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Evaluation of gametogenic potential of vitrified human umbilical cord Wharton's jelly–derived mesenchymal cells

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Abstract

Background aims. Vitrification as an advanced cryopreservation method is recommended for cell storage toward future applications. The purpose of this report was to appraise whether gametogenic potential of these cells is altered by vitrification. *Methods.* A two steps method was applied for hUCM cells vitrification. An n-hUCM group of hUCM cells served as control. In order to differentiation of hUCM cells into male germ cells, the cells were induced by retinoic acid, testosterone and testicular-cell-conditioned medium. To evaluate induced hUCM cells toward germ cells, we used immunocytochemistry and karyotyping methods. *Results.* v-hUCM cells similar to n-hUCM cells formed flat cells after gametogenic induction, and showed protein expression of germ-cell-specific markers DAZL, VASA (DDX4) and SCP3. Karyotyping pattern remained unchanged in the either groups. *Conclusions.* The analysis of these results demonstrates that vitrification does not alter differentiation potential of hUCMs to male germ like cells. These results may set an in vitro pattern to study germ-cell formation from hUCM cells and also as a potential source of sperms for male infertility.

Key Words: *gametogenic potential, mesenchymal cells, umbilical cord, vitrification*

Introduction

Human umbilical cord matrix–derived mesenchymal (hUCM) cell culture has advanced in recent years. Proliferative capacity, multipotentiality and noninvasive procedure to access and isolate hUCM cells are among the advantages of these cells (1). Invasive procedures for harvesting of bone marrow stem cells (BMSCs) (2) and adipose tissue–derived MSCs (3), the low rate of successfully isolating umbilical cord blood mesenchymal stromal cells (MSCs) (4), reduction of the quantities, differentiation potential and frequency of BMSCs with aging (2,5,6) and ethical reasons regarding the use of of embryonic stem cells (ESCs) (7) create limitations in the use of these stem cells. In contrast, MSCs isolated from Wharton's jelly of the human umbilical cord is closely lacking of imperfections, as previously mentioned (8).

To bank cells for future applications in clinics and laboratories, cryopreservation is critical (9). Vitrification (glass-like solidification of a solution) is more desirable than is conventional slow freezing. In vitrification, the defects of slow freezing such as damage caused by cytoplasmic ice crystal formation (10), the requirement of an expensive programmable freezer and time-consuming procedures are eliminated (11). As a cryopreservation method, vitrification has been successfully applied for human cord blood hematopoietic progenitor cells (12), human embryonic stem cells (hESCs) (13,14) and human amnion-derived MSCs (15). We have previously reported on the vitrification of hUCMs (16).

Trials for application of stem cells in reproductive medicine have been an important concern for many years. In the past decade, researchers have reported *in vitro* differentiation of ESCs (17–22) and adult stem cells such as BMSCs (23), fetal porcine skin stem cells (24) and clonal pancreatic stem cells (25) into germ line

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cells. One study was carried out wherein hUCM cells were induced toward male germ cells (26). Human UCM cells are being considered as a non-immunogenic (27) source of stem cells capable of differentiating into various cell types suitable for experimental (28,29) and clinical studies, especially regenerative medicine (30). Whether vitrification would alter differentiation capacity of hUCM cells has not been investigated. Regardless of existent results, there is long way to achievement of final goals. Therefore, in the present study, we evaluate the gametogenic potential of hUCM cells after vitrification.

Methods

Isolation and culture of hUCM cells

All materials were purchased from Sigma-Aldrich Co (Sigma-Aldrich, St. Louis, MO, USA) except for the cases mentioned in the text. Ethics approval was obtained from the ethics committee at Kerman University of Medical Sciences, Kerman, Iran. After informed consent was given by mothers in the Afzalipour hospital gynecology ward in Kerman University of Medical Sciences, fresh human umbilical cords (UCs) from normal, full-term newborn infants were obtained during cesarean section. UCs were transferred to the laboratory in Hanks' balanced salt solution supplemented with 100 IU/mL penicillin and 50 μg/mL streptomycin sulfate, and tissue processing was performed in less than 24 h from acquisition. After removal of blood vessels, the Wharton's jelly was scraped from the amnion and apportioned to 2- to 3-mm pieces. These pieces were explanted onto 35 × 10 mm petri dishes (Falcon BD, Franklin Lakes, NJ, USA) and cultured in a general culture medium of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (PA Biologicals, Sydney, Australia), 100 IU/mL penicillin and 50 μg/mL streptomycin sulfate in a humidified 37°C incubator with 5% CO_2 in air. After removal of Wharton's jelly pieces subsequent to cell bud appearance, cell culture was continued to >80% confluence.

Flow cytometry analysis

For evaluation of surface antigen expression, approximately 1×10^5 4th-passage viable hUCM cells were suspended in washing buffer containing 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). After centrifugation, 4% paraformaldehyde was added and samples were incubated for 15 min at 4°C. Subsequent to washing, the cells were suspended in 10% normal goat serum in PBS. Samples were then incubated for 15 min at 4°C. After cell washing, the cells were labeled with the following antibodies: CD44–fluoresceine-isothiocyanate (FITC), CD90-FITC, CD45-FITC and CD34-FITC for 1 h at 4°C. Finally, the cells were suspended in 4% paraformaldehyde and the examination was conducted. A fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest software was used for antibody binding evaluation.

Evaluation of the differentiation potential of hUCM cells

Cultured cells (at passages 4–5) at a density of 1×10^3 cells/cm^2 on glass coverslips were incubated in adipogenic differentiation medium, osteogenic differentiation medium and chondrogenic differentiation medium kits (Invitrogen, Carlsbad, CA, USA). Each medium was changed every 3–4 days. After completion of the differentiation period, histochemical staining was performed. Cells induced with the osteogenic, adipogenic and chondrogenic formulas were stained with alizarin red S and alkaline phosphatase, oil red O and toluidine blue, respectively, to reveal these differentiations.

Vitrification of hUCM cells

Vitrification was performed by application of a two-step exposure to vitrification solution on hUCM cells at passages 2–4, described previously (15). Initially, a pellet of 1×10^6 hUCM cells was mixed with 50 μL of equilibration solution including 20% ethylene glycol (EG), which was based on DPBS (Dulbecco's phosphate-buffered saline) containing 20% FBS for 5 min. Afterward, the cell pellet was suspended in 500 μL of vitrification solution, including 40% EG, 18% Ficoll 70 and 0.3 mol/dm^3 of sucrose, which was based on DPBS containing 20% FBS for 40 seconds. Finally, suspended hUCM cells were immediately loaded into 1.5-mL cryovials (Nunclon, Roskilde, Denmark) and plunged directly into liquid nitrogen. After 24 h (31), the cryovial was submerged in a 37°C water bath. The warmed cells were suspended serially in 0.5, 0.25 and 0 mol/L sucrose in DPBS containing 20% FBS. The cells were plated at a density of 3×10^3 to 5×10^3 cells/cm^2 in a culture flask and subcultured to >80% confluence.

Testicular cell–conditioned medium preparation

Testicular cell cultures (TCCs) were prepared according to a previously reported protocol (20). Briefly, 10 testes from 1- to 3-day-old newborn mice were ripped into pieces in trypsin–ethylenediaminetetraacetic acid (EDTA) solution. Subsequent to 5-min trypsinization, the suspension was centrifuged for 3 min at 300*g*. The sediment was mixed with 1 mL of 10% FBS in DPBS and was incubated at room temperature for 10 min. The top 0.8 mL was then