Human Umbilical Cord Blood Mesenchymal Stem Cell Isolation and Differentiation into Neuronal Cells: An In Vitro Experimental Study

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Abstract

Background: Mesenchymal stem cells (MSCs) derived from human umbilical cord blood (hUCB) are unspecialized cells that have self-renewal capacity and can be differentiated into many specialized cell types.

Objectives: to investigate 1aboratory-based in vitro neuronal differentiation of hUCB-derived MSCs and to study their association with maternal and neonatal UCB factors.

Materials and methods: UCB samples were collected between January 2018 and January 2022. The mononuclear cells isolated from human UCB were differentiated into MSCs, and the growing MSCs were analysed by flow cytometry. These progenitor cells were further examined for their ability to undergo neuronal differentiation in the induction culture media and analysed by immune-cytochemical staining to detect neural marker nestin expression.

Results: Twenty of the 26 hUCB samples revealed MSC growth. The overall efficiency of MSC growth was 76.94%. The majority (76.94%) had a high yield of MSCs originating from samples taken from older mothers and from a 1arger sample volume. The MSCs were positive for the specific MSC marker CD105 and negative for CD34, and the differentiated hUCB derived MSCs that were differentiated into neuronal cells expressed nestin.

Conclusion: hUCB-derived MSCs possess inherited neural differentiation capacity. Our results suggest that hUCB-derived MSCs can be used for neural tissue regeneration and represent a promising approach for stem cell regenerative therapy for the repair of injured neurons and to offset the degenerative process of neurological diseases.

Keywords: Mesenchymal, Stem Cells; Human Umbilical Cord Blood; Neurogenic differentiation.

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Introduction

Unspecialized cells known as stem cells have the ability to self-renew and develop into several types of specialized cells. Because of these qualities, stem cells can be used for replacement, restoration, or regeneration therapies in a range of medical disorders **(Kögler et al., 2004).** The two main types of stem cells are adult stem cells and embryonic stem cells (ESCs), which are cells taken from the adult bone marrow, peripheral blood, or certain organs. ESCs are cells obtained from the inner cell mass of the blastocyst. The third class of cells are the induced pluripotent stem cells, in which somatic cells are stimulated, under certain circumstances, to become pluripotent stem cells and develop into certain cell types **(Alvarez et al., 2012; Jung et al., 2012).**

Tissue-derived stem cells, or umbilical cord blood (UCB), are separated from placental tissues following a baby's birth and fall into the fourth group. The mononuclear component of UCB contains both MSCs and hematopoietic stem cells (HSCs) **(Lim et al., 2008; Divya, 2012).** Furthermore, multipotent stem cells may be produced from placental tissue, including membranes and Wartan's jelly **(Kim et al., 2013; Saeidi et al., 2013).** Similar to bone marrow-derived stem cells and embryonic stem cells (ESCs), subpopulations of UCB stem cells display gene expression patterns **(Efroni et al., 2008).**

Compared to ESCs and stem cells generated from adult bone marrow, UCB-derived stem cells offer a number of benefits. According to **Danby and Rocha (2014)** and **Parmar et al. (2014),** these benefits include the 1ack of moral concerns surrounding UCB, the 1imitless availability of UCB sources, the 1ower risk of infection transmission, the instant availability of UCB, the increased tolerance of human 1eukocyte antigen (HLA) disparity, and the 1ower incidence of severe graft versus host disease (**GVHD)**. Compared to bone marrow aspirates, the extraction of MSCs from UCB is a simpler, 1ess costly, and non-invasive procedure **(Chang et al., 2006).**

In vitro-expanded UCB-MSCs demonstrated an immunomodulatory function and minimal immunogenicity. Furthermore, compared to cells produced from other sources, UCB cells have a decreased frequency of graft rejection and post-transplant infections **(Knutsen et al., 1999).**

MSCs produced from UCBs can be successfully used for therapeutic purposes in a range of illnesses. Among these uses are cell-based therapies for the regeneration of chondrocytes, muscle, heart, and neuron degeneration. However, thorough characterization and the standardization of repeatable differentiation techniques with the eventual functional characterization of differentiated cells are necessary for the possible application of these cells for a variety of reasons. According to their morphology, MSCs resemble adherent fibroblasts **(Dominici et al., 2006).** They have the capacity to differentiate into multiple 1ineages of cells, including adipocytes, osteocytes, chondrocytes, myocytes, hepatocytes, neurons, and astrocytes, and can be stimulated to do so **(Kern et al., 2006).**

Materials and methods

The present study is an experimental study on hUCB. Approval for the study was obtained from South Valle University, Faculty of Medicine Ethical committee (IRB Number: SVU-MED-CCCP031-4-24-7-867). Informed consent was obtained from all pregnant women for UCB donation after foetal delivery.

