Rapid Induction of Mesenchymal Stem Cells to Neural Cells

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Abstract

Background:-Human umbilical cord blood- mesenchymal stem cells(HUCB-MSCs) hold great promise *in vitro* neural differentiation and therapy for neurodegenerative disorders. It has demonstrated that the number and differentiating potential of bone marrow mesenchymal stem cells (MSCs) decrease with age. Therefore, the search for alternative sources of MSCs is of significant value.

Objective:-To determine the possibility of obtaining clonally expanded UCB-MSCs and evaluate their rapid induction and differentiation into neural cells *in vitro.*

Methods:-MSCs were isolated from HUCB by combining gradient density centrifugation with plastic adherence. In order to identify factors that able to lead to neural differentiation, the cultured cells were treated with retinoic acid (RA) and B-mercaptoethanol (BME). Differentiating characterization of UCB-MSCs were detected by immunocytochemistry analysis.

Results:-UCB-MSCs appeared like the fibroblast cell and these cells were extensively expanded in culture. The results showed that UCB-MSCs were positive for CD71and CD90 but were negative for CD34. The immunocytochemistry staining indicated that the differentiated cells give positive response for nestine marker. The result confirmed for neural progenitor differentiation of MSCs.

Conclusion: The results of this study confirmed that UCB provides a great source of stem cells for using in treatment of neurodegenerative disorders of the central nervous system.

***KEY words:-** mesenchymal stem cells, umbilical cord blood, neuronal induction, in vitro.*

Introduction

Human umbilical cord (HUC) has been suggested to represent another promising source of mesenchymal stem cells (MSCs) (Wang *et al.*,2004; Hernandes *et al*.,2020).

During pregnancy, the mother and fetus are connected by the umbilical cord which is composed of umbilical vessels (two arteries and one vein) and specialized mucous connective tissue called Wharton̕s jelly, all covered by the amniotic epithelium (Can &karahuseginoglu,2007).

Umbilical cord blood (UCB) is considered one of the most abundant sources of non embryonic stem cells. The collection of 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 cells 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 allogenic lymphocytes *in vitro*(LE Blance *et al.*,2003).

In the past decade, the studies showed that human UCB contains hematopoietic stem cells (HSCs) and MSCs, both of which can be used as alternative sources to bone marrow for cell transplantation and therapy. The HSCs of UCB have already been proven useful in treating various hematological disorders. On the other hand, the identify of MSCs had remained elusive until they were recently isolated as a homogenous cell population by a number of different laboratories .

The most common source of MSCs has been the BM, but aspirating BM from the patient is an invasive procedure and in addition, it has been demonstrated that the number and the differentiating potential of BM-MSCs decreases with age. Therefore, the search for alternative sources of MSCs is of significant value, so far, little success has been reported in the literature about the isolation, characterization and differentiation of MSCs from UCB [13].

It was reported that UCB- derived could differentiate into mesoderm cell lineages such osteoblasts, hepatocytes, neuron like cells [2] and cardiomyocytes. Previous reports have shown that UCB-MSCs can be trans differentiated into neural linage by treating with nerve growth factor and retinoic acid. This multilinage differentiation capacity, the expression of neural properties and overlapping genetic programs for hematopoiesis and neuropoiesis (Tershikh *et al*, 2001) suggest that UCB cells may have the ability to trans differentiate into neural cells.

In 2003, Buzanska *et al.* succeeded in isolating and establishing a neuronal stem progenitor cell line from HUCB, and they were compared early and late passage for growth kinetics and the ability to differentiate into neurons, astrocytes and oligodendrocytes. In another study, demonstrate that isolated adherent cells expressing MSCs –related antigens such as Sh2 , CD29 and CD13 from a mononuclear cell fraction of UCB (Jeong *et al.*,2004).

Material and Methods

Cord Blood Cells Separation:

All CB sample were obtained freshly from discarded placenta of full term normal vaginal deliveries, blood was kept in anticoagulant treated sterile bag or tubes. Cord blood was diluted 1:1 with PBS, the diluted blood was carefully overlaid on Ficoll – paque at a ratio 3:1 in 10 ml sterile conical tubes, the specimens were centrifuged on a cooling centrifuge for 20 minutes at 2000 rpm at 4C°. After density gradient centrifugation, the resulting MNCs were retrieved from buffy coat layer and washed with PBS at 2000 rpm for 5 minutes at 4C°

Culturing and expansion of mesenchymal stem cells *in vitro*

The final suspension of MNCs was cultured in IMDM + 10% FCS at final concentration 1-2 X 10^6 cells/ml. Cultured were maintained at 37C° in humidified atmosphere containing 5% CO_2.

