ORIGINAL RESEARCH PAPER

Volume-7 | Issue-2 | February-2018 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

HUMAN UMBILICAL CORD BLOOD: A POTENTIAL SOURCE FOR MESENCHYMAL STEM CELLS

Gynaecology

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ABSTRACT

Mesenchymal stem cells (MSCs) have raised immense hopes for their vast applications in regenerative medicine. It has paved its way to a large number of clinical trials for a broad range of diseases. So far, several studies have provided promising results, but more research is required for their further validation due to varying outcomes. This might be due to varying sources of cells, heterogeneity in MSCs cultures and absence of reliable protocols for isolation and enrichment of the naïve stem cell fraction. MSCs can be isolated from many tissues under different culture conditions but appropriate cell source is needed to be investigated for their efficient applications in clinical practice. Human umbilical cord blood (hUCB) is the richest source of non-embryonic stem cells. The collection of MSCs from hUCB is easier, less expensive and non-invasive. UCB-MSCs are less immunogenic, and therefore do not elicit the proliferative response of allogeneic lymphocytes in vitro and they elicit a lower incidence of graft rejection and post-transplant infections compared with other sources. Hence the present study has been taken in consideration for standardization of appropriate *in vitro* protocols of MSCs isolation and enrichment from hUCB .

KEYWORDS

Introduction

Umbilical cord blood (UCB) is considered one of the most abundant sources of non-embryonic stem cells. UCB is an interesting source of these cells because the collection process is painless and non-invasive, it causesno harm to the mother or infant, and it is a material usually discarded **[Bieback et al. 2009; Erices et al. 2000; Gang et al. 2004;Lee et al. 2004].** Evidence has emerged that MSCs may be used for supporting new clinical concepts in cellular therapy. However, MSC isolation from UCB from full-term deliveries is laborious, time-consuming, and results in a low yield of cells **[Bieback et al. 2009**]. Some crucial steps in the isolation process of MSCs from UCB are known to be the time between collection and isolation, the net volume of blood, and the mononuclear cell (MNC) count **[Bieback et al. 2009]**. Other parameters that might relate to a successful isolation of these cells have not been described.

The collection of mesenchymal stem cells (MSCs) from UCB that is discarded at the time of birth is an easier, less expensive and non-invasive method than collecting MSCs from bone marrow aspirates. These MSCs attract special interest due to these specific advantages over embryonic and adult stem cell counterparts, since there are also no ethical issues associated with UCB. Another important characteristic of UCB-MSCs is that they are less immunogenic, and therefore do not elicit the proliferative response of allogeneic lymphocytes *in vitro*. UCB-MSCs expanded *in vitro* also retain low immunogenicity and an immunomodulatory effect. Moreover, cells derived from the UCB elicit a lower incidence of graft rejection and post-transplant infections compared with other sources. Considering these facts, UCB-derived MSCs can therefore be effectively utilized for therapeutic applications of various diseases. These applications include cell-based therapy to replenish degenerated neurons, cardiac cells, muscle cells, chondrocytes, and so forth. However, the potential application of these cells for various purposes requires extensive characterization, and requires standardization of reproducible differentiation protocols with ultimate functional characterization of the differentiated cells.

MSCs are a multipotent stem cell population found originally in the bone marrow **[Pittenger et al. 1999]**. These cells have the inherent ability of differentiating into osteogenic, adipogenic and chondrogenic lineages as well as non-mesodermal lineages including neural and hepatic lineages **[Pittenger et al. 1999; Krause et al. 2001].** MSCs have been also isolated from UCB. UCB-derived MSCs show high morphological and molecular similarities to bone marrow derived MSCs including the lacking of hematopoietic surface antigens CD133, CD34 and CD45 **[Erices et al. 2000; Bieback et al. 2004; Lindner et al. 2010].** Although the isolation of MSCs from UCB has been shown to be laborious because of their low number, compared to the bone marrow, cord blood derived MSCs demonstrate higher proliferation capabilities than bone marrow MSCs **[Bieback et al. 2004].** Cord blood MSCs have been successfully differentiated *in-vitro* into osteogenic, chondrogenic, neural and hepatic lineages [**Kang et al. 2005].** UCB-MSCsin all the media exhibits a specific markerprofile, positivity for CD13, CD44, CD73, CD90, CD105, CD106, and CD166, and negativity for CD14, CD34, CD45, and HLA-DR. The expression levels of positive markers in MSCs are similar among different kinds of media.

