for 15 min and stained with crystal violet. The total number of migrated cells in 10 randomly selected fields (×100) was counted.

Statistical Analysis

Data were demonstrated by mean ± standard deviation ($\bar{x}$ ± SD). Statistical analyses were processed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

The Influence of MPA and 17β-E2 on Cell Growth

When EPCs were cultured with 17β-E2 (1×10^{-8} mol/L) alone, the cell growth increased than that of the control group. When EPCs were cultured with MPA (10^{-5} or 10^{-6} mol/L) alone, the OD value decreased than that of the 17β-E2 group though the cell appearance seemed no different than that of the control group[4].

When EPCs were treated with MPA (10^{-4} mol/L or 10^{-3} mol/L) combined with 17β-E2 (1×10^{-8} mol/L) for 24 h, the cells became round, started to float and the cell growth was inhibited compared with the control group. As shown in Figure 1, the cell growth was the lowest in the group treated with MPA of 10^{-3} mol/L combined with 17β-E2 than in other groups. However, no difference was reported between the following two groups: MPA (10^{-5} mol/L or 10^{-6} mol/L) combined with 17β-E2 and 17β-E2 alone. The data suggested that when EPCs were stimulated by MPA combined with 17β-E2, the cell growth status was decided by the concentration of MPA. Higher concentration of MPA (≥ 10^{-4} mol/L) could decrease the cell growth speed.

The Influence of MPA and 17β-E2 on Cell Cycle

When EPCs were treated with MPA (1×10^{-4}, 2.5×10^{-4}, and 5×10^{-4} mol/L) combined with 17β-E2 (1×10^{-8} mol/L), the cell cycle distribution changed. The S-phase ratio increased while the G2-phase ratio decreased compared with the

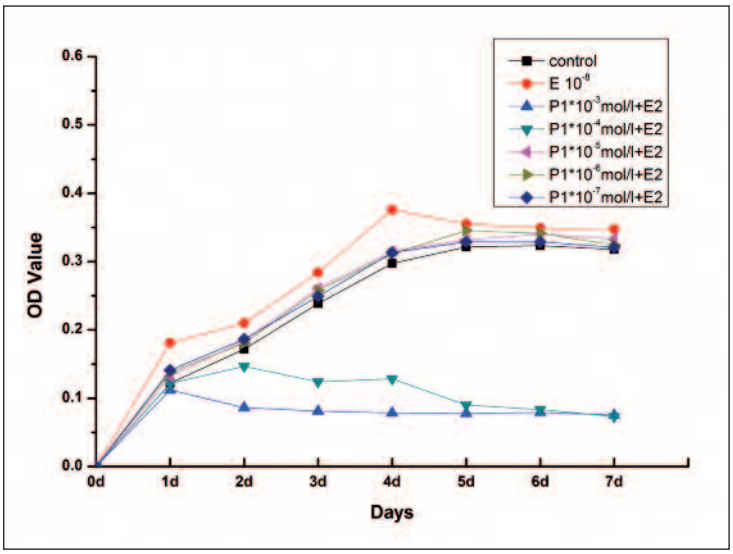

Figure 1. Influence of MPA and 17β-E2 on cell growth (E: 17β-E2; P: MPA).

control group. When MPA at the concentration of 2.5 × 10^{-4} mol/L combined with 17β-E2, the S-phase ratio of EPCs was the highest than that of the control group (*$p < 0.05$, vs. control group). The S-phase ratio was higher than that of the control group (*$p < 0.05$, vs. control group) even at the MPA concentration of 5×10^{-4} mol/L. The data suggested that MPA combined with 17β-E2 could induce the S-phase cycle arrest.

The Influence of MPA and 17β-E2 on apoptosis

The influence of different concentrations of MPA combined with 17β-E2 on apoptosis is shown in Figure 3. No change was reported in the apoptosis rate than that of the control group after treating EPCs with MPA (10^{-4} mol/L) combined with 17β-E2. When the concentration of MPA was increased to 2.5 × 10^{-4} mol/L or 5 × 10^{-4} mol/L, the apoptosis rate increased compared with the control group (*$p < 0.05$; **$p < 0.01$ respectively) and the MPA (10^{-4} mol/L) combined with 17β-E2 group (#$p < 0.05$; (##$p < 0.01$ respectively).

