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RESEARCH ARTICLE

IN-VITRO CULTURE, CHARACTERIZATION AND DIFFERENTIATION OF HUMAN UMBILICAL CORD DERIVED CELLS

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ABSTRACT

Stem cells, undifferentiated cells with tremendous self renewal capacity and potency, act as repair system of body. These cells are confined to their niche in the organ systems. Umbilical cord is one of the most common sources of stem cells. Umbilical cord derived stem cells shows expression of pluripotency markers and share similar growth kinetics to embryonic stem cells. It is easily accessible in large quantities and can be used for various applications such as model systems in veterinary and human research, transgenesis and regenerative medicine. Here, we report the isolation of stem cells from human umbilical cord. Immunophenotypic characterization using flow cytometry suggests their mesenchymal origin. When cultured in adipogenic medium, these mesenchymal stem cells could differentiate into adipose, thus, indicating their potency.

Key words: Mesenchymal stem cells, Umbilical cord, Flow cytometry.

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INTRODUCTION

Stem cells are naive cells which have ability to differentiateto any kind of specialized cells. The rigorous research in this field has been opened up the avenue to revolutionize the medicine technology (Ma *et al*., 2006; Liu *et al.,* 2015).In view of their unique ability of secreting growth factors and pluripotency, mesenchymal stem cells (MSCs) are more promising for researchers for tissue engineering and cell therapy (Chatzistamatiou *et al.,* 2014, Asgari *et al.,* 2015, Shimazu *et al*., 2015). Unlike embryonic stem cells, MSCs are immunologically competent and therefore, have a remarkable clinical potential in regenerative medicine (Kita *et al.,* 2005, Ishige *et al.,* 2009, Corrao *et al.,* 2013). Moreover, MSCs are currently being used as a model for human diseases and tested in several clinical trials (Ribeiro *et al.,* 2013, Nagamura-Inoue *et al.,* 2014). Several studies have demonstrated the use of MSCs to treat autoimmune diseases and reducing tumor progression. Furthermore, MSCs have been implicated in tissue regeneration, as cell vehicles for gene therapy and for engraftment of hematopoietic stem cells (Qiao *et al*., 2008, Chen *et al*., 2009, Maleki *et al.,* 2014). MSCs exist in many tissues and organs such as the umbilical cord blood and matrix, adipose tissue, synovial membranes, embryonic tissue, and amniotic fluid (Chang *et al.,* 2014, Odabas *et al*., 2014). Among them, bone marrow is a rich source for MSCs but the procedure of tissue collection from donors is intricate as well as painful and, therefore, not suitable for clinical use (De Schauwer *et al*., 2013, Pirjali *et al*., 2013, Martins *et al*., 2014). Moreover, the differentiation potential of MSCs depends on the age and fitness of the donor (Iftimia *et al.,* 2013, Nazari-Shafti *et al*., 2015**).** To overcome those problems, tissue such as umbilical cord, is preferred as a potent source of MSCs, that can be isolated using short enzymatic digestion, expanded and differentiated into desired cell type, preserved cryogenically and further can be revivedas per requirement (Salehinejad *et al.,* 2012, Pawelec *et al.,* 2014, Cui *et al.,* 2015). In the present study, the isolation of MSCs from human umbilical cord is reported. MSCs were derived using enzymatic dissociation of umbilical cord tissue and expanded *in vitro.*

MATERIALS AND METHODS

Collection of umbilical cord samples

Umbilical cords (UC) were collected in a Phosphate buffered saline (PBS) supplemented with antibiotic solution within 5 hrs from birth with prior informed ethical and legal consent. Whole UC tissue was washed properly with PBS in a biosafety cabinet. A 10 cm UC was cut into three equal pieces. Each piece was cut into small pieces of about 1 cm in length and washed thoroughly to remove blood and blood clots, Umbilical arteries, veins, and cord adventitia were removed to obtain Wharton's jelly (Fig. 1A and B). It was dissected into small pieces of approximate 1 mm^3 and used to initiate primary culture using both tissue explant and the enzymatic digestion methods (Yang *et al.,* 2014, Buy l*et al.,* 2014).