Cells where allowed to adhere overnight and non adherent cells were washed out with medium changes.

Cultured were maintained, to get hold of developing colonies of adherent fibroblastoid cells. Fibroblastoid cells were recovered between days (8-10) after initial plating using 0.25% trypsin-EDTA, recovered cells were replaced at a ratio of 1:3 in the same conditions. The cells began to proliferate and formed a monolayer of fibroblasts cells for the next (6-8) days.

Immunophenotypic analysis of mesenchymal stem cells

After fibroblastoid cells were dispersed with trypsin-EDTA, the cells were re cultured in multi-well tissue culture plates at a density of 1X10^4 cells/well in IMDM+ 10%FCS.

The cells were allowed to developing a monolayer of adherent cells within 4-5 days, then the medium was aspirated and the multi-well plates washed two times with PBS for 10 minutes, then washed with PBS and they were leaved to dry, after that the plates were ready for Immunophenotypic procedure.

Neuron like cells differentiation

Mesenchymal stem cells were induced for neurogenic differentiation, the fibroblastoid cells were re-cultured in multi-well tissue culture plates at a density of 1X104 cells/well in IMDM+10%FCS for 1 day. After twenty four hours, medium were replaced with pre-induction medium consisting of IMDM +10%FCS+1m B-mercaptoethanol (BME) for two days.

To initiate neural differentiation, the pre-induction medium were removed, and the cells were washed with PBS and transferred to neuronal induction medium composed of 0.5mm retinoic acid (RA) and 100 mm B-mercaptoethanole in IMDM+10%FCS for 5 days .

The morphology of cells was observed under inverted microscope every 1 day, when the shapes of cells no longer changed, the expression of nestine was confirmed by immunocytochemistry analysis.

Results

-Culturing and expansion of mesenchymal stem cells from umbilical cord blood

The cells isolated by gradient density centrifugation showed heterogeneity during the first 6 days. The adherent cells were observed on days 3 and 6 days. The spindle -shaped cells appeared at the bottom of culture flasks, and many round cells were suspended in the medium. Cells were completely removed from the medium. The adherent cells began to proliferate, and the numerous fibroblast like-cells could be observed and gradually grow to form small individual colonies displaying fibroblast-like morphology as well as a small round cells can also seen (Fig.1,2).

When the cells grow to 70-80% confluence, the cells were ready for the first passage (Fig. 3). The passage MSCs behaved similarly to those in primary cultures after (6-8) days (Fig. 4).

Immunophenotypic analysis of mesenchymal stem cells *in vitro*

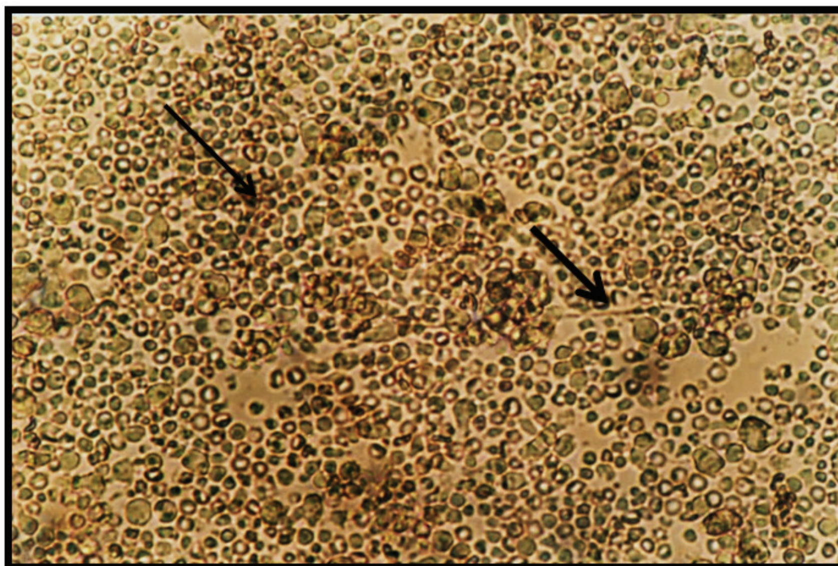
After the first passage, the isolated cells were prepared for examination and analysis by immunocytochemistry. The cultured cells from UCB expressed positive response for MSCs surface markers CD71 and CD90. However the cells were negative for hematopoietic lineage marker CD34. These results indicated that the isolated cells in this study represented HUCB-MSCs and were not mixed with cells of hematopoietic origin (Fig.,5,6,7).