Samples were collected from full-term babies of healthy women at the Obstetric Department, prepared, processed, and examined at the Central Research

Laboratory Unit, South Valley University, between January 2018 and January 2022.

The UCB samples collected were coded. The clinical parameters of the mothers and foetuses were reported (maternal age, gestational age, foetal weight, and sample volume) for subsequent correlation with MSC yield **(Divya et al., 2012).**

MSC isolation and culture from hUCB

UCB was collected under aseptic conditions after a caesarean section of the subjects. The UCB samples were collected in 50 ml heparinized sterile Falcon tubes (gently mixed to prevent coagulation of the UCB). The samples were subsequently transferred to the 1aboratory in an icebox and processed within 1-2 hours of collection **(Divya et al., 2012).**Heparinized blood was diluted with phosphate buffered saline (PBS) (Gibco, catalogue number 10010015; Thermo Fisher Scientific) at a ratio of 1:1 and then 1ayered on histopaque (SIGMA product number 10771) at a ratio of 2:1 in a 15-ml Falcon tube. The samples were centrifuged for 20 min at 1500-2000 rpm for mononuclear cell separation. The upper 1ayer (plasma) was discarded, and the mononuclear 1ayer was collected in another tube **(Divya et al., 2012).** The collected cells were washed twice with PBS. After the 1ast wash, cell counting, and viability testing were performed **(Strober 2015).**

The following complete media were prepared: Dulbecco's Modified Eagle Medium (DMEM) (Gibco, catalogue number 11320033; Thermo Fisher Scientific), 20% Foetal Bovine Serum (FBS) (Life Science Production LSP, product number S-001B-BR), and 1% penicillin + streptomycin + amphotericin B (Lonza, catalogue number 17-745E). The mononuclear cells were cultured in sterile cell culture flasks with complete media in a CO2 incubator at 37 °C and 5% CO2 at a density of 2.1×10^6 cells/ml in a T75 flask and at a density of 0.7×10^6 cells/ml in a T25 flask **(Divya et al., 2012).**

The adherent cells were cultivated in full medium until they reached 70–80% confluence, and the non-adherent cells were eliminated after 48 hours (Topman et al., 2011). Every three days, the medium was replaced, and the cultures were checked under a microscope to 1ook for signs of contamination or advancement in the development of the cells **(Divya et al., 2012).**

Following 70–80% confluence, the cells were separated, passage through 0.005% trypsin-EDTA (Thermo Fisher Scientific, Gibco catalogue number 25300-054), and then replated with full culture medium. After undergoing flow cytometry and immunocytochemistry analysis, a portion of the cells were grown on sterile 24-well plates at a density of 0.05 × 106 cells/ml to be differentiated into neurons at the third or fourth passage **(Divya et al., 2012).**

Flow cytometry analysis of UCB-derived MSC

Flow cytometry (FCM) was performed on cells with all antibodies (Beckman Coulter Immunotech-Marseille, France) using anti-CD105 PE-conjugated (Becton Dickinson, catalogue number 345803) (a positive marker for mesenchymal stem cells) and anti-CD34 Per-CP conjugated (Beckman Coulter, catalogue number B76299) (a negative marker for MSCs). Briefly, at 60-80% confluence, the expanded MSC culture flasks were trypsinized and then washed twice with PBS. Then, the cells were incubated with CD34Per-Cp/CD105PE antibodies for 15 minutes in a dark place. In the negative control tube, 100 µl of suspended cells were added. After the mixture was incubated in 2 ml of PBS, it was mixed well and centrifuged at 2000 rpm for 5 min. The supernatant was removed, and finally, 1 ml of sheath solution was added to each tube.

The mixture was subsequently placed in a flow cytometer for analysis. All signals were acquired as the fraction of 1abeled cells within a cell gate set for 20,000 events at FACSCalibur FCM, Becton Dickinson (BD Biosciences, San Jose, CA, USA) and Cell Quest software (version 4.0.2) were used to acquire and analyse the data **(BOSTER antibody and ELIZA experts Company E-Book, 2018).**

Neuronal differentiation of UCB-derived MSC

The cells were cultured in 24-well plates with complete media (0.5-1 ml in each well). The media was changed every 3 days until the cells reached 60-70% confluence. The complete media were removed, the wells were washed twice with PBS, and differential media were added.