The Influence of MPA and 17β-E2 on Migration

The Transwell assay was used to evaluate the influence of MPA combined with 17β-E2 (1 × 10^{-8} mol/L) on cell migration. When EPCs were stimulated by the MPA concentration of 10^{-4} mol/L combined with 17β-E2, the migra-

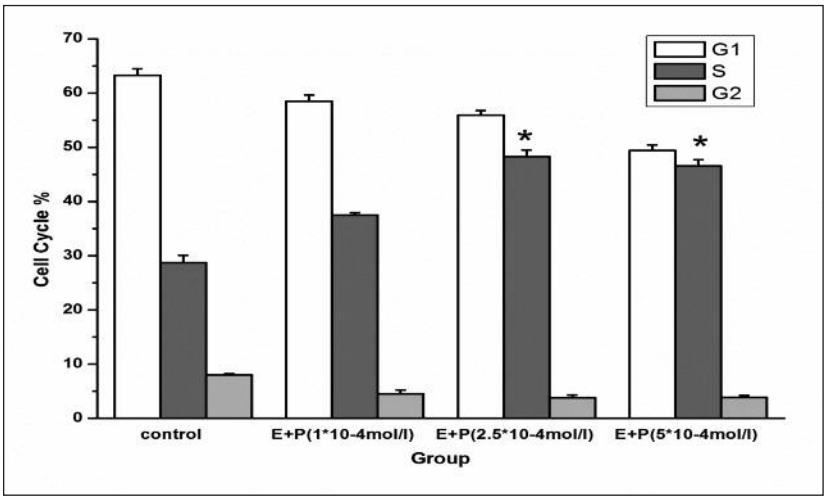

Figure 2. Influence of MPA combined with 17β-E2 (1 × 10^{-8} mol/L) on the cell cycle of EPCs. The S percent of the cell cycle under the influence of E+P (2.5 × 10^{-4} mol/L) increased than that of the control group (*$p < 0.05$, vs. control group). The S percent of cell cycle under the influence of E+P (5 × 10^{-4} mol/L) increased than that of the control group (*$p < 0.05$, vs. control group) (E+P: MPA combined with 17β-E2).

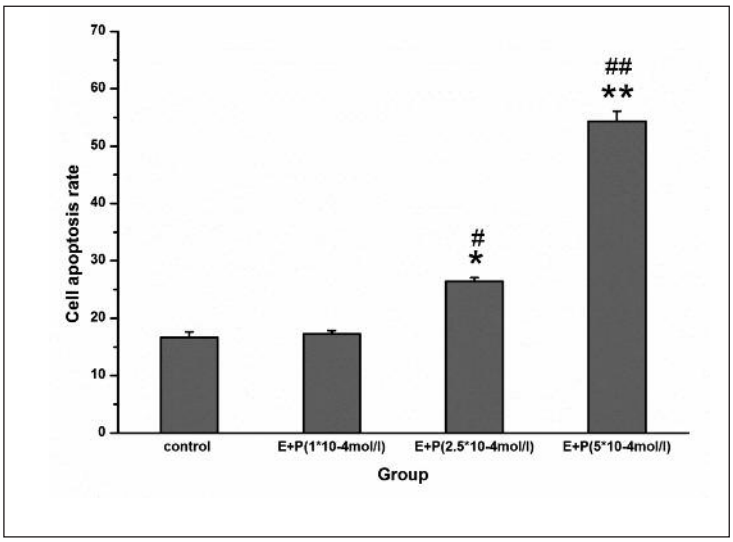

Figure 3. Influence of MPA combined with 17β-E2 (1×10^{-8} mol/L) on the apoptosis of EPCs. ($\bar{x} \pm s$, n = 3, $^{*}p < 0.05$, vs. control group; $^{**}p < 0.01$, vs. control group; $^{\#}p < 0.05$, vs. MPA (1×10^{-4} mol/L) + 17β-E2 (1×10^{-8} mol/L) group; $^{\#\#}p < 0.01$, vs. MPA (1×10^{-4} mol/L) + 17β-17β-E2 (1×10^{-8} mol/L) group) (E+P: MPA combined with 17β-E2).

tion number of EPCs decreased than that of the control group ($^{*}p < 0.05$). When EPCs were stimulated by the MPA concentration of 2.5×10^{-4} mol/L combined with 17β-E2, the migration number of EPCs decreased than that of the control group ($^{**}p < 0.01$). The same phenomenon was observed when EPCs were stimulated by the MPA concentration of 5×10^{-4} mol/L combined with 17β-E2 ($^{**}p < 0.01$). When EPCs were stimulated by the MPA concentration of 2.5×10^{-4} mol/L combined with 17β-E2, the cell migration number decreased than when stimulated by the MPA concentration of (10^{-4} mol/L combined with 17β-E2 ($^{\#}p < 0.01$). When EPCs were stimulated by the MPA concentration of 5×10^{-4} mol/L combined with 17β-17β-E2, the cell migration number decreased than when stimulated by the MPA concentration of 1×10^{-4} mol/L combined with 17β-17β-E2 ($^{\#\#}p < 0.01$).

