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Original article

Human menstrual blood–derived stem cells promote the repair of impaired endometrial stromal cells by activating the p38 MAPK and AKT signaling pathways

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ABSTRACT

Multiple studies have confirmed that human menstrual blood–derived stem cells (MenSCs) have potential applications in regenerative medicine or cell therapy. However, the contribution of MenSCs to endometrial repair is currently unknown. We evaluated the protective effects of MenSCs on impaired endometrial stromal cells (ESCs), as well as the signaling pathways involved in this process. Mifepristone was used to damage human ESCs, which were subsequently cocultured with MenSCs. The proliferation, apoptosis, and migration of ESCs were assessed, together with the expression of related signaling proteins including total p38 mitogen-activated protein kinase, P-p38, total protein kinase B (AKT), P-AKT, β-catenin, and vascular endothelial growth factor (VEGF). MenSCs significantly recovered the proliferation and migration ability of impaired ESCs, inhibited ESC apoptosis, and upregulated protein expression of P-AKT, P-p38, VEGF, and β-catenin. Our findings suggest that MenSC-based therapies could be promising strategies for the treatment of endometrial injury, and that AKT and p38 signaling pathways may be involved in this process.

1. Introduction

The human endometrium is a dynamic organ that regularly undergoes remarkable periodic proliferation, differentiation, breakdown, and renewal throughout a woman's reproductive years. The vital role of the endometrium in female fertility is well recognized [1]. Damage to the endometrium can result in failed embryo implantation and pregnancy loss [2]. Endometrium remodeling is thought to be modulated by ovarian steroids, as well as by various growth factors and cytokines [3]. In addition, several drugs have been found to affect normal development of the endometrium [2,4].

Mifepristone is a remarkably active antiprogesterone and antiglucocorticoid agent in humans [5]. Studies have shown that mifepristone can directly impair the endometrium *in vitro* and *in vivo* [6,7]. Mifepristone downregulates the expression of vascular endothelial growth factor (VEGF), thus inhibiting vascular and tissue remodeling in the endometrium [8]. Preventing and reversing the endometrial damage caused by mifepristone is a clinical challenge.

Endometrial mesenchymal stem cells are known to be involved in endometrial regeneration. Stem cells located in the functionalis of the endometrium are normally shed in menstrual blood, and are known as menstrual blood–derived mesenchymal stem cells (MenSCs) [9]. MenSCs are a novel cell population that can be safely isolated to provide a stable source of stem cells [10,11]. Compared with bone marrow–derived mesenchymal stem cells, MenSCs have many advantages including high proliferative capacity, minimal ethical considerations, and easy accessibility [12–14]. MenSCs have the potential to undergo targeted migration to impaired organ sites, and are believed to be involved in endometrial angiogenesis [15,16].

Angiogenesis is vital for endometrial growth and embryo implantation. VEGF functions as an angiogenic factor and plays a central role in vascular development in the endometrium during embryo implantation [17]. Moreover, VEGF is essential for endometrial neoangiogenesis during postmenstrual/postpartum endometrial repair [18]. The molecular mechanisms regulating angiogenesis and VEGF expression have been extensively studied [19,20]. Among these mechanisms, the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and p38 mitogen-activated protein kinase (MAPK) signaling pathways have emerged as the most notable [19,21]. Several lines of evidence suggest that phosphorylation of AKT affects VEGF expression [19,20]. Moreover, it has been reported that human endometrial stem cells can repair myocardial infarction in rats by activating the AKT signaling pathway

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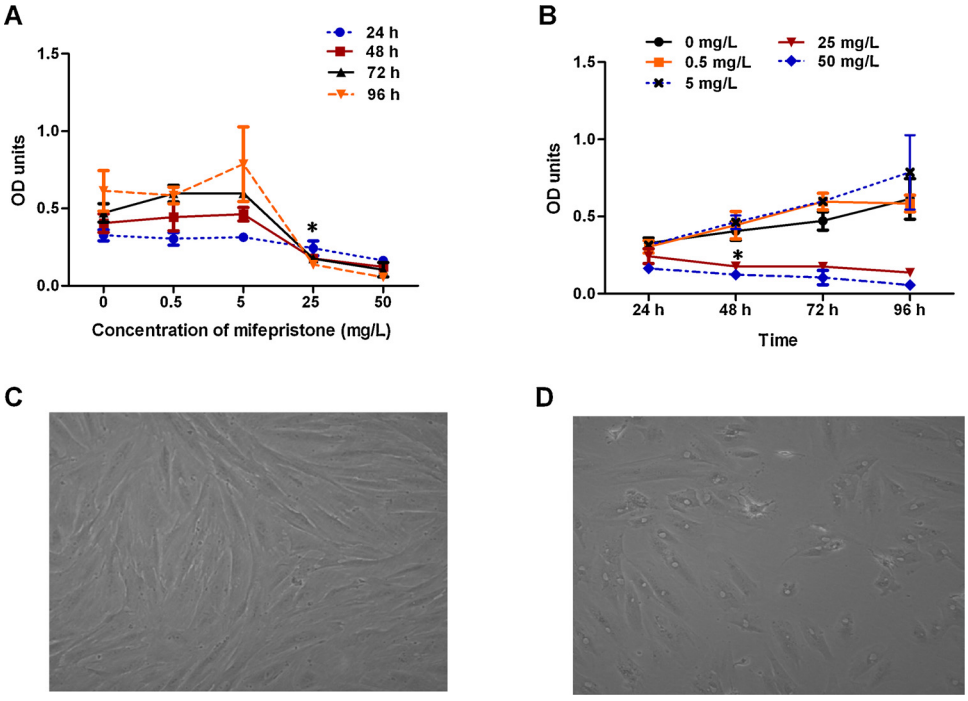