Therefore UCB-derived hMSCs, can be routinely isolated and expanded in culture, would be the most practical tool for stem cell-based therapy and transplantation.In the present study, we have attempted to isolate adherent cells of a mesenchymal phenotype from human UCB sample and assessed the identity of cells. The establishment of a reproducible adult stem cell culture system, like MSCs, is important to elucidate the role of molecular markers. The present study was undertaken to standardize appropriate *in vitro* protocols for MSCs isolation and enrichment from hUCB based on

cellular perception for their further applications.

Materials & Methods

UCB collection and sample selection for study

The study was approved by Institutional Review Board of Deccan College of Medical Sciences, Hyderabad.UCB was collected from the umbilical cord vein in 1 IU of heparin tube with informed Consent of the mother.The cord blood was processed within in 1 h of collection for MNCs isolation.

Mono Nuclear Cells (MNCs) Isolation from cord blood

5 ml of cord blood was diluted with 5 ml of saline in a 15 ml falcon tube. This diluted cord blood was transferred slowly onto 3 ml of ficol so as to form a layer. This was centrifuged at 3000 rpm for 30-45 min. A buffy coat of MNCs will be formed. This buffy caot has transferred into another tube and 5 ml of saline was added. Centrifuged at 3000 rpm for 10 min. Supernatant was discarded and to the pellet added 5 ml of saline. Centrifuged at 3000 rpm for 10 min. Then the pellet was dissolved in 1 ml of DMEM media supplemented with 10% FCS and with antibiotics. Further the cells are assed for viability.

Cell viability assessment

Trypan blue assay

Dilution of the cell suspention was prepared by using 0.2% trypan blue solution. Cells were incubated at RT for 2-3 min. The counting chambers of hemocytometer were loaded with 10 µl of cells mixed in trypan blue and covered with glass coverslip. Using bright field optics cells were counted in all counting chambers. Non-viable cells (stained in deep blue) as well as viable cells (unstained) are counted separately. Percentage of viable cells and concentration was determined using following formula:

% viability = (no. of viable cells/no. of total cells) × 100

FDA Assay

1x 10^4 cells were taken in a microcentrifuge tube (1.5 ml). 5 µl of FDA (1 µg/ml) was added to the cells and were incubated at RT for 5min. Cells were covered using glass coverslips, mounted and observed under the inverted fluorescent microscope and analyzed in Axiovert Version 4.2 (Carlziews, Germany)

MTT Assay

The initial concentration of cells was taken as 3×106 cells/ml. Serial dilution was prepared till the cells concentration reaches 3×103 cells/ml. 100 µl of cells were seeded in triplicates into 96 well microplate. 30 µl of MTT (1 mg/ml) was added in each sample including negative control. Samples were mixed thoroughly and incubated for 4 hr at 37˚C in CO2 incubator at 5% CO2 and 100% humidity. After 4 hr, 50µl of Isopropanol was added in the cells to stop the reaction. Optical Density of cells were determined in a microplate reader at 540-570nm.

Enrichment of human UCB-MSCs

Isolated MNCs were cultured in DMEM-F12 media with 10% FCS and 100 µl/mL Penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), and 0.25 mg/mL amphotericin B (Invitrogen). MNCs with plastic adherent property can attach to the petriplatewith in 24-48 hr. Once they got attached the medium is replaced with a fresh media for every 2nd OR 3rd day. MNCs will start proliferating and change their morphology from spherical to spindle shaped MSCs within a week.

Morphological assessment

For morphological characterization MSCs were cultured on collagen coated petri-dishes at 1.2 X 10^4 cells/cm^2 density in DMEM-F12 medium, supplemented with 10% Fetal calf serum (FCS, Invitrogen), 100 µl/mL Penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), and 0.25 mg/mL amphotericin B (Invitrogen). Morphological characterization of cultured cells was done using phase contrast confocal microscopy (Carl Zeiss, Germany).

Assessment of cell proliferation using MTT

The initial concentration of cells was taken as 3×106 cells/ml. Cells were seeded in triplicates into 96 well microplate. 30 µl of MTT (1mg/ml) was added in each sample including negative control. Samples were mixed thoroughly and incubated for 4 hours at 37˚C in CO2 incubator at 5% CO2 and 100% humidity. After 4hours, 50 µl of Isopropanol was added in the cells to stop the reaction. Optical Density of cells was determined in a microplate reader at 540-570 nm. Readings are taken at regular intervals of time at 0-day, 7-dayS, 14 - days.