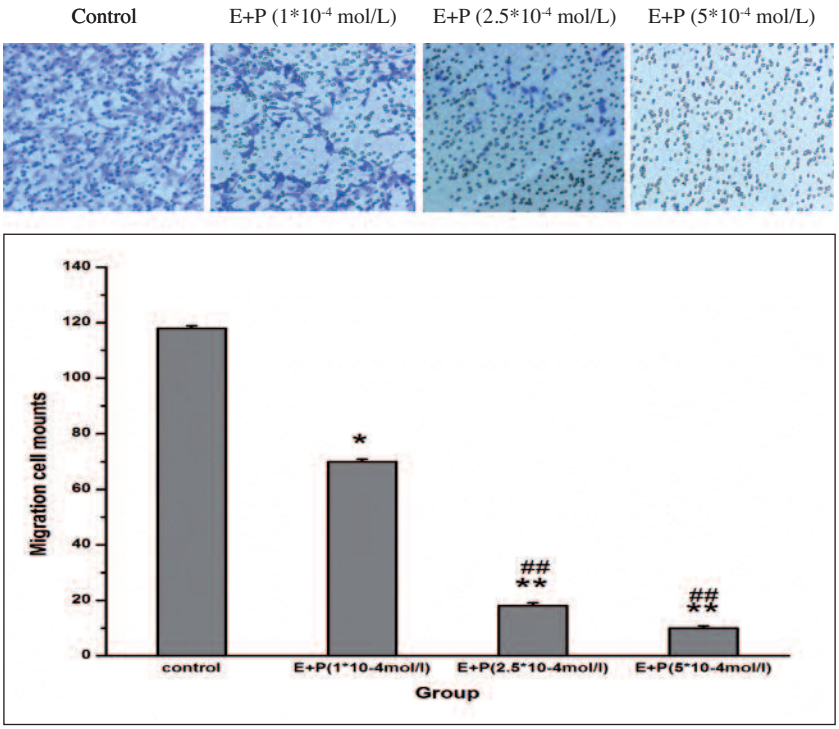

Figure 4. Influence of MPA combined with 17β-E2 (1×10^{-8} mol/L) on EPC migration ($^{**}p < 0.05$, vs. control group; $^{\#\#}p < 0.01$, vs. MPA [1×10^{-4} mol/L] +17β-E2 group) (E: 17β-E2; P: MPA; E+P: MPA combined with 17β-E2).

Discussion

Many females can be influenced by the common action of estrogen and progesterone. The estrogen can improve the harmful influence induced by dangerous factors, act on the different stages of apoptosis signal pathway, and decrease the apoptosis rate. When the estrogen concentration was 1×10^{-8} mol/L, it enhanced the proliferation of EPCs significantly, while increased the concentration of estrogen above 10^{-8} mol/L did not enhance proliferation of EPCs in dose-dependent manner. The question whether 17β-E2 can reverse the action of MPA on EPCs still remains elusive. Our study indicated that the higher concentration of MPA (>1×10^{-5} mol/L) combined with 17β-E2 (10^{-8} mol/L) can inhibit EPC proliferation and migration, prompt cell apoptosis, and change cell cycle distribution. It means, the higher concentration of MPA can inhibit the cell biology function of EPCs.

At the same time, the lower concentration of MPA has no influence on EPC proliferation, but can partly inhibit the effect of 17β-E2 on EPC proliferation. 17β-E2 prompts the homing of EPCs to the ischemia or vascular injury by prompting EPC mobilization and proliferation, repairing vessel endothelial cells, and increasing angiogenesis.

On the basis of the preliminary work, when the MPA concentration was less than or equal to 1×10^{-5} mol/L had no obvious influence on EPC proliferation. When the MPA concentration was equal to or greater than 1×10^{-4} mol/L, it promoted cell apoptosis, inhibited cell proliferation, induced the S phase cycle arrest, and downregulated the ability of cell migration. The effect of 17β-E2 (1×10^{-8} mol/L) alone on EPCs can enhance cell proliferation.

When EPCs were stimulated by the MPA concentration less than or equal to 1×10^{-5} mol/L combined with 17β-E2, cell proliferation decreased. Cell proliferation and cell migration decreased when EPCs were stimulated by the MPA concentration less than or equal to 1×10^{-4} mol/L combined with 17β-E2. Thus, the two medicines acting together can change the cell cycle and induce cell apoptosis. These results indicate that the MPA concentration less than or equal to 1×10^{-4} mol/L can reverse the active effect induced by 17β-E2; however, the molecular mechanism still need further research.

Eigeliene et al.[8] showed that 17β-E2 combined with MPA caused to the following characteristic changes in the breast explants: stimulated epithelial proliferation, lowered apoptosis ratio, and decreased the relative number of epithelial cells expressing ERα, ERβ, and PR[9]. MPA seems to impairs the 17β-E2 signaling in the endothelial cells. 17β-E2 was shown to markedly increase the expression of PR and MPA was shown to downregulate the expression of PR[10].

Conclusions

All these studies indicate that the MPA can downregulate the effect of 17β-E2. However, our result demonstrated that the effect of MPA combined with 17β-E2 on EPCs is decided by MPA concentrations. The molecular mechanism of the effect of different MPA concentrations combined with 17β-E2 on EPCs still need further study, which may provide a new dimension to the tumor therapy.

Conflict of interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

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