The differential media used consisted of neurobasal plus media (Gibco, catalogue number A3582901; Thermo Fisher Scientific), fibroblast growth factor (FGF) (Gibco, catalogue number PHG0024; Thermo Fisher Scientific) (10 ng/ml), FBS (1%) and penicillin, streptomycin, and amphotericin B (10 µl/ml) **(Divya et al., 2012).**

Then, 0.5-1 ml of media was added to each well. The media was changed every 3 days for 10 days. The cells were examined with a microscope to determine morphological changes (if present) indicating neuronal differentiation (neuron 1ike cells) in the form of retraction of the cytoplasm toward the nucleus and several cytoplasmic extensions. Then, the cells were subjected to immunocytochemical staining to detect the presence of Nestin.

Immunocytochemistry

An Econo Tek HRP anti-polyvalent antibody (DAB) ready to use (Scy Tek 1aboratories, Utah, United States) was used according to the manufacturer's instructions to detect nestin antibodies in differentiated cells. Media were removed from the cultured 24-well plates. The wells were washed twice with PBS. The cells were fixed with 4% paraformaldehyde in PBS. Then, 0.5 ml of the solution was added to each well and 1eft for 7-10 minutes at room temperature. The paraformaldehyde solution was aspirated, and the cells were washed three times with PBS to reduce nonspecific background staining due to endogenous peroxidase activity. The samples were incubated with hydrogen peroxide for 3-5 minutes and then washed twice with washing buffer (0.005% Tween 20 in PBS). The block was incubated with the superblock for 5-10 minutes at room temperature. The sample was washed once with a washing buffer. A primary antibody against Nestin was applied (Thermo Fisher, catalogue number PA5-11887) after dilution at 1:200 in PBS and incubation for 1 hour at room temperature. One well from each sample was incubated with PBS without a primary antibody as a negative control. The solution was aspirated, and the wells were washed with washing buffer three times. Econo Tek biotinylated anti-polyvalent was applied, and the samples were incubated for 10 minutes. The sample was washed three times in washing buffer. Econo Tek HRP was applied, and the samples were incubated for 10 minutes. The samples were rinsed three times in washing buffer. Four drops (200 µl) of DAB chromogen were added to the DAB substrate, mixed by swirling, and added to each well. The mixture was incubated for 5-10 minutes at room temperature. The samples were rinsed in distilled water, 1eft to dry, and then examined with a microscope.

Statistical analysis

The data analysis was performed using SPSS (version 26; Chicago, IL, USA). Descriptive statistics are expressed as the means ± standard deviations (SD), minimum, maximum, and range. The results were approximated to the nearest two

decimals. Regression analysis used for predictors affecting MSC count. A P- value of ≤ 0.05 was considered significant.

Results

Twenty-six hUCB samples were collected. Maternal age, gestational age, foetal weight, sample volume, mononuclear cell count, and MSC count are summarized in **(Table.1).**

Table.1.Demographic characteristics of the collected samples

Variables	Range	Mean	SD
Maternal Age (years)	(22-34)	27.92	3.86
Gestational Age (week)	(37-40)	38.39	1.02
Foetal Weight (gram)	(2250-3900)	3124.62	514.93
Sample Volume (ml)	(27-55)	43.12	8.49
Mononuclear Cells per ml ($\times 10^6$)	(1.42-3.56)	2.65	0.52
MSCs count in 0 passage per ml ($\times 10^6$)	(0-2.42)	1.51	0.87

Failure to obtain MSCs from samples represented (23.06%) either due to contamination or no growth detected from the start.

The contamination rate was 11.53% (3 out of 26 flasks, representing 50% of the failure rate); growing bacteria caused the death of the cell culture after growth was detected. Contamination was diagnosed by microscopic examination. Strict sterile precautions should be followed to avoid such mishaps. Contamination significantly interfered with cell culture growth **(p = 0.008)** as seen in **(Table.2),** resulting in 50% of the failure of the growing samples.

The other 50% of failure to obtain MSCs was due to an unknown reason (no cell growth detected; no adherent cells in the flask were detected after the first 48 hours of incubation), representing **11.53%** (3 out of 26 flasks).