Fig. 1. Proliferation and morphology of ESCs after mifepristone treatment. ESCs (passage 3) were treated with mifepristone at (A) different concentrations (0, 0.5, 5, 25, and 50 mg/L) and (B) for different amounts of time (24, 48, 72, and 96 h). A. The proliferation of ESCs was decreased significantly after treatment with 25 mg/L and 50 mg/L mifepristone. $^{*}P < 0.05$ *vs.* 5 mg/L mifepristone treatment. B. The proliferation of ESCs was significantly inhibited by mifepristone treatment at 25 mg/L and 50 mg/L for 48, 72, and 96 h. $^{*}P < 0.05$ *vs.* 24 h treatment duration. Results are representative of three separate experiments conducted in triplicate using samples from three different patients. C, D. Under treatment with 25 mg/L mifepristone for 48 h, ESC morphology changed from a spindle-shape (C) to an irregular shape with cytoplasmic vacuolization (D).

[22]. However, the contribution of MenSCs to endometrial repair has not been elucidated. The aim of the current study was to assess the protective effects of MenSCs on proliferation, apoptosis, and motility of ESCs that are impaired by mifepristone and to investigate the precise mechanisms involved in this progress.

2. Materials and methods

2.1. Tissue collection

This study was approved by the ethics committee of Sir Run Run Shaw Hospital, an affiliated hospital of the College of Medicine, Zhejiang University. Informed consent was obtained from all study participants. Proliferative phase endometrial samples (n = 3) were obtained from premenopausal patients undergoing laparoscopic or laparotomic hysterectomy for various benign gynecological indications such as uterine myomas or metroptosis. Exclusion criteria included any endometrial abnormality (hyperplasia, polyps, or cancer) and administration of any hormones or gonadotropin-releasing hormone (GnRH) agonist therapy within 3 months of surgery. Endometrial tissue samples were collected under sterile conditions and transferred to DMEM/F12 medium (Gino Biological, Hangzhou, China) at 4 °C. All experiments involved three independent samples and were performed at least in triplicate.

2.2. Isolation and culture of primary endometrial stromal cells

Endometrial stromal cells (ESCs) were isolated as previously described [23]. Briefly, collected tissue samples were minced into 1-mm^3 fragments and digested with 2–3 ml of 0.1% type-I collagenase (Gibco, USA) and DNase (Invitrogen, USA) at 37 °C for 45 min. The enzymatic digestion reaction was terminated by addition of 2-3 ml of DMEM/F12 medium containing 10% fetal bovine serum (FBS; Gibco, USA). The stromal cells were separated using two filtration steps and collected by two rounds of centrifugation at 500 × g for 5 min. The supernatant was removed and cell pellets were suspended in 10% FBS-DMEM/F12 medium and seeded on sterile plastic culture dishes. Immunohistochemical staining for the intermediate filament protein vimentin confirmed that the purity of stromal cells was as high as 95% [23].

2.3. MenSC culture and identification

Human menstrual stem cells (MenSCs) were obtained from Professor Xiang (Zhejiang-California International NanoSystems Institute, Zhejiang University, Hangzhou, China) and cultured in endometrial/menstrual stem cell culture medium (S-Evans Biosciences, China), as described by Mou [14]. In addition, MenSCs were confirmed in our laboratory before the experiment [24,25]. MenSCs were spindle-shaped; positive for CD44, CD90, CD73, and CD29; and negative for CD117, CD34, CD45, and HLA-DR. MenSCs at passage 3-4 were used for subsequent experiments.

2.4. Treatment with mifepristone and ESC/MenSC coculture

ESCs were seeded into 6-well plates at a density of 3×10^5 cells/ml in DMEM/F12 medium with 10% FBS. After 24 h, the medium was changed to DMEM/F12 medium with 2% FBS and a subgroup of ESCs was treated with 25 mg/L mifepristone (Sigma, USA) at 37 °C for 48 h. At the end of treatment the medium was replaced with fresh medium (2% FBS) lacking mifepristone. Inserts containing a 0.4-μm pore size PET membrane (Corning, NY, USA) with MenSCs (1×10^5 cells/ml) were placed in the upper part of the 6-well plates and cocultured with the stromal cells for 24, 48, 72 and 96 h. Before we determined the optimum dense of MenSCs, the effect of MenSCs with different cell dense (1×10^5, 5×10^4 and 1×10^4 cells/ml) on proliferation of ESCs at different time points (24, 48 and 72 hours) were examined (Supplementary figure S1). Endometrial epithelial cells (1×10^5 cells/ml) as a control of non-stem cells were used to coculture ESCs (Supplementary Figs. S2 and S3).

2.5. Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology, China). Briefly, ESCs were seeded into 96-well culture plates (8×10^3 cells per well) and treated with different concentrations of mifepristone (0, 0.5, 5, 25, and 50 mg/L; 100 μl/well) for 24, 48, 72 and 96 h. At the end of each experiment the