ICC staining of human UCB-MSCs using CD90-FITC at day 14

Cultured UCB-MSCs were stained with positive mesenchymal marker CD90-FITC according to manufacture instruction.

Results

Human Umbilical cord blood collection

UCB was collected into bags containing citrate-phosphate-dextrose or heparinas anti-coagulant under sterile conditions and processed within 24 h.

Isolation of mononuclear cells

MNCs were isolated from UCB by Ficoll-paque density gradient centrifugation.

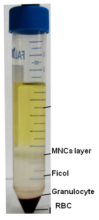


Figure 1: Ficoll-paque density gradient separation of MNCs from UCB

Trypan Blue Exclusion Assay

In this assay, we observed viable cells which don't uptake any color and non-viable cells (dead cells) taking trypan blue dye with appearance in dark blue color. The cells were spherical in shape with approximately <10µm in diameter.

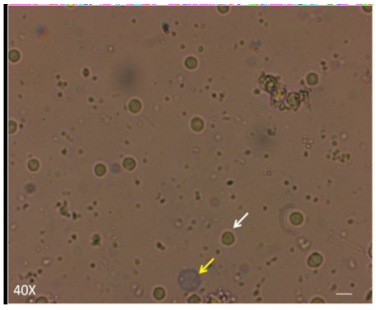


Figure 2: Trypan blue-exclusion assay showing viable cells (indicated by white arrow) and dead cells stained in blue (indicated by yellow arrow)

FDA cell viability assay in isolated human UCB-MNCs

The cell membrane integrity and viability of human UCB-MNCs were identified by using fiourescentdiacetate assay (FDA) where enzymatic activity of esterases on FDA was identified by incubating cells with 1mg/ml FDA. The cells fluorescing green under fluorescence microscope were termed as viable cells and remaining non-viable. Most of the cells had intact membrane showing circular-oval shape with no dye diffusion. The cell membrane showing dye diffusion were found damaged cell membrane and contained pores. cell membrane showing dye diffusion were found damaged cell membrane and contained pores.

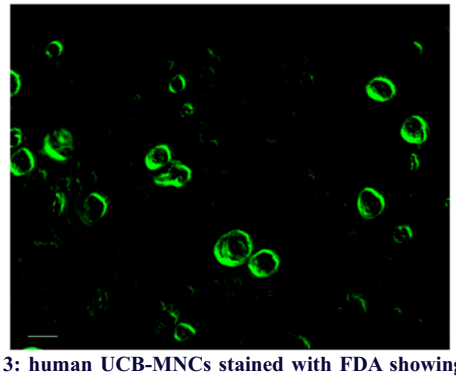


Figure 3: human UCB-MNCs stained with FDA showing intact membrane and high percentage viability under fluorescence

microscope (Magnification: 40X, Scale bar: 10X)

Enrichment and characterization of human UCB-MSCs

When we cultured morphology of cells was changed from spherical to spindle shape from day1 to day 14. The cells were starting adhere on petri-plate at day 3 and changed their morphology completely at day 14.

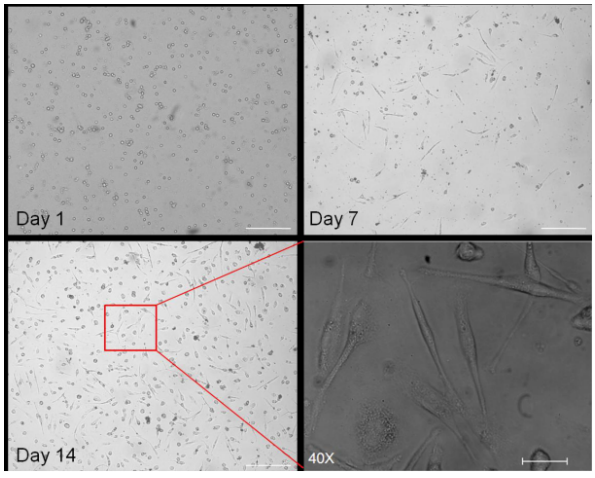


Figure 4: Proliferating human UCB-MSCs (A) Day 1(upper left panel) MNCs, (B) Day 7 (upper right panel) proliferating MSCs, (c) Day 14 (lower left panel) confluent MSCs, (D)

Day 14 (lower right panel) proliferating MSCs under higher resolution (40X: magnifications)

ICC staining of human UCB-MSCs using CD90-FITC at day 14

Cultured UCB-MSCs were stained with positive mesenchymal marker CD90-FITC. We observed most of the cells showed positive staining for CD90. The fibroblast or spindle shaped phenotype of cells flourished in green with intracellular staining of Cd90.