Table 2: The effect of sample contamination on cell culture growth

Variables		Cell Culture Growth		Total	p value*
		Failure	Success		
Sample Contamination	**No**	3	20	23	**0.008***
	Yes	3	0	3	
Total		6	20	26	

* Chi-square test; **bold** = significant

Twenty of the 26 samples in total, demonstrated increased and further described MSC development. MSC growth efficiency as a whole was 76.94%. The majority (76.94%) exhibited a high output of MSCs that came from higher sample volumes and samples collected from older moms (**Table.3**).

Table 3. The effect of predictor variables on MSCs count at 0 passages per ml ($\times 10^6$)

Predictor variables	Unstandardized Coefficients		Standardized Coefficients Beta	t	*P*-value	95.0% Confidence Interval for B	
	B	Std. Error				Lower Bound	Upper Bound
Maternal age	0.118	0.040	0.522	2.980	0.**007***	0.036	0.201
Gestational age	0.025	0.287	0.029	0.086	0.932	-0.572	0.622
Foetal weight	0.000	0.001	-0.189	-0.586	0.564	-0.001	0.001
Sample volume	0.040	0.016	0.388	2.432	**0.024***	0.006	0.074

***Bold**: significant; Multiple 1inear regression analysis.

The MSCs exhibit specific characteristics, including adherence to plastic flasks and a spindle-like morphology as seen in **(Fig.1).**

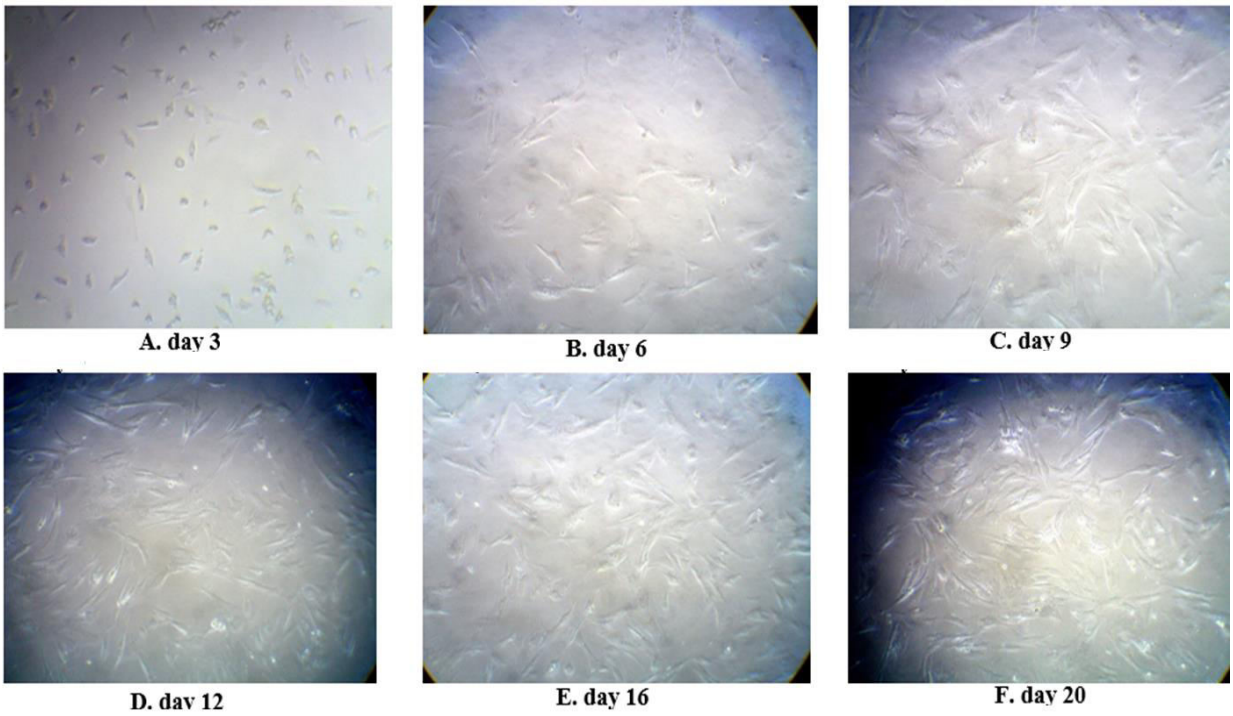

Fig.1.Inspection progress of MNCs growth and MSCs characteristics (adherence to plastic flask and spindle 1ike morphology) after culturing. It reveals increase in cells confluence over days tell reach 70-80% confluence at day 20.