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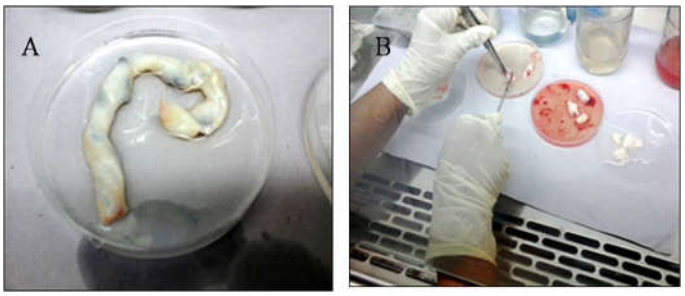

Fig. 1. Processing of umbilical cord tissue for cell culture. A) Human umbilical cord (10cm) collected from clinic. B) Initiation of primary cell culture from chopped tissue

Initiation of primary culture: Properly cut tissue explants were seeded into in Dulbecco's modified Eagle's medium (DMEM) supplemented with 40% fetal bovine serum (FBS) and 0.1% antibiotic-antimyco solution and incubated at 37 °C with 5 % CO2 and saturated humidity. Primary culture was also initiated using enzymatic tissue dissociation method that involved 0.1 % collagenase IV and trypsintreatment. Cell suspension was further seeded in a DMEM with 10% FBS and incubated at 37 °C with 5 % CO2.

Expansion and cryopreservation of UC derived cell line: To subculture the UC derived cells**,** monolayer established after initiation of primary culture either using explants or enzymatic digestion methods was treated with 0.25 % trypsin and 0.02 % EDTA for 5 min at 37°C. Cells viability assay was performed and then cells were, re-seeded at appropriate density in DMEM with 10% FBS and incubated at 37°C with 5% CO2. Growth kinetics of derived cell line was studied using trypan blue. Derived cell line was checked for contaminants and cryopreserved in liquid nitrogen.

Characterization of UC derived cell line: Cells, when reached to 60% confluency, were stained with Giemsa using standard protocol (Bradley *et al.,* 2014). To evaluate ploidy of UC derived cell line, chromosomal spreads were prepared using literature-based procedure (Bradley *et al.,* 2014, Ruan *et al*., 2014) and stained with Giemsa. For immunophenotyping, hUCMSCs were harvested and labeled with mouse anti-human monoclonal antibodies phycoerthyrin (PE) conjugated CD90 and CD105 and Fluorescein isothiocynatate (FITC) conjugated CD73. The labeled cells were incubated for 1hr on ice, washed with FACS buffer and analyzed using FACS, Calibaur (Yang *et al.,* 2014).

Study of differential potential of umbilical cord derived stem cells: Upon 80% confluency, hUCMSCs were harvested and cultured in adipocyte differentiation medium AdipoXL basal medium (HiMedia) for minimum 14 days with appropriate media changes after every 3-4 days. The differentiation into adipocytes was confirmed using Oil O Red staining using standard protocol (Uranio *et al*., 2011, Buyl *et al.,* 2014).

RESULTS

Isolation and expansion of hUC derived cells: hUC derived cells were successfully isolated and cultured *in vitro.* An adherent layer of heterogeneous cells in the primary culture was yielded after 15 days of incubation at 37ºC. After the first passage of culture, highly proliferative fibroblast-like cells were seen to be predominantly present in culture flask.

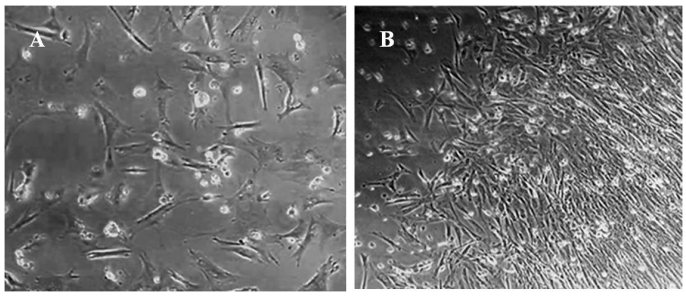

Fig. 2. Primary cell culture of UC derived cells: Cells observed on 3rd A) and 12th day using inverted phase contrast microscope B) (100 X magnification).