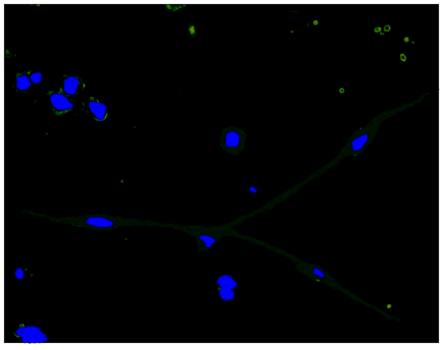


Figure 5: ICC staining for proliferating cells as monolayer at Day 14. Spindle shaped structure was stained with CD90-FITC showing morphology of MSCs

MTT cell proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assay was performed at day 3, 7 and 14 to assess hMSCs proliferation efficiency. Briefly; 3.1 X 105 cells were seeded in each well of 96 well cell culture plate in different groups as described above. Cells were allowed to proliferate for 14 days and assessed for MTT reduction at different time periods. Formazan crystals were dissolved using acidified Isopropanol solution. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, USA) and compared in each group.

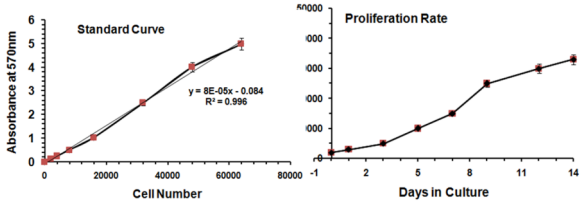


Figure 6: MTT cell viability assay showing straight graph of serially diluted cells (standard curve) and proliferating cells at different time points. Values indicate Optical density readings taken after MTT Assay

Discussion

Umbilical cord blood (UCB) is considered one of the most abundant sources of non-embryonic stem cells **[Erices et al. 2000]**. The collection of mesenchymal stem cells (MSCs) from UCB that is discarded at the time of birth is an easier, less expensive and non-invasive method than collecting MSCs from bone marrow aspirates **[Chang et al. 2006]**. These MSCs attract special interest due to these specific advantages over embryonic and adult stem cell counterparts, since there are also no ethical issues associated with UCB. Another important characteristic of UCB-MSCs is that they are less immunogenic, and therefore do not elicit the proliferative response of allogeneic lymphocytes in vitro **[Le Blanc et al. 2003]**. UCB-MSCs expanded in vitro also retain low immunogenicity and an immunomodulatory effect. Moreover, cells derived from the UCB elicit a lower incidence of graft rejection and post-transplant infections compared with other sources **[Knutsen et al. 1999]**. Considering these facts, UCB-derived MSCs can therefore be effectively utilized for therapeutic applications of various diseases. These applications include cell-based therapy to replenish degenerated neurons, cardiac cells, muscle cells, Osteocytes, chondrocytes, and so forth.

In the present study we have isolated UCB-MNCs using Ficoll-paque and cultured *in vitro* to proliferate into MSCs. Cell counting and cell viability was assessed by three different methods. In trypan blue exclusion assay we observed viable and non viable cells, FDA assay membrane integrity and MTT mitochondrial dehydrogenase enzymatic activity, together representing high percentage of viable and intact cells having rich metabolic activity.

Isolated MNCs were cultured *in vitro,* MNCs with plastic adherent property can attach to the petriplate within 24-48 hr. Once they got adhere MNCs will start proliferating and change their morphology from spherical to spindle shaped MSCs within a week. Further Morphological characterization of cultured cells was done using phase contrast confocal microscopy (Carl Zeiss, Germany). In ICC staining UCB-MSCs cultured as monolayer stained with mesenchymal specific marker CD90. In molecular characterization, cultured cells at day 3, 7 and 14 of UCB-MSCs were harvested by trypsinization and used for total cell RNA isolation. We observed 3 bands as 28s, 18s and 5s on agarose gel showing intact high quality RNA. cDNA also synthesized and appeared as intact single band on agarose gel. MTT cell proliferation assay showed linear graph for long-term proliferation of UCB-MSCs representing highly increased metabolism and cell proliferation from day 3 to 14.

Although isolation and characterization is a preliminary step in stem cell research, it is certainly a crucial step and the data obtained will aid in conducting further research on efficacy of *ex vivo* expanded stem cells for various cell-based therapies.

Acknowledgment

None

Conflict of Interest

None

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