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CELLULAR AND MOLECULAR EVALUATION OF IN VITRO QUALITY PARAMETERS OF HUMAN UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS

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ABSTRACT

Introduction: Establishing a reproducible adult stem cell culture system, such as mesenchymal stem cells (MSCs), is critical for elucidating the function of molecular markers associated with these cells' undifferentiated state. In this study, we describe some important parameters to be considered for a successful isolation, culture, and characterization of MSCs from human umbilical cord blood (hUCB).

Methods: Five hUCB samples were collected from healthy female subjects who were free from infectious diseases and genetic disorders. Mononuclear cells (MNCs) were counted, and viability was determined using MTT assay. MNCs were cultured in DMEM supplemented with 10% fetal bovine serum and enriched through culture and characterized using morphometric, and molecular analysis.

Results: The minimum number of cells was 12.5 million and highest number of cells was 20.6 million from all five samples. In initial culture of MSCs from hUCB, various morphologic phenotypes were seen, although the cells eventually developed a homogeneous fibroblast-like shape at day 14 showing >80% confluency. Spindle-shaped clonogenic MSCs expressed a high level of CD90, CD105, and CD73, while negative expression of CD34. Our study provided evidence of expansion of enriched MSCs in culture from day 1 to day 21 as supported by data of CD90, CD105 and CD73 expression levels in a time-dependent manner.

Conclusion: Our results suggest that the expanded hUCB harbor an enriched source of MSCs that express pluripotent stem cell markers and lack hematopoietic markers after culture and forms the basis for using hUCB as eminent source of MSCs, which can be used for different therapeutic applications.

KEYWORDS : Human umbilical cord blood, mesenchymal stem cells, enrichment

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells that form in the early stages of development in the mesoderm. MSCs were originally identified in bone marrow and have now been shown to be isolated from a range of human tissues, including adipose tissue, nerve tissue, the umbilical cord, and amniotic fluid (**Kobolak et al., 2016; Zhan et al., 2019**). The number of bone marrow MSCs (BM-MSCs) is exceedingly low, while the risk of viral infection is extremely high. Furthermore, the quantity of stem cells, as well as their capacity to grow and differentiate, decreases with age, limiting their therapeutic usefulness (**Kern et al., 2006**). Research shows that MSCs are abundant in human umbilical cords.

Human umbilical cord has always been thought of as a waste product after delivery, there is no ethical debate about isolating MSCs from it compared to bone marrow. Umbilical cord – mesenchymal stem cells (UC-MSCs) have similar morphological, immunophenotype, proliferation, multi-directional differentiation, and ability to promote differentiation of hematopoietic stem cells (HSCs) as that of BM-MSCs (**Thaweesapphithak et al., 2019**), but UC-MSCs have higher proliferative capacity and lower HLA-ABC and HLA-DR expression than BM-MSCs. Furthermore, UC-MSCs with a wide range of stem cells are readily available, convenient to collect, and store. UC-MSCs are predicted to be a good replacement for BM-MSCs.

MSCs may be extracted from a variety of tissues, including bone marrow (BM), umbilical cord (UC), adipose tissue, dental pulp, and umbilical cord blood (UCB). MSCs may be used to support novel therapeutic ideas in cellular treatment, according to evidence. MSCs separation from UCB from full-term births, on the other hand, is time-consuming and yields a limited quantity of cells (**Bieback et al. 2004; Secco et al. 2008**). The period between collection and isolation, the net amount of blood, and the mononuclear cell (MNC) count are all recognized to be important factors in the isolation of MSCs from UCB (**Bieback et al. 2004**). Other factors that might influence the effective isolation of these cells have yet to be identified.

Establishing a reproducible adult stem cell culture system, such as MSCs, is critical for elucidating the function of molecular markers associated with these cells' undifferentiated state. Because of the non-invasive nature of collection and its widespread availability, the effective isolation of MSCs from UCB is of significant interest. However, there have been reports of difficulty in isolating and differentiating these cells. In this study, we describe some important parameters to be considered for a successful isolation, culture, and characterization of MSCs from UCB. We characterize MSCs by their morphometric appearance and gene expression profile before and after enumeration in culture. We have identified transcripts which are reported to be selectively expressed in undifferentiated MSCs population in culture.