All MSCs were negative for CD34, with a mean value of 2.65% and ±SD of 3.199, while they were positive for CD105, with a mean value of 20.916% and ±SD of 12.4398 as seen in **(Fig. 2).**

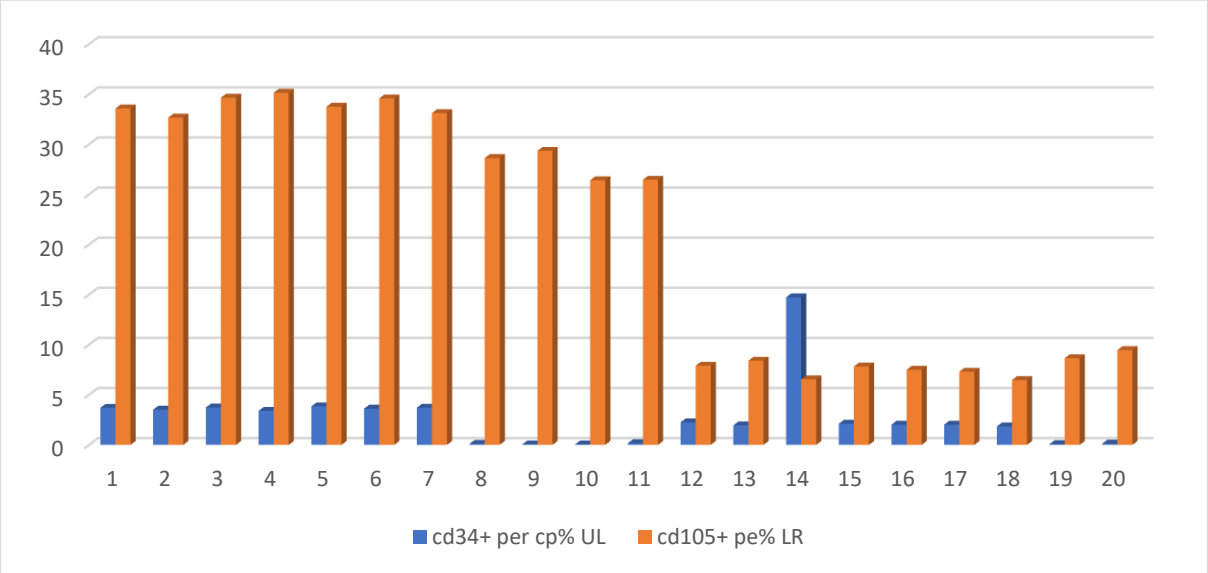

Fig.2. Flow cytometry analysis of MSC markers (cells were CD34- and CD105+)

All the MSC samples that underwent neurogenic differentiation using the one-step protocol revealed changes in cell morphology from flat spindle-shaped cells to neuronal-like cells, which included retraction of the cytoplasm toward the nucleus and several cytoplasmic extensions as seen in **(Fig.3)** and positivity for the nestin antibody by immunocytochemical analysis as seen in **(Fig.4).**