Neuron like cells differentiation *in vitro*

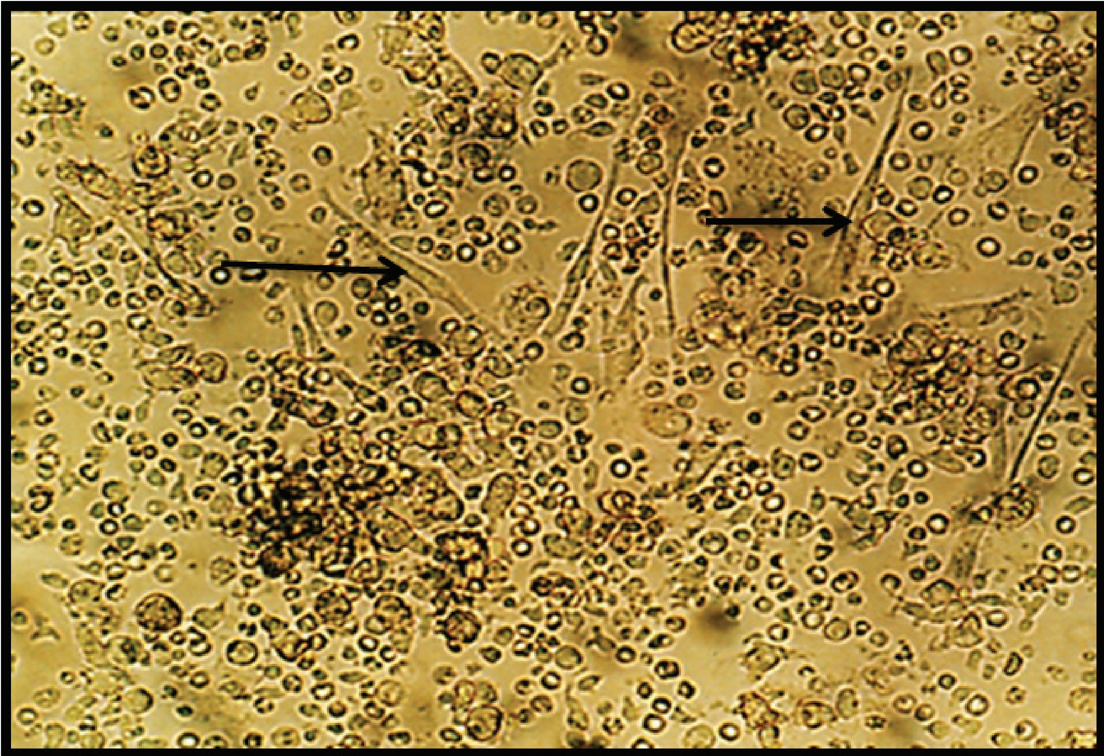
Undifferentiated MSCs displayed a flat morphology with short processes. To identify conditions that may induce differentiation towards a neural phenotype, the multistep induction method were used. When the cells were treated with (BME) after (1-2) days, most of cells became bipolar and extended long processes while other cells were rounded (Fig.,8).

When the cultures treated with neuronal induction medium consisting of (IMDM+10%FCS+RA+BME), the cells acquired the morphology of neuron cells exhibiting a retractile cell body with extended neuron like structures (Fig.,9).

