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Effects of human adipose-derived mesenchymal stem cells combined with estrogen on regulatory T cells in patients with premature ovarian insufficiency

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ABSTRACT

Objective: To investigate the effects of human adipose-derived mesenchymal stem cells (hADSCs) combined with estrogen on regulatory T cells (Tregs) in patients with premature ovarian insufficiency (POI).

Methods: hADSCs were isolated by enzymatic digestion and identified by flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from POI patients and healthy controls. PBMCs were cultured in the following experimental groups: the control group, hADSC group, estrogen group and combined group. The PBMCs in the hADSC group were co-cultured with hADSCs at concentrations of 1×10^4, 2×10^4, or 1×10^5 cells/well, and the estrogen group was co-cultured with 10^{-9}, 10^{-8}, or 10^{-7} mol/L 17β-estradiol. Cell proliferation was measured with the CCK-8 assay. The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was measured by flow cytometry. The expression levels of Foxp3, TGF-β1 and IFN-γ were measured by real-time PCR.

Results: Treatment with hADSCs, estrogen and their combination promoted Tregs differentiation of PBMCs from POI patients and healthy controls. An increase in the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was observed when PBMCs were co-cultured with hADSCs, 17β-estradiol and their combination. Foxp3 and TGF-β1 mRNA expression was higher and IFN-γ mRNA expression was lower in the hADSCs, estrogen and combined groups than in the control group.

Conclusion: Combined treatment with hADSCs and estrogen played an immunomodulatory role by promoting Tregs proliferation, thereby potentially improving impaired ovarian function.

1. Introduction

Premature ovarian insufficiency (POI) refers to the clinical syndrome of ovarian recession in women before the age of 40 and is characterized by menstrual disorders with high gonadotropin and low estrogen levels [1]. The etiology of POI is complicated and includes genetics, autoimmune components, iatrogenic injury and infection [2]. Approximately 30% of POI cases are associated with autoimmune dysregulation [3]. Recent studies have shown that the proportion of Tregs in patients with POI is decreased [4]. Thus, POI is closely related to cellular immunity. Currently, POI treatment primarily focuses on hormone supplementation; however, long-term use of hormones may result in poor patient compliance and does not fundamentally solve the problem of anovulatory fertility. This disease seriously affects the quality of life of females during their reproductive years and can even affect family harmony and social stability. Therefore, POI treatment remains challenging for clinicians [5].

Tregs are a subtype of T cells with immunosuppressive and immunomodulatory functions, and their normal physiological function is essential for the homeostasis of the immune system [6]. The transcription factor forkhead box P3 (Foxp3) is specifically expressed in Tregs and plays an important role in their development and function [7]. Tregs play a pivotal role in immunotherapy and have been reported to be involved in the prevention and treatment of many autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [8,9]. Therefore, effective methods for promoting the proliferation of Tregs may be essential for the successful treatment of POI.

Mesenchymal stem cells (MSCs) are widely used in cell-replacement therapy and regenerative medicine due to their self-renewal and multidirectional differentiation potential. In addition to their pluripotency potential, MSCs possess specific properties of low immunogenicity and immunoregulation [10,11]. MSCs have been widely used in numerous animal experiments, which have shown that MSCs can effectively improve chemotherapy-induced POI [12–15]. However, few studies have

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addressed the application of MSCs in the prevention and treatment of immune-induced POI.

Human adipose-derived MSCs (hADSCs) are easily obtained, abundant and multipotent. Therefore, hADSCs can be used as seed cells for tissue engineering. In addition, relevant research has shown that 17β-estradiol can play an immunomodulatory role by regulating the ratio of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, promoting the expression of Foxp3, and enhancing the immunosuppressive activity of Tregs [16]. Therefore, we generated a co-culture system by combining hADSCs and 17β-estradiol with peripheral blood mononuclear cells (PBMCs) from patients with POI and measured the levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs and related cytokines to explore the possible mechanism of action of the combination of hADSCs and 17β-estradiol on the immunoregulatory effects of Tregs in POI patients.

2. Methods

2.1. Patients

Twenty-five patients with POI and twenty sex- and age-matched healthy individuals from Zhujiang Hospital were enrolled in the study. The study was approved by the Zhujiang Hospital of Southern Medical University Ethics Committee, and informed consent forms were signed by patients. The POI patients met the following criteria: 1) age < 40 years old; 2) amenorrhea > 4 months; 3) at least two blood tests (interval of 1 month or more) showing serum follicle-stimulating hormone (FSH) > 25 IU/L; 4) no use of hormone drugs within 3 months; 5) karyotypes of 46, XX; and 6) a lack of genetic, iatrogenic or infectious factors. Twenty milliliters of heparin-anticoagulated blood was drawn from each patient at 9 a.m. on the 2nd to 4th day of the menstrual period after a 12-hour fasting period, and PBMCs were isolated with lymphocyte separation medium (Tian Jin Hao Yang Biological Manufacture Co., Ltd., Tianjin, China).