Between 2nd and 3rd passages, the adherent cells were composed almost of bipolar fibroblast-like cells that could later reach to confluency (Fig. 2). Growth kinetics and FACS analyses studies revealed that approximately 14% of cells were in proliferative stage. Morphological and growth pattern characteristics of isolated cells closely resembled those of previously reported hMSCs isolated from umbilical cord (Fig.3).

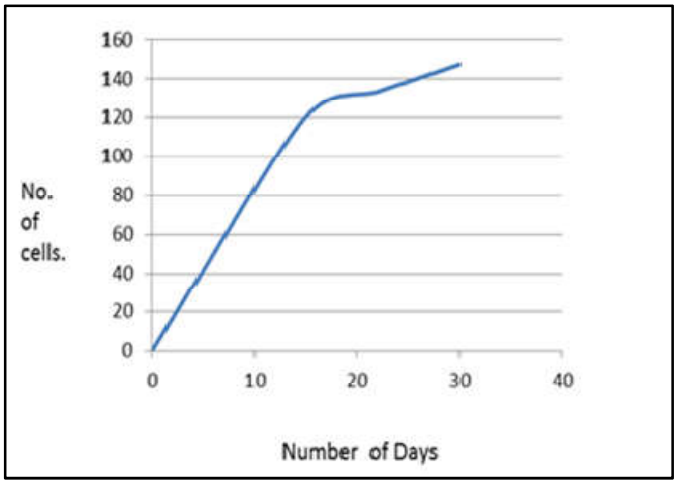

Fig. 3 Growth curve of hUC derived cells

Characterization of hUC derived cell line: In order to analyze the morphology of hUC derived cell line, monolayer was stained with Giemsa. The stained monolayer revealed the heterogeneous morphology of hUC derived cells. Most of the cells exhibited fibroblast like while few shows ovoid and epitheloid morphology as well (Fig. 4 A). Further, Giemsa based chromosomal analysis of hUC derived cell line showed normal ploidy (2n=46) and structural pattern of chromosomes (Fig. 4 B). To characterize the expanded hUC derived cell line, we checked the expression profile of MSC-specific surface markers such as CD73 and CD9 (Fig. 5 A and B). FACS analysis showed the expression of CD73 and CD90 in 39.15 % and 7.71% of cells, respectively. The variation in coexpression of CD73 and CD90 in different batches was the initial indication that the composition of all the expanded hUC derived cell line may not be similar even though all of them showed an adherent, fibroblastic morphology.

Adipogenic differentiation of hUC derived cells: Mesenchymal Stem Cells (MSC) are fibroblastoid multipotent adult stem cells with a high capacity for self-renewal. The directed differentiation of hUC derived MSCs into adipocytes was employed in adipoXL (Himedia) medium. Adipocytes confirmation was done using Oil O red stain (Fig. 6).