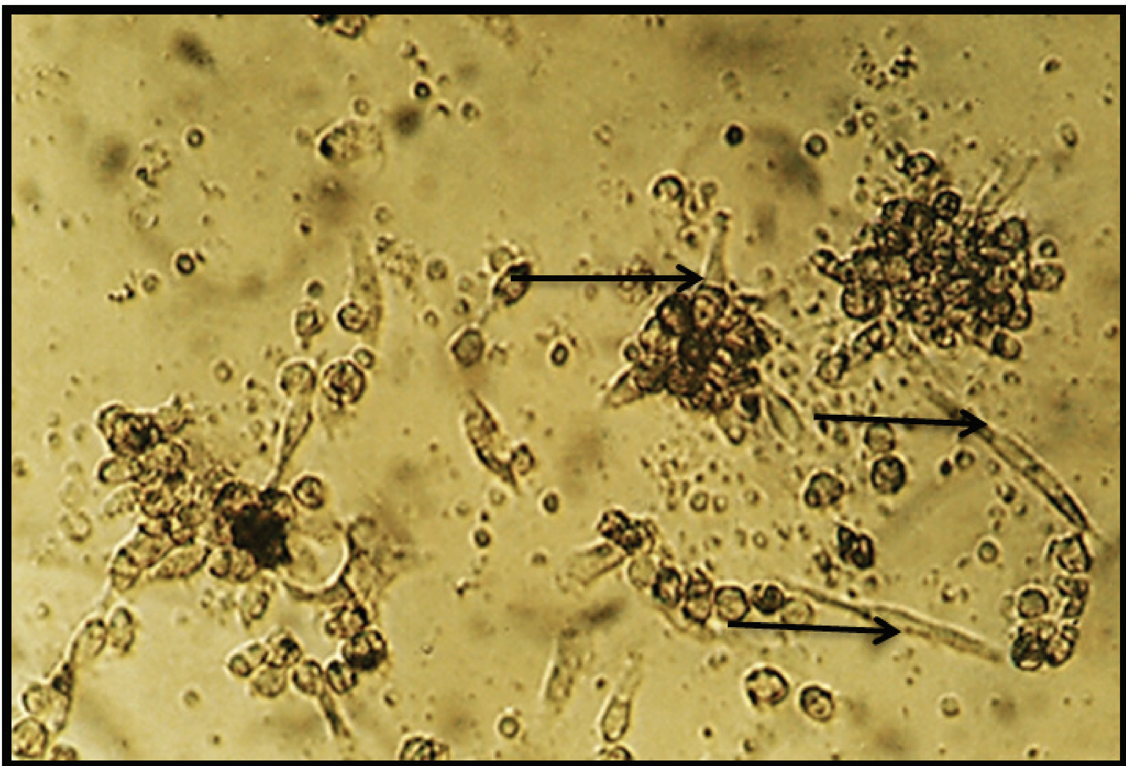
The morphological changes increased progressively, so in the areas of higher cell density, the differentiated cells were arranged into a network like structure (Fig.,10). After seven days of neuronal induction, differentiated cells exhibiting neural-like morphology(Fig.,11,12) and cells positive response for nestine marker, which is generally used as a specific marker for early neural progenitors, could be observed in the HUCB-MSCs cultures(Fig.,13,14).



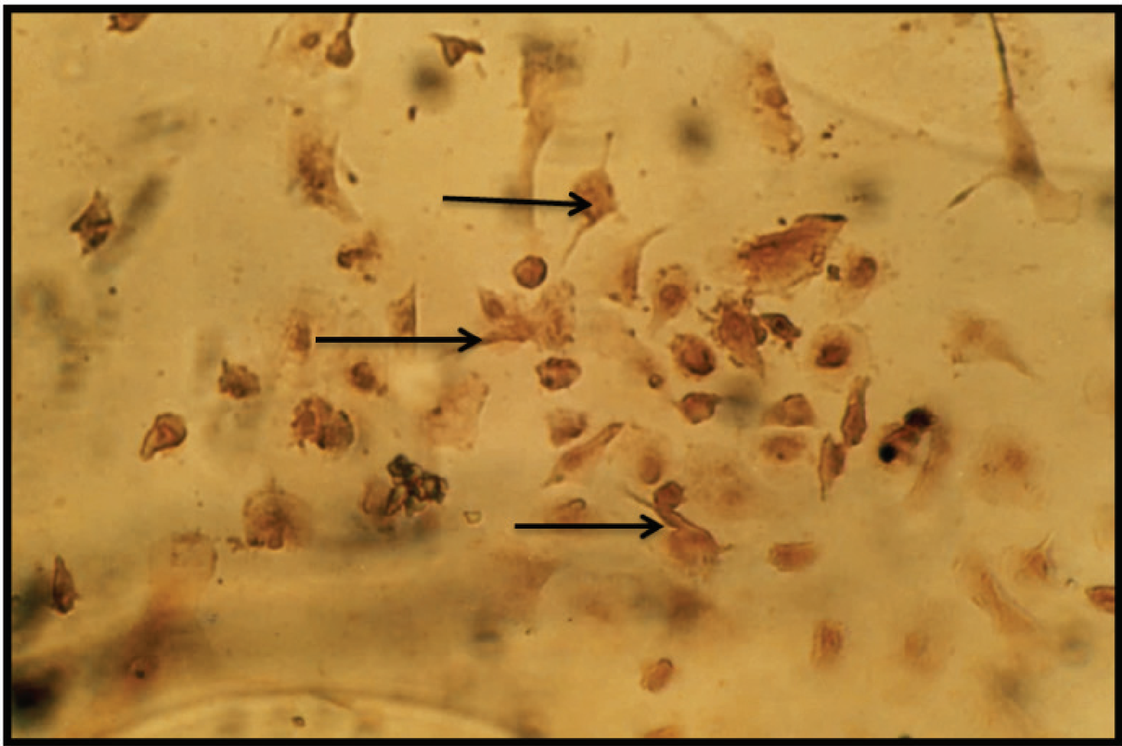
Figure(1):The morphology of UCB-MSCs which cultured in IMDM+10% FCS showed that the cells after 3 days of culturing ,most of cells are floated (thin arrow) and the remaining of cells began to adherent on the culture flask (thick arrow) and the cells appeared as a spindle – like shape (X160)