2.2. Isolation of hADSCs

Adipose tissue was obtained from the Department of Gynecology and Obstetrics at Zhujiang Hospital of Southern Medical University. Under sterile conditions, the adipose tissue was washed repeatedly with phosphate-buffered saline to remove blood and was minced into paste by eye scissors. Then, 0.1% type I collagenase (Sigma, USA) was added to the tissue, which was then placed in a constant-temperature shaking bath at 37 °C for 60 min. Next, the digested solution was centrifuged at 1200 rpm for 5 min. The resulting cell pellet was resuspended in L-DMEM containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 IU/mL); plated in T25 culture flasks; and incubated at 37 °C in a 5% CO_2-saturated humidified incubation chamber. The complete medium was changed every 3 days. When the adherent hADSCs reached 80% confluence, the cells were trypsinized with 0.25% trypsin-EDTA solution and passaged at a ratio of 1:3. The third generation of cells was used for subsequent experiments.

2.3. Characterization of hADSC surface marker expression and differentiation potential

The third generation hADSCs were digested with trypsin to generate a 1×10^6-cells/mL single-cell suspension. Then, anti-human monoclonal antibodies, including CD29, CD34, CD105, CD45, CD44 and HLA-DR (Becton Dickinson and Company, USA), were added to the cell suspension, which was then incubated at room temperature for 30 min and analyzed by flow cytometry.

The methods used for adipogenic and osteogenic differentiation were performed as described by Gao et al. [17]. Briefly, the third generation hADSCs were inoculated in a 12-well culture plate coated with 0.1% gelatin at a density of 5×10^3 cells per well. The conditioned medium was changed every 3 days. After 21 days of induction, Alizarin Red S staining and Oil Red O staining were used to analyze the ability of the hADSCs to differentiate into adipocytes and osteocytes.

2.4. Co-culture of PBMCs with hADSCs and estrogen

PBMCs stimulated with phytohemagglutinin (PHA, 5 μg/mL) were co-cultured with various concentrations of hADSCs (1×10^4, 2×10^4, and 1×10^5 cells/well) and 17β-estradiol (10^{-9}, 10^{-8}, and 10^{-7} mol/L). First, hADSCs were made into cell suspensions of three concentrations, 1×10^4, 2×10^4, and 1×10^5 cells/well, and were then added to 24-well plates at 500 μL per well, and the medium was discarded after the cells were allowed to adhere to the well surface for 24 h. Suspensions of PBMCs from healthy individuals and POI patients were prepared at a concentration of 1×10^5 cells/well. PHA-stimulated PBMCs were added to culture plates containing hADSCs adhered to the surface at 500 μL per well, and the cells were co-cultured in a cell culture incubator for 72 h. The experimental groups were as follows: PHA + PBMCs, PHA + PBMCs + 1×10^4 ADSCs, PHA + PBMCs + 2×10^4 ADSCs, and PHA + PBMCs + 1×10^5 ADSCs. In parallel, PHA-stimulated PBMCs of healthy individuals and POI patients were seeded into 24-well plates at a concentration of 1×10^5 cells per well, treated with 17β-estradiol (Sigma, USA) at doses of 10^{-9}, 10^{-8}, or 10^{-7} mol/L and incubated for 72 h. The experimental groups were as follows: PHA + PBMCs, PHA + PBMCs + 10^{-9} mol/L 17β-estradiol, PHA + PBMCs + 10^{-8} mol/L 17β-estradiol, and PHA + PBMCs + 10^{-7} mol/L 17β-estradiol. Then, we selected the most effective concentrations of hADSCs and estradiol for co-culture with PBMCs and created the following experimental groups: the control group, hADSC group, estrogen group, and combined group. After 72 h of co-culture, PBMCs were harvested for RNA expression analysis and flow cytometry.

2.5. Cell proliferation assay

After co-culture for 72 h, the PBMCs in the supernatant were collected and seeded to 96-well plates in three replicate wells at a density of 1×10^5 cells per well. Then, 10 μL of CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the cells were incubated for 4 h at 37 °C without light exposure. Absorbance (A) values were measured at 450 nm with a microplate reader.

2.6. Analysis of Foxp3, TGF-β1 and IFN-γ mRNA expression by quantitative real-time PCR

Total RNA from PBMCs was extracted with Trizol reagent (Takara Bio Inc., Otsu, Japan), and the RNA concentration was measured. cDNA was synthesized by reverse transcription according to the product specifications, and PCR reactions were performed using the SYBR Prem IX Ex TaqTM kit (Takara Bio Inc., Otsu, Japan). The reaction conditions were as follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 20 s, and a final step at 65 °C for 15 s. The primers were synthesized by Shanghai biochemical corporation as follows: β-actin forward, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and reverse, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′; Foxp3 forward, 5′-CCC AGG AAA GAC AGC AAC CTT-3′ and reverse, 5′-CAA ACA GGC CGC CGT CTG GAG CC-3′; TGF-β1 forward, 5′-CTT CAA TAC GTC GAC ATT CGG G-3′ and reverse, 5′-GTA ACG CCA GGA ATT GTT GCT A-3′; IFN-γ forward, 5′-ACA GCA AGG CGA AAA AGG ATG-3′ and reverse, 5′-TGG TGG ACC ACT CGG ATG A-3′. β-actin was used as an internal control. The relative expression level of each gene was calculated with the $2^{-\triangle\triangle Ct}$ method.

2.7. Flow cytometric analysis

Cells were stained with CD4-FITC and CD25-APC (eBioscience, San Diego, CA, USA) for 30 min at 4 °C, washed and fixed with a fixation/permeabilization buffer for 30 min, permeabilized with a permeabilization buffer, and blocked with rat serum for 15 min. Lastly, cells were