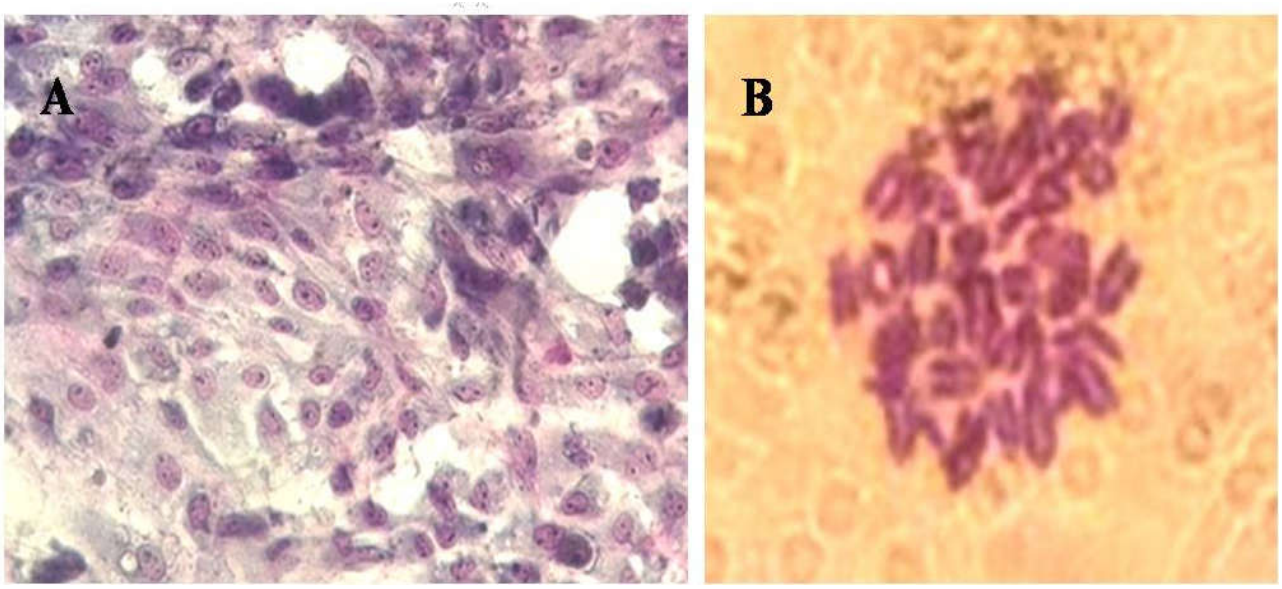

Fig. 4. Characterization of hUC derived cell line. A. Morphology of umbilical cord derived stem cells using Giemsa stain; 100X magnification. B. Chromosome study of isolated stem cells; 1000 X magnification