METHODS

This study has been approved by Institutional Review Board of Deccan College of Medical Sciences, Hyderabad, Telangana, India. All the study participants provided their informed consent forms, and the study related procedures were conducted in accordance with the ethical guidelines of Indian Council of Medical Research, Govt. of India.

Sample Collection and Transportation

Five human UCB samples were collected from healthy female subjects who were free from infectious diseases and genetic disorders. Human UCB (30 mL) was collected in a 50mL polypropylene tube containing 30 IU/mL heparin during cesarean from Owaisi hospital and transported to laboratory for further processing.

MNCs Isolation from hUCB Samples

A total of 5mL of UCB was diluted in 1X phosphate buffer saline (PBS)in 1:1 ratio and carefully layered over Ficol-paque density gradient medium (1.073 g/mL). Each tube was centrifuged at 1100 × g/min for 30min to separate different layers. MNCs were transferred from interface layer to fresh tube, suspended in two volumes of PBS, and collected by centrifugation at 1100 × g/min. Each cell pellet was washed twice with 1X PBS and resuspended in 500µL. Cell concentration and viability in each sample was determined by Trypan blue exclusion assay.

Enumeration of Human MSCs

MNCs were mixed with low glucose Dulbecco's Modified Eagle Medium (DMEM-LG) supplemented with heat inactivated 10% fetal

bovine serum, 1X antibiotic and antimycotic solution. 2 X 10^4 MNCs were seeded into a T-25 flask and incubated at 37°C in 5% CO_2 atmosphere with 95% humidity. Fresh culture medium was added every after third day and floating or non-adherent cells were removed on each occasion. Cells were allowed to grow up to 21 days. Cells from each UCB sample were cultured up to 6 passages after reaching to 80% confluency. At each passage, cells were removed from culture flask using 0.25% trypsin and sub-cultured at 1:3 ratio each time to expand through successive passages.

MSCs Characterization

Morphometric analysis

Enriched MSCs population during first time culture from each sample was characterized by morphometric appearance using microscopic imaging at different time points from day 1 to day 21.

Molecular Analysis

Relative quantification of MSCs positive markers CD73, CD90, CD105 and negative markers CD34 and CD45 were performed using SYBR Green-based real-time quantitative polymerase chain reaction (RT-qPCR) with the help of a RT-qPCR system (S1000, Bio-Rad, USA). Briefly, total ribonucleic acid (RNA) was extracted from cells at day 1, 3, 7, 14, and 21 using GITC method (Chomoczynski, 1987) and converted into complementary DNA (cDNA) using MMLV reverse transcriptase II. A total of 5ng cDNA was used to conduct RT-qPCR in triplicates using expand GAPDH specific primers as endogenous control.

MSCs Growth Rate Analysis

MSCs growth rate analysis was performed using MTT cell proliferation assay from day 1 to day 21 at first passage and day 1 to day 14 at subsequent passages for all five UCB samples. Briefly, at each time point 2mg/mL MTT was added in each sample parallel cultured in 96 well plates in triplicates and incubated for 4h at 37°C. Following to incubation, dark blue formazan crystals formed in actively metabolizing cells were dissolved in dimethyl sulfoxide (DMSO) and absorbance (optical density) was measured 570nm wavelength using a microplate reader.

Statistical Analysis

All the values were recorded and presented as mean±SD. Student t-test and one-way ANOVA was used to compare the groups for generating statistical significance between each group. A p value <0.05 was set for statistical significance for all the parameters among groups.

RESULTS

Cell Count and Viability from Each hUCB Sample

Ficol-paque density gradient separation of MNCs from a total of five UCB samples was performed to segregate other cellular components and further to enrich MSCs population through *in vitro* culture. Density gradient separation clearly showed absence of erythrocyte components and other debris and counterparts producing pure population of MNCs harboring MSCs. Total number of viable and dead cells were counted using trypan blue exclusion assay with the help of hemocytometer (**Table 1**).

