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Human endometrial stem cells: High-yield isolation and characterization

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Abstract

Background. Menstrual blood is only recently and still poorly studied, but it is an abundant and noninvasive source of highly proliferative mesenchymal stromal cells (MSCs). However, no appropriate isolation method has been reported due to its high viscosity and high content of clots and desquamated epithelium. *Methods.* We studied three different isolation approaches and their combinations: ammonium-containing lysing buffer, distilled water and gradient-density centrifugation. We tested the proliferative capacity, morphology, surface markers and pluripotency of the resulting cells. *Results.* Our isolation method yields up to four million nucleated cells per milliliter of initial blood, of which about 0.2–0.3% are colony-forming cells expressing standard mesenchymal markers CD90, CD105 and CD73, but not expressing CD45, CD34, CD117, CD133 or HLA-G. The cells have high proliferative potential (doubling in 26 h) and the ability to differentiate into adipocytes and osteocytes. Early endometrial MSCs (eMSCs) express epithelial marker cytokeratin 7 (CK7). CK7 is easily induced in later passages in a prohepatic environment. We show for the first time that a satisfactory and stable yield of eMSCs is observed throughout the whole menstrual period (5 consecutive days) of a healthy woman. *Discussion.* The new cost/yield adequate method allows isolation from menstrual blood a relatively homogenous pool of highly proliferative MSCs, which seem to be the best candidates for internal organ therapy due to their proepithelial background (early expression of CK7 and its easy induction in later passages) and for mass cryobanking due to their high yield and availability.

Key Words: *cell therapy, cryobank, differentiation, endometrial stem cells, hepatocytic differentiation, iCELLigence, menstrual blood, mesenchymal–epithelial transition, mesenchymal stromal cells*

Introduction

Regenerative medicine calls for new sources of stem cells. Although adult HLA-compatible stem cells (SCs) are more promising for regenerative medicine than embryonic stem cells or induced pluripotent stem cells (iPSCs) because of the teratogenic potential of the latter, their standard sources, bone marrow and fat tissue, are not readily available, sharply limiting the range of potential donors and HLA-compatibility choice. Menstrual blood is an abundant and noninvasive source of high-quality cells available from young healthy female donors of any HLA type, and they are equally suitable for recipients of both genders because the transplantability of donor cells and tissues does not depend on the donor's gender [1,2]. The HLA-antigens most responsible for graft loss are HLA-DR (first 6 months after transplantation), HLA-B (first 2 years) and HLA-A (long-term survival) [3]. It has been shown in many studies that endometrial mesenchymal stromal cells (eMSCs), like other mesenchymal stromal cells (MSCs), do not express tissue-specific HLA markers of first-line rejection (HLA-DR) and mildly express markers responsible for late rejection HLA A, B and C [4,5]. These cells can differentiate into various tissue types—neuronal [6,7], muscle [8], and so on. They have high clonogenicity and the ability to pass up to 68 doublings or 26 passages before the emergence of senescence marker β-galactosidase [8,9] as well as higher proliferative and telomerase activity than MSCs derived from other sources, comparable only with embryonic stem cells [10].

However, menstrual blood is a relatively new and poorly studied source [11] for which no appropriate isolation method has been developed. Current

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isolation methods deal with small sample volumes (<10 mL of menstrual blood) because of the high cost of reagents or the necessity of high dilution of the initial biosample, while the SC yield per mL of the sample is low [12]. The existing protocols of SC isolation from blood-containing tissues, when applied to menstrual blood, have several disadvantages: (i) erythrolysis with water or ammonium salts [13–15] has low efficiency due to high viscosity and osmolarity of menstrual blood mucus. It is difficult to achieve a safe osmolar balance using pure water and furthermore lysing solutions do not help to separate desquamated flat endometrium that prevents adhesion of eMSCs; (ii) affinity chromatography and cell isolation using gradient centrifugation [16–18] have high cost for large sample volumes along with significant erythrocyte contamination and clotting at the Ficoll surface significantly decreasing the yield of eMSCs; (iii) toxic effects of hemoglobin emerging from unseparated erythrocytes without performing an erythrolysis stage [7,10] make it necessary to dilute the sample to volumes too large to handle; and (iv) uterus biopsy [19,20] has high invasiveness.