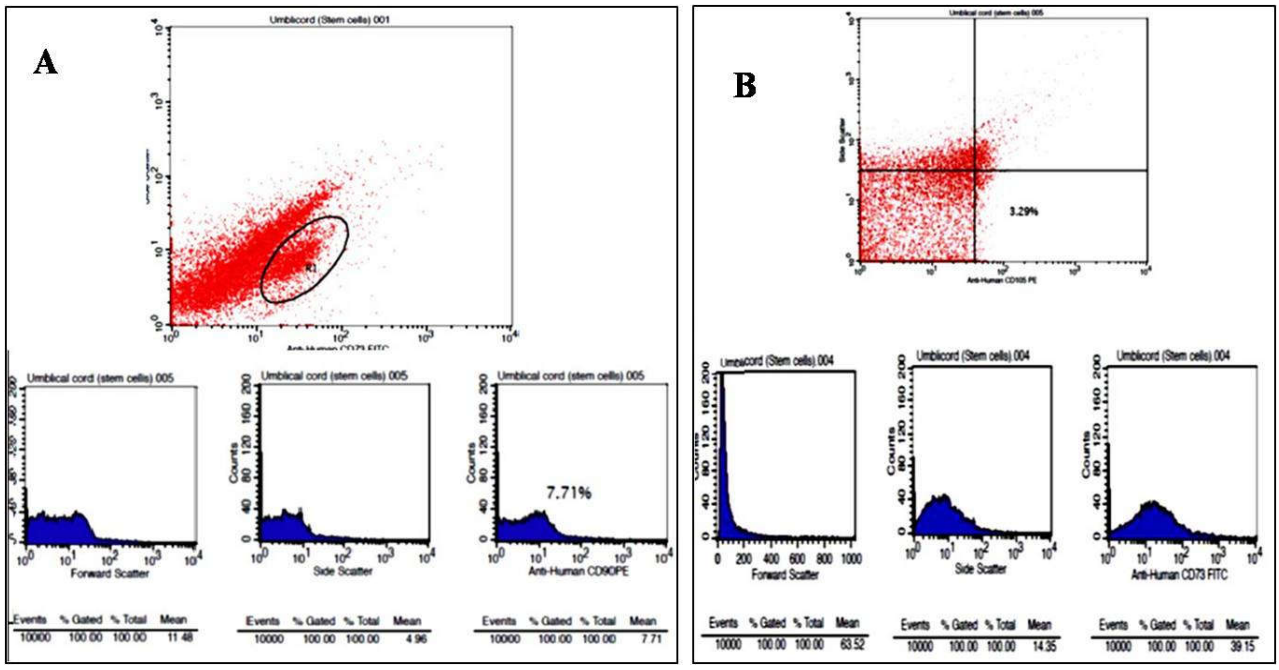

Fig. 5. Immunophenotyping of hUC derived stem cells. FACS analysis of CD 90 (A) and 73 (B) expressing cells

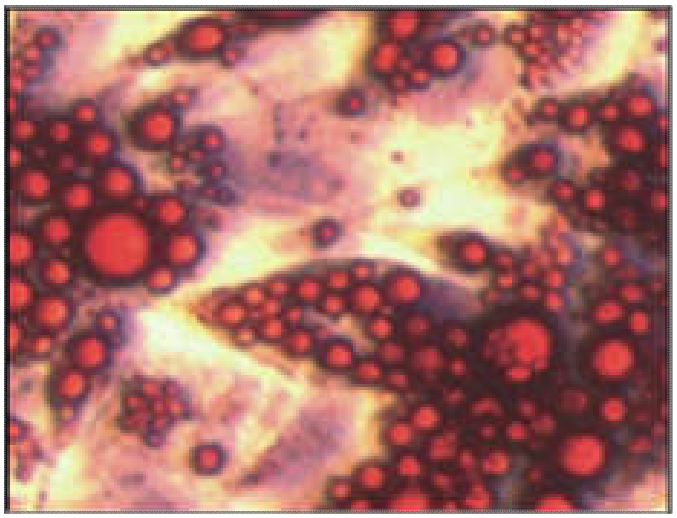

Fig. 6. Oil O red staining of cells. hUC derived cells were differentiated to adipocytes using AdipoXL (HiMedia) basal media

DISCUSSION

We found that yield of cells was better with enzymatic digestion of UC as compared to explants method. In explants culture, after around 15 days in culture, fibroblast-like cells could be found around the tissue explants which further reached 90 % confluence. The cell morphology was relatively homogeneous (data not shown). For the cells that had been isolated by the enzyme digestion method, spindle-like or polygonal fibroblast-like cells could be observed, growing in a dispersed pattern after 3 days in culture. After 9–12 days in culture, the cells were approximately 90 % confluent. We have also done media optimization using different concentrations of serum (data not shown). Cell viability assay revealed that 15% serum provides better nutrient supplement and 90% confluency was observed in comparatively lesser time period (Hayward *et al*., 2013). Growth kinetics studies were carried out based on number of live cells which revealed 1:2 spilt ratio. We could find that total cell count gets increased with passage number and days of incubation. The cells were subcultured when they attained maximum density. Each culture flask was passaged for approximately four times. The differentiation of cells was confirmed by oil red staining. The differentiation was seen after 8-9 days of induction. The differentiation of stem cells into adipocytes confirmed the potency of stem cells in the culture. Isolated UC-MSCs shows rapid proliferation according to proliferation assay and are positive for MSCs markers such as CD73 and CD90 (Haywar*det al.,* 2013). However, more sophisticated methods such FACS sorting or cell cloning is essential to get pure homogenous population of MSCs. hUC derived cells, isolated during this work, have the potential to be used for several studies such as drug testing, cytotoxicity assays and virological research.

Conclusion

Cells from human umbilical cord are isolated using explants and enzymatic digestion method. These cells are harvested and passaged. Shortcoming with the explants method is the inability to determine the number of cells that have been isolated from the cord at passage, because the cells continue to outgrow from the explants even after the cells have been harvested. Human umbilical cord derived cells with fibroblastic morphology are successfully expanded and maintained for 2 months in laboratory conditions. During the study, isolation of hUC derived MSCs with multipotency potential is successfully attempted.

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