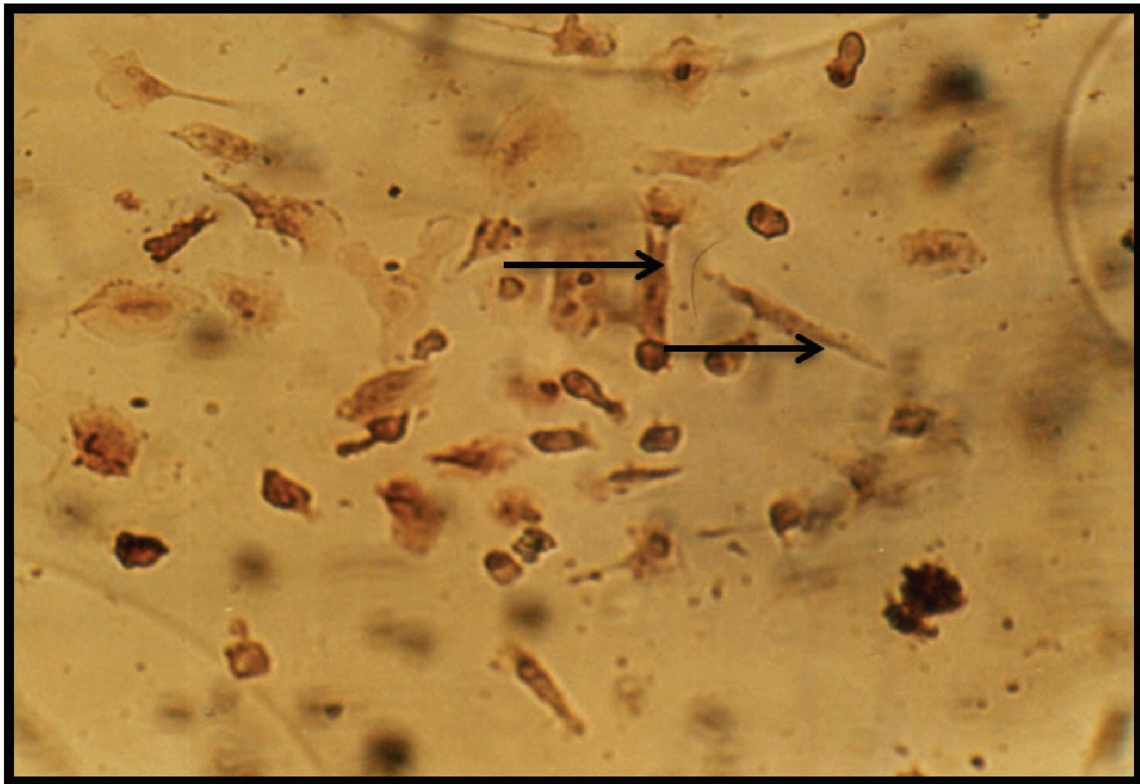
Figure(2): The cells after 6 days in culture, the cells began to proliferated and numerous fibroblast like cells and gradually grow to form small individual colonies (arrows)(X160)