Table1 Total Number and Percentages of Viable Cells Isolated Using Ficol-paque Density Gradient Centrifugation from Five Different Cord Blood Samples

Subject ID	Gender	Age (Year)	No. of Cells (Millions)	Cell Viability (%)
UCB-S01	F	26	16.1	94.1±4.1
UCB-S02	F	28	18.2	96.4±3.2
UCB-S03	F	32	12.5	94.5±3.6
UCB-S04	F	34	12.6	93.2±4.1
UCB-S05	F	30	20.6	97.6±2.4

Enrichment and Characterization of Human MSCs

Enrichment of MSCs was first confirmed using morphological assessment with the help of light microscopy under 10X magnification starting from day 1 to day 21. In vitro culture of isolated MNCs using ficol-paque density gradient separation of human UCB cells showed change in their morphology from day 3 in serum containing medium.

Spherical-shaped cells started appearing as fibroblast-like appearance from day 3 and became fully spindle shaped at day 14. The spindle fibers of cells lengthened at day 21 and showed typical morphological features of MSCs (**Figure 1**).

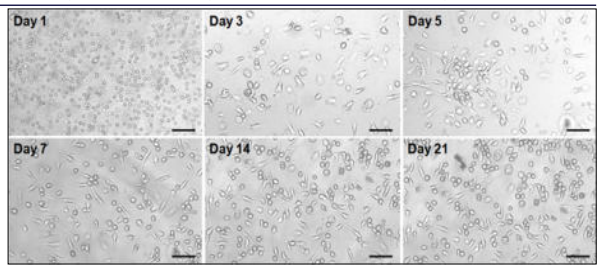

Figure 1: Morphological Assessment of Cultured MNCs from Day 1 to Day 21 Showed Gradual Changes in Cell Size and Shape and Appeared Fully in Spindle Morphology at Day 14 And Day 21 Under Phase-contrast Microscopy. Scale Bar: 50µm, Magnification: 10X

Molecular Characterization

Molecular characterization of human MSCs was performed using both qualitative and quantitative PCR analysis.

Qualitative PCR analysis at day 1 of MSCs specific makers revealed strong expression of CD34 (MSCs negative marker) and weaker expression of CD90, CD73 and CD105 (specific to MSCs markers), while at day 14 strong positive expression for CD90, CD73 and CD105 (specific to MSCs markers) and negative for CD34 (MSCs negative marker) on agarose gel at day 14 was observed (**Figure 2**).

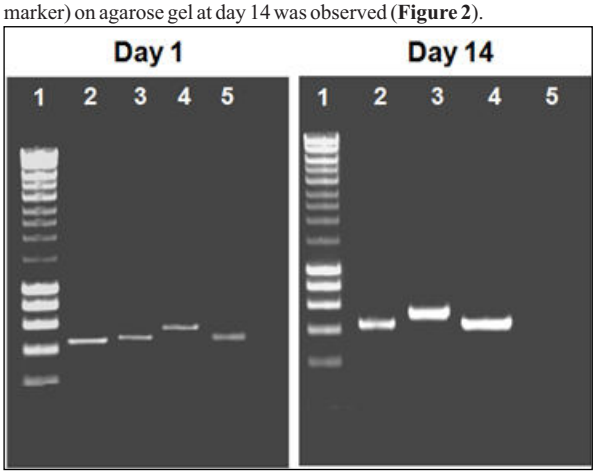

Figure 2: Qualitative PCR Showing Difference in Expression of Positive and Negative MSCs Specific Markerson Agarose Gel after Ethidium Bromide Staining (M indicates DNA marker of 50bp)

Quantitative PCR analysis of in vitro enriched MSCs at day 1, 3, 7, 14 and 21 for specific positive (CD90, CD73 and CD105) and negative markers (CD34) using SYBR-Green based relative gene expression analysis showed significantly higher fold values for positive markers whereas non-significant expression levels were observed for CD34 (**Figure 3**).