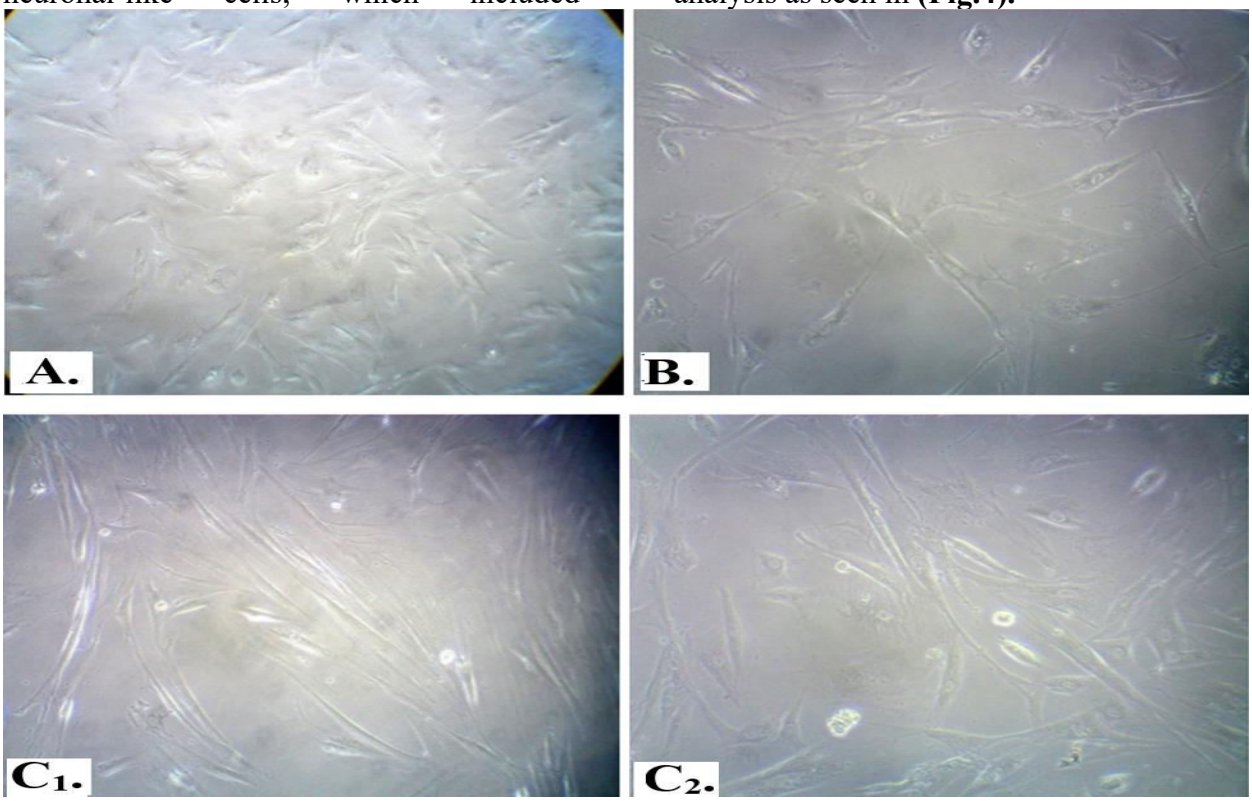

Fig.3. Neurogenic differentiation of MSCs. Changes in cell morphology from flat spindle-shaped cells to neuronal-like cells, which included retraction of the cytoplasm toward the nucleus and several cytoplasmic extensions. A. MSCs before differentiation are flat-spindle-shaped. **B.** MSCs on day 5 of differentiation reveal a slight retraction of cytoplasm toward the nucleus. **C.** MSCs day 10 reveals retraction of the cytoplasm toward the nucleus and several cytoplasmic extensions.