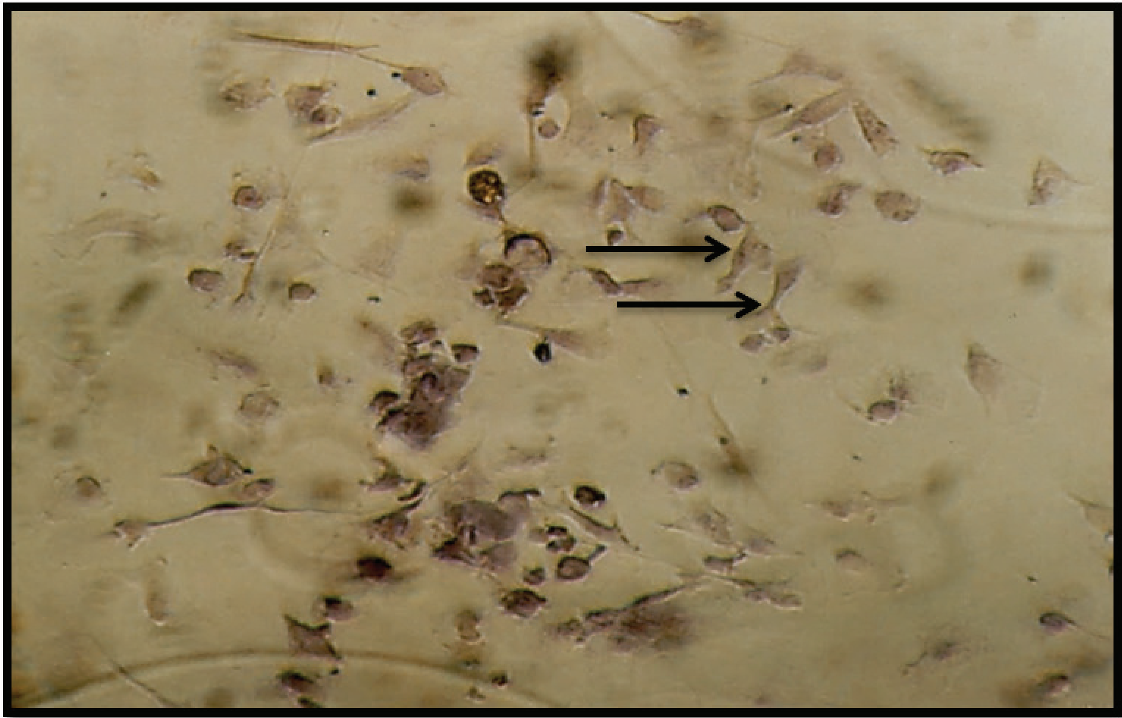
Figure(4): The cells after 3 days from the first passage of culturing showed that cells appeared spindle-like cells (arrows) (X160).



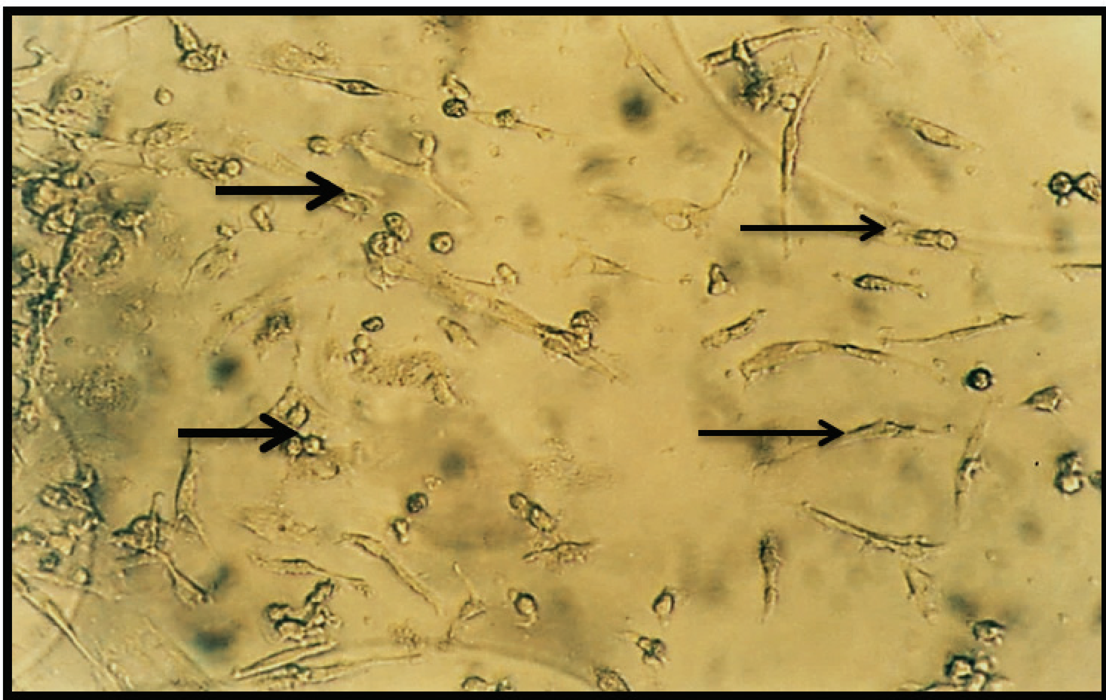
Figure(5): Immunophenotypic analysis of MSCs on the end of the first passage , the cells were positive response for CD 71 marker and stained with brown color (arrows)(X160).