In this work we (i) developed an optimized method for eMSC isolation and (ii) characterized the quantitative yield, morphology, differentiation potential, antigen phenotype and proliferative capacity of the cells.

Materials and methods

Overall protocol for eMSC isolation

Written consent was obtained from two donors. The study of human volunteer samples was approved by the Local Ethics Committee of Sechenov First Moscow State Medical University. Blood samples were collected on the 1st, 2nd, 3rd, 4th and 5th days of the menstrual cycle. Cell morphology was evaluated using light microscopy, and cells were counted using a Goryaev chamber. We developed an optimized protocol for eMSC isolation as follows:

(1) A sterilized (5 min in boiling water) 25-mL menstrual cup (MeLuna Classic with stem handle) was used to collect menstrual blood (up to 20 mL of blood can be obtained during 3–5 h of collection; see Figure 1 for details).
(2) The collected blood was transferred into a 50-mL falcon tube with sterile heparin sulfate, 20 U/mL of blood (500 U/mL solution, Sigma) immediately after removing the cup from the vagina. Samples were kept at 4°C for no longer than 12 h before reaching the processing laboratory. All of the following steps were conducted in a sterile room using sterile solutions.
(3) Blood was separated from plasma by centrifuging for 5 min at 380*g* (1500 rpm).

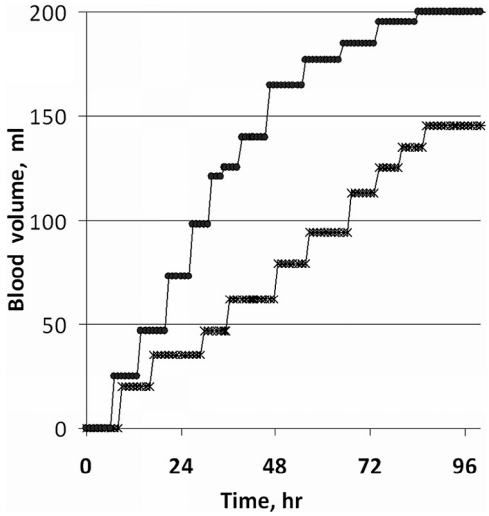

Figure 1. Endometrial blood collection during two different menstrual periods. Steps correspond to times of blood collection (15–20 mL per cup 4–6 times per day).

(4) The red pellet (usually half of the initial blood volume) was divided into 9 mL aliquots and put into 50-mL falcon tubes, to which four volumes of lysis buffer was added (~9 mL of pellet + 36 mL of lysis buffer) and left for 3–4 min with shaking at room temperature until dark red color was obtained. The lysis buffer composition was as follows: 114 mmol/L NH_4Cl (3.05 g/500 mL), 7.5 mmol/L $KHCO_3$ (0.375 g/500 mL), 100 mmol/L ethylenediaminetetraacetic acid (EDTA; 18.5 mg/500 mL) and 500 mL H_2O.
(5) Then ~5 mL of sterile distilled water was added per 50-mL tube for an additional 2 min of vigorous shaking to enhance lysis. Becausee intracellular electrolytes were already released due to partial lysis, the osmolarity could not been significantly decreased with water.
(6) The cells were filtered from clots through a cell strainer (100 µmol/L pore size, SPL, Catalog Number 93100) and centrifuged for 5 min at 380*g* (1500 rpm) at room temperature.
(7) From this procedure, about 1 mL of light-pink pellet was obtained.
(8) The pellet was resuspended in 5 mL of growth medium per tube, and the cells were counted in a Goryaev chamber. The growth medium composition was as follows: minimum essential medium alpha GlutaMAX (Gibco, Catalog Number 32571-028), 80 µU/mL Humulin (Eli Lilly), 1.6 nmol/L prednisolone sodium phosphate (MJ Biopharm Pvt. Ltd.), 20 mmol/L N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid pH 7.2–7.4 (Gibco, Catalog Number 15630080), 20 ng/mL fibroblast growth factor (Sigma), 10% fetal bovine serum (FBS; PAA Laboratories,