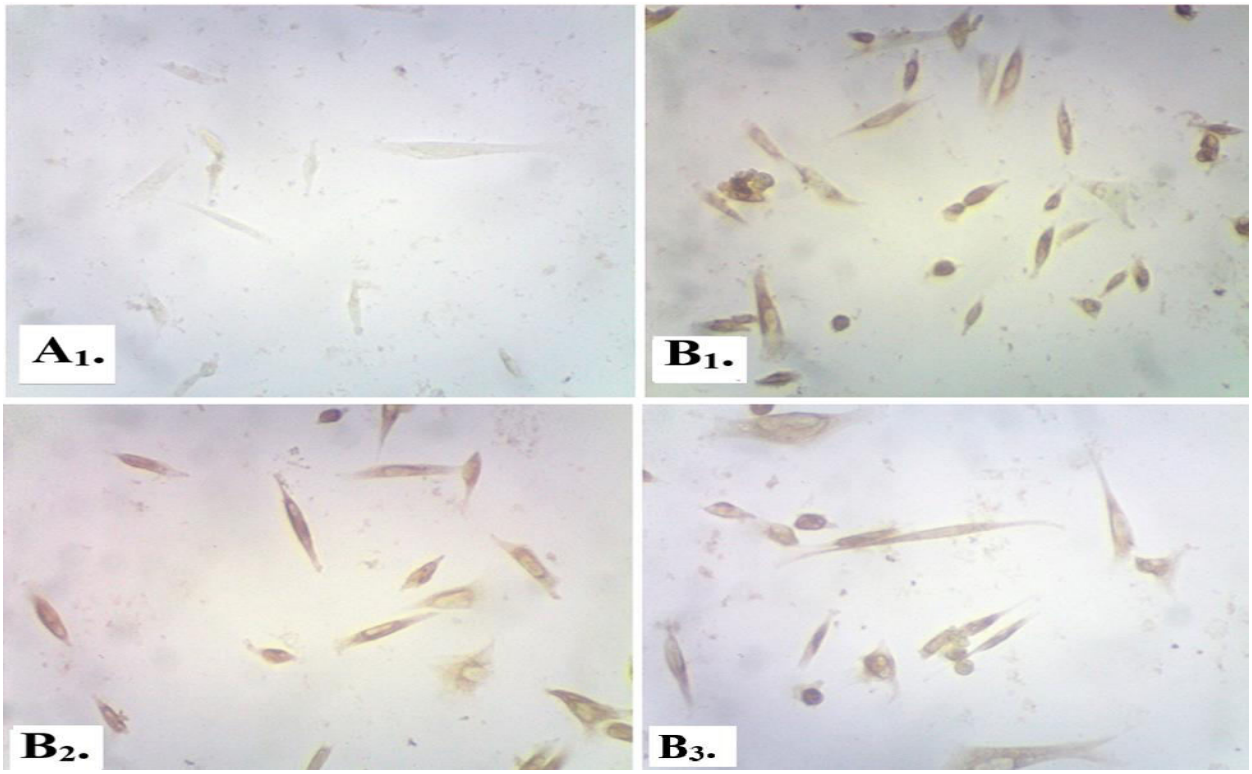

Fig.4. Immunocytochemical analysis of nestin antibody in the differentiated MSCs showing a positive reaction (brown pigments in the cytoplasm). A. Negative control: showing no pigmentation of cells. **B.** Positive reaction (brown pigments in the cytoplasm).

Discussion

Regenerative medicine is an evolving field that holds the promise of regenerating damaged tissues and organs in the body by replacing damaged tissue or organs **(Mao and Mooney, 2015).**

Human neural stem cells, derived from aborted embryos or fetal brain tissue, have the potential to treat brain injuries caused by trauma, neuronal degeneration, and stroke. However, they face two challenges: immunogenicity from embryonic or fetal brain tissue, which can cause immune rejection in transplant patients, and 1imited sources, 1eading to ethical disputes. A wide range of cells with appropriate sources and non-graft rejection could solve these problems, potentially 1eading to the development of human neural stem cells **(Ma et al., 2005; Wang et al., 2010).**

MSCs isolated from umbilical cord blood can potentially differentiate across mesodermal 1ineages, such as chondrogenic, osteogenic, or adipogenic 1ineages **(Goodwin et al., 2001),** or across ectodermal 1ineages, such as neurogenic 1ineages **(Lee et al., 2004).** From cord blood, UCB-MSC-derived neurons are readily produced and have potential use in the treatment of neurodegenerative diseases. But according to recent research, there is a small fraction of robust, quickly developing MSCs that may be produced from UCB **(Rebelatto et al., 2008).** Furthermore, many methods have been employed to produce neurons from UCB-MSCs. Improved therapeutic outcomes might result from using MSCs that have been stimulated to differentiate into neural stem cells (NSCs) in vitro before being injected into people or animals. Animal tests have been conducted **(Ma et al., 2005; Wang et al., 2010).**

MSCs were discovered in a novel source when researchers separated "fibroblast 1ike cells" from the human umbilical cord in the 1990s. The umbilical

cord has received increasing attention after being thought of as worthless medical trash in the past. Human umbilical cord MSCs (hUC-MSCs) have gained popularity in recent years because of their ability to develop into neural cells, easier access, and greater supplies than bone marrow. Mesenchymal stem cells (hUC-MSCs) generated from human umbilical cord matrix are thought to be useful for neural 1ineage differentiation in the treatment of neurodegenerative illnesses **(Ozygała et al., 2023).**

The isolation of MSCs from umbilical cord blood and their neural differentiation potential have been extensively studied. UC-derived MSCs demonstrated good differentiation capacity towards osteogenesis and adipogenesis **(Zhai et al., 2023).**

In our work, we used DMEM media supplemented with FBS to extract and develop MSCs from UCB, and we used a one-step technique using neurobasal medium supplemented with FGF-2 (10 ng/ml) and 1% FBS for 10 days to accelerate their differentiation into neuronal cells **(Divya et al., 2012).** MSC growth efficiency as a whole was 76.92%. The majority (76.92%) exhibited a high output of MSCs that came from bigger sample volumes and samples collected from older moms. Sample contamination was the cause of 23.06% of the growth failure (11.53%).

The growing bacteria caused the death of the cells cultured after growth was detected. Contamination was diagnosed by microscopic examination (showing flouting bacilli and hyphae). Strict sterile precautions were followed, and penicillin/streptomycin/amphotericin B was used instead of penicillin/streptomycin only. The other 11.53% failure of growth was for an unknown reason (no adherent cells were detected in the flask after the first 48 hours of incubation).