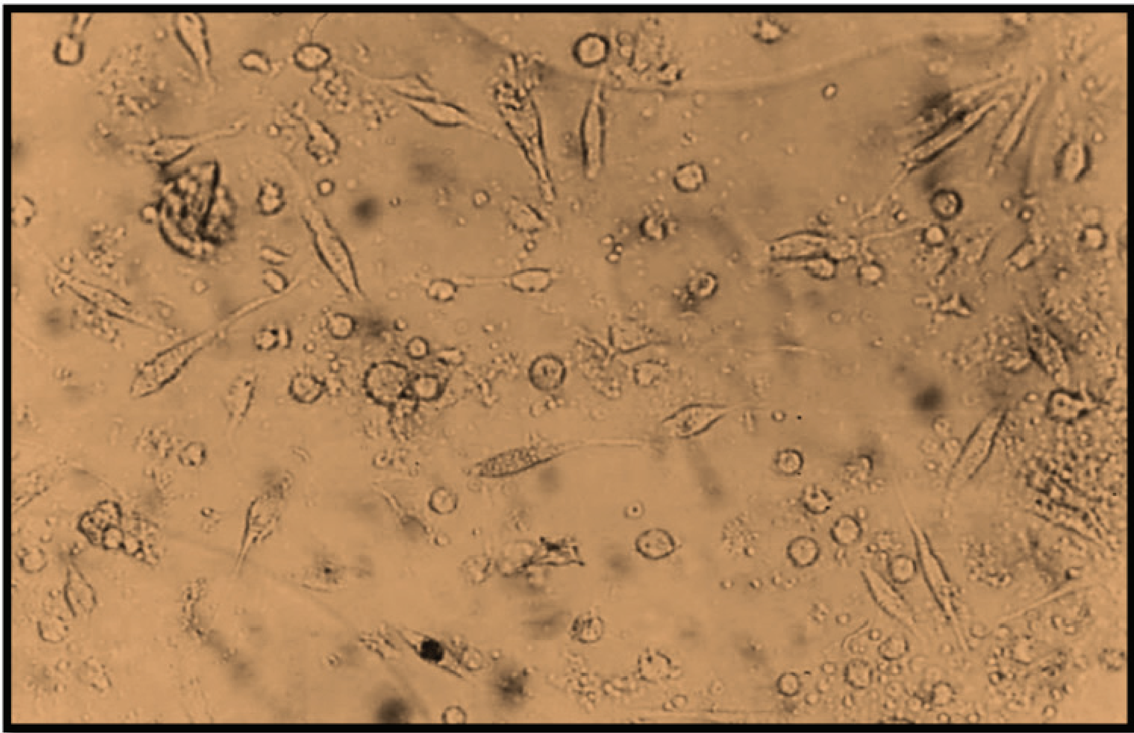
Figure(6): Immunophenotypic analysis of MSCs on the end of the first passage , the cells were positive response for CD 90 marker and stained with brown color (arrows)(X160).