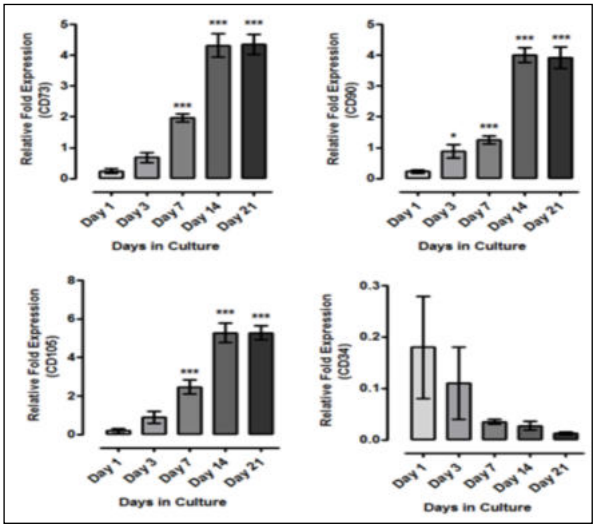

Figure 3: Relative fold values of quantitative gene expression changes

for positive and negative markers specific to human MSCs in culture with time showing significantly higher expression levels at day 14 and day 21 for **(A-C)** CD73, CD90 and CD105 (***p<0.0001) whereas no significant increase in **(D)** CD34 was observed (p>0.05).

MSCs Growth Rate Assessment

Quantitative evaluation of growth rate and changes in viability of cultured human MSCs with increasing the time was estimated using MTT cell proliferation and viability assays. The cell proliferation assay revealed significant increase in number of human MSCs during in vitro culture with the time and was significantly higher at day 14 and day 21 (**Figure 4A**). However, MTT cell viability assay revealed no significant change in viable nature of MSCs during in vitro culture with the time.

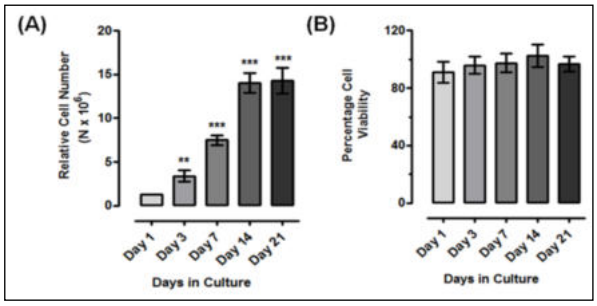

Figure 4: (A) MTT cell proliferation assay showing significant increase in number of cultured human MSCs at day 7 (**p<0.001), day 14 (***p<0.001) and day 21 (***p<0.001) as compared to day 1. **(B)** No significant change in viability of in vitro enriched human MSCs was observed with the time (p>0.05)

DISCUSSION

MSC is a potential cell type for enabling novel cellular treatment approaches. UCB is one of the greatest sources of adult stem cells accessible. UCB has been proven to contain endothelial progenitor cells as well as MSCs in addition to hematopoietic stem cells. Although bone marrow is still the most frequent source of off-the-shelf MSCs, UCB is a viable option that may be obtained noninvasively and with few ethical issues. However, the low projected frequency of effective isolation and variability of successful isolation pose significant hurdles for clinical studies using UCB-derived MSC. For humans, a wide range of isolation and culture of MSC from UCB have been documented. This study explores whether UCB may represent a suitable source of MSCs for pre-clinical and clinical use and analyzes several in vitro parameters useful to better define the quality of UCB-derived MSC prior to their application.

In our study, MSCs were extracted from five human UCB samples and compared for cell number and viability before culture. The minimum number of cells was 12.5 million and highest number of cells was 20.6 million. Although cell number varied from different samples, no significant difference in cellular viability was reported (minimum 93.2±4.1 % and maximum 97.6±2.4 %). MSCs enrichment was done by culturing isolated MNCs on plastic culture dishes in DMEM supplemented with 10% FBS. The FBS content was adjusted by 10% to give the optimal growth for these cells, which exhibited excellent efficiency in maintaining the culture and led to the rapid amplification of cell stock.