The isolation of MSCs was confirmed by their adherence to plastic flasks, fibroblast-like appearance, and flow cytometry (CD $105^{+}/34^{-}$). All MSCs were negative for CD34, with a mean value ± SD of 2.65% ± 3.20, while they were positive for CD105, with a mean value of 20.92% ± 12.44.

We used DMEM media supplemented with FBS in culturing MNCs isolated from umbilical cord blood to get MSCs and their further expansion. The study revealed a success rate of MSC isolation at 76.92% (20/26 samples revealed MSCs). **Zhang et al. (2011)** used DMEM media; and had a success rate of 47.8%, which is 1ess than our study (76.92%)**. Divya et al. (20121)** used the same media as our study and revealed a success rate of 20% (9/45 samples generated MSCs). **Nguyen et al. (2022)** used different xeno-free and serum-free media products to select GMP-standard culture conditions for UCB-MSC isolation and expression. Among the different culture kits used, they found that only stem MACS media revealed a suitable ability for MSC isolation from UCB with a success rate of 90%, which is higher than our study (76.92%)**. Schubert et al. (2018)** had a study that revealed the same results as **Nguyen et al. (2022)**: equine adipose MSCs grew in stem MACS media. The majority of samples in our study (76.92%) had a high yield of MSCs originating from samples taken from older mothers (within the study age range) and from a 1arger sample volume. It was not affected by gestational age or foetal weight. **Divya et al. (2012)** revealed that newborns with a higher mean body weight and a shorter gestation time were the source of the majority of UCB that produced a high production of MSCs and had extremely high neural differentiation potential. Babies with comparable clinical characteristics have produced a 1arge output of MSCs, according to earlier publications

(Ballen et al., 2001; Strohsnitter et al., 2008). This discovery is tentative, but it indicates a favourable association and warrants more research.

Jang et al. (2004), reveal that administering b-mercaptoethanol and retinoic acid to MSCs caused a significant alteration in their cellular morphology, changing them from fibroblastic to an elongated process with a spindle form that resembles the neuronal phenotype. According to other research, in order for hUCB-MSCs to transdifferentiate into neurons, they must undergo four stages of therapy with a variety of growth factors **(Lee et al., 2004).** Two distinct methods were employed by **Divya et al. (2012)** to induce neuronal development in hUCB-MSCs. According to Lee OK et al. (2004), the first was a four-step induction technique. Direct neural differentiation is the only stage in the second protocol **(Divya et al., 2012).**

When considered collectively, these findings suggest that hUCB-MSCs have a markedly different neurogenic potential, which requires appropriate attention. A one-step approach supplemented with FGF-2 (10 ng/ml) and 1% FBS was used in our work to stimulate MSCs to develop into neural cells over a period of ten days **(Divya et al., 2012).** The cytoplasm retracted toward the nucleus and several cytoplasmic extensions extended, indicating that differentiated cells had the morphological traits of neuronal cells. Additionally, they tested positive for Nestin by immunocytochemistry, an intermediate filament protein produced in rapidly proliferating progenitor cells of neuronal tissue that is growing and regenerating **(Goldman et al., 1999).**

Positive cells were detected by the presence of brown pigmentation in the cytoplasm, while negative cells and negative control cells had no pigmentation.

The 1imitations of the current study include the small sample size, and additional studies with 1arger sample sizes are recommended to obtain more robust results. Other 1imitations included the excessive cost of exported solutions, the 1ong duration of shipping, and the unfavourable environment of airport health quarantine, which might 1ead to passing beyond the expiration date or even spoiling of the materials. The authors hope that the Egyptian government might put in place facilitating rules to overcome these issues.

The cells were cultured at the Nano-physics Laboratory at the Central Research Laboratory Unit, South Valley University. The authors recommend establishing a stem cell 1aboratory in the faculty of medicine, SVU, which might greatly facilitate the job of research in this field.

The results of the present study might open the door for other experimental studies for the treatment of neurological diseases that might benefit from the production of neuronal cells.

Conclusion

hUCB-derived MSCs possess inherited neural differentiation capacity. Our results suggest that soon, hUCB-derived MSCs can be used for neural tissue regeneration and represent a promising approach for stem cell regenerative therapy for the repair of injured neurons and to offset the degenerative process of neurological diseases.

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