Several culture media have been tested for maintenance of MSCs such as MEM, DMEM, RPMI-1640 and Basal Medium Eagle (BME), supplemented with FBS, generally at concentration of 10-20%. The choice of the medium culture is important to success of MSC growth in the in vitro cell culture (**Tapp et al., 2009**). MSC were highly dependent on serum for growth. Cells cultured in 10% FBS reached to 80-85% confluence similar to the earlier study (**Im et al., 2005**). In initial culture of MSC from human UCB, various morphologic phenotypes were seen, although the cells eventually developed a homogeneous fibroblast-like shape at day 14 showing >80% confluency.

Spindle-shaped clonogenic MSC expressed a high level of CD90, CD105, and CD73, while negative expression of CD34. CD34 is a hematopoietic stem cell surface marker that is found in lymph nodes, bone marrow, and different endothelial cells. The absence of CD34 staining proved that the cells were not generated from circulating stem cells (**Pittenger et al., 1999, Peng & Huard 2004**). Our study provided evidence of expansion of enriched MSCs in culture from day 1 to day 21 as supported by data of CD90, CD105 and CD73 expression levels in a time-dependent manner. The cell number was also enhanced in culture with time and was highest at day 14. No significant difference was observed in cell number between day 14 and day 21 representing that cell reaches to highest confluency at day 14 and could be considered as highest growth time point for human UCB-MSCs.

CONCLUSION

Our results suggest that the expanded hUCB harbor a enriched source of MSCs that express pluripotent stem cell markers and lack hematopoietic markers after culture. Human UCB cells possess an inherent stromal cells property. These pluripotent MSCs reach to >80% confluency at day 14 in culture with significant increase in cell number and continuous increasing expression of specific markers. Our findings therefore form the basis for using human UCB as eminent source of MSCs, which can be used for different therapeutic applications.

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Conflict Of Interest: None

REFERENCES

1. Kobolak, J., Dinnyes, A., Memic, A., Khademhosseini, A., and Mobasheri, A. (2016). Mesenchymal stem cells: identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche. Methods 99, 62–68.
2. Zhan, X. S., El-Ashram, S., Luo, D. Z., Luo, H. N., Wang, B. Y., Chen, S. F., et al. (2019). A Comparative study of biological characteristics and transcriptome profiles of mesenchymal stem cells from different canine tissues. Int. J. Mol. Sci. 20:1485.
3. Kern, S., Eichler, H., Stoeve, J., Klüter, H., and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24, 1294–1301.
4. Thaweesapphithak, S., Tantrawatpan, C., Kheolamai, P., Tantikanlayaporn, D., Roytrakul, S., and Manochantr, S. (2019). Human serum enhances the proliferative capacity and immunomodulatory property of MSCs derived from human placenta and umbilical cord. Stem Cell Res. Ther. 10:79.
5. Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22:625–634.
6. Chomczynski, P., &Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, *162*(1), 156–159. https://doi.org/10.1006/abio.1987.9999
7. Secco M, Zucconi E, Vieira NM, Fogaca LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M. Mesenchymal stem cells from umbilical cord: do not discard the cord! NeuromusculDisord. 2008;18:17–18.
8. Tapp H., Hanley E.N., Patt J.C. & Gruber H.E. 2009. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. Exp. Biol. Med. 234:1-9.
9. Im GI., Shin Y.W. & Lee K.B. 2005. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis and Cartilage 13:845-853.
10. Pittenger M.F., Mackay A.M., Beck S.C., Jaiswal R.K., Douglas R., Mosca J.D., Moorman M.A., Simonetti D.W., Craig S. &Marshak D.R. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143-147.
11. Peng H. & Huard J. 2004. Muscle-derived stem cells for musculoskeletal tissue regeneration and repair. Transpl. Immunol. 12:311-319.