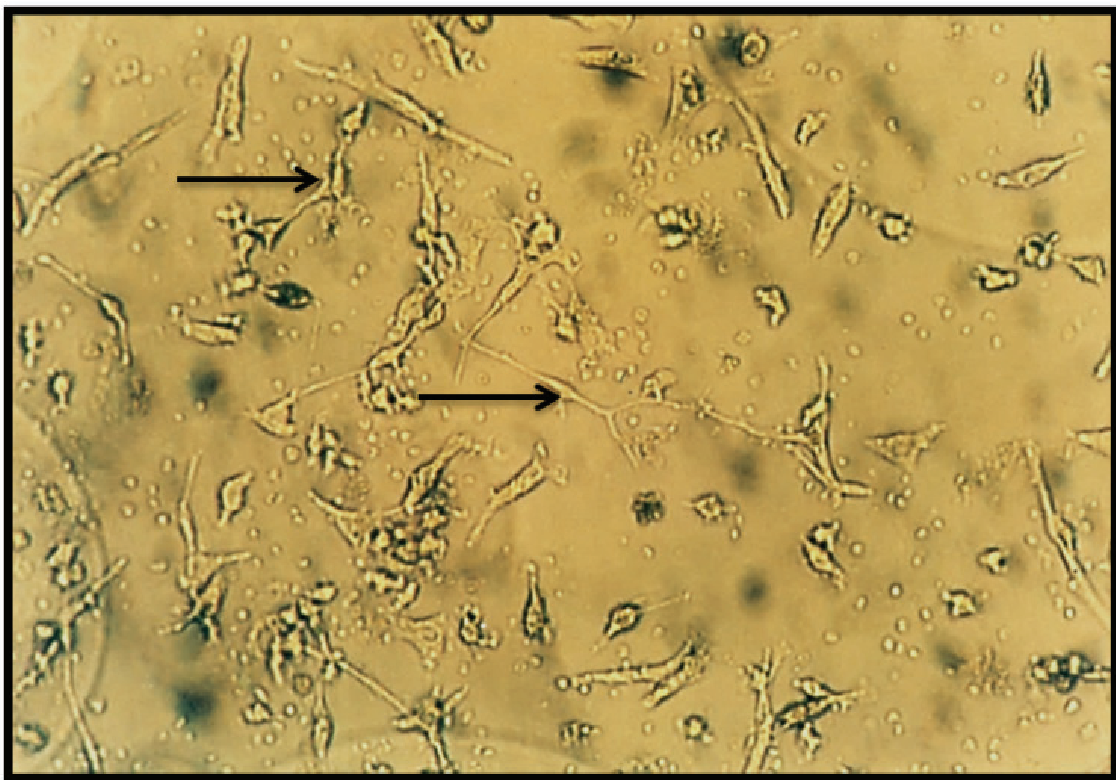
Figure(7): Immunophenotypic analysis of MSCs on the end of the first passage , the majority of cells were negative response for CD34 marker and this stained with blue color of counter stain hematoxylin(arrows) (X100.8)



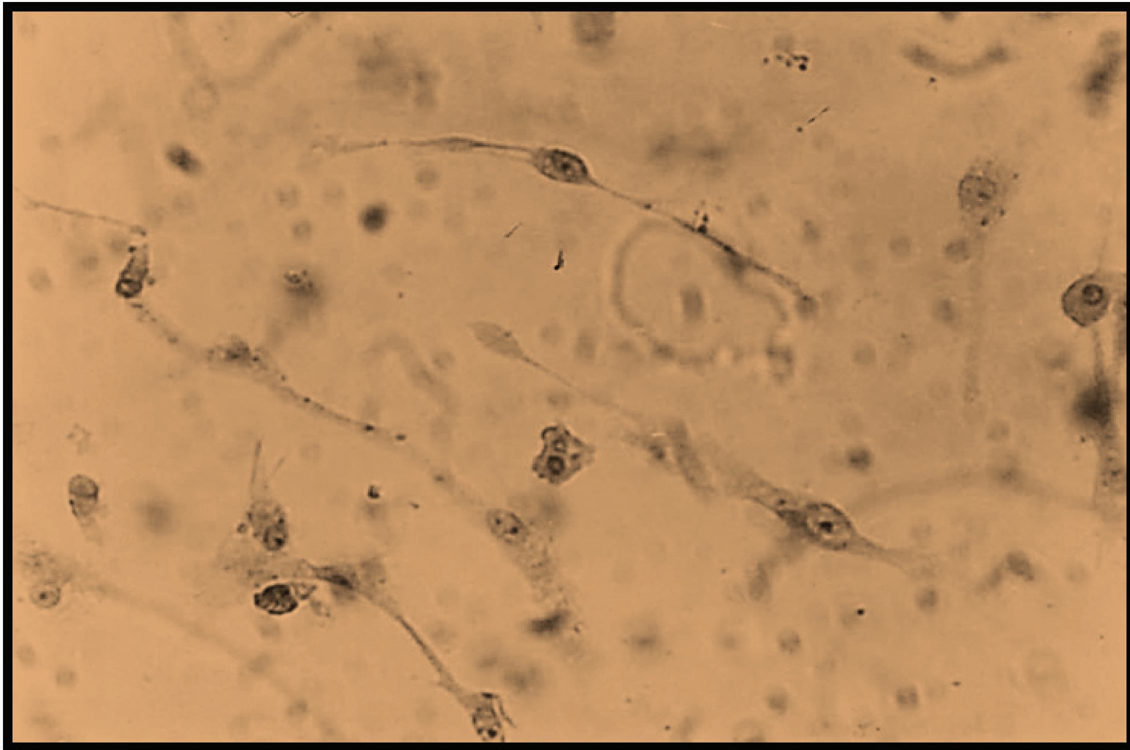
Figure(8):The morphology of differentiated MSCs in vitro after treatment with BNE, the cells after one day of culturing showed that the cells become bipolar and extended to processes(thick arrows) while other cells were rounded (thin arrows)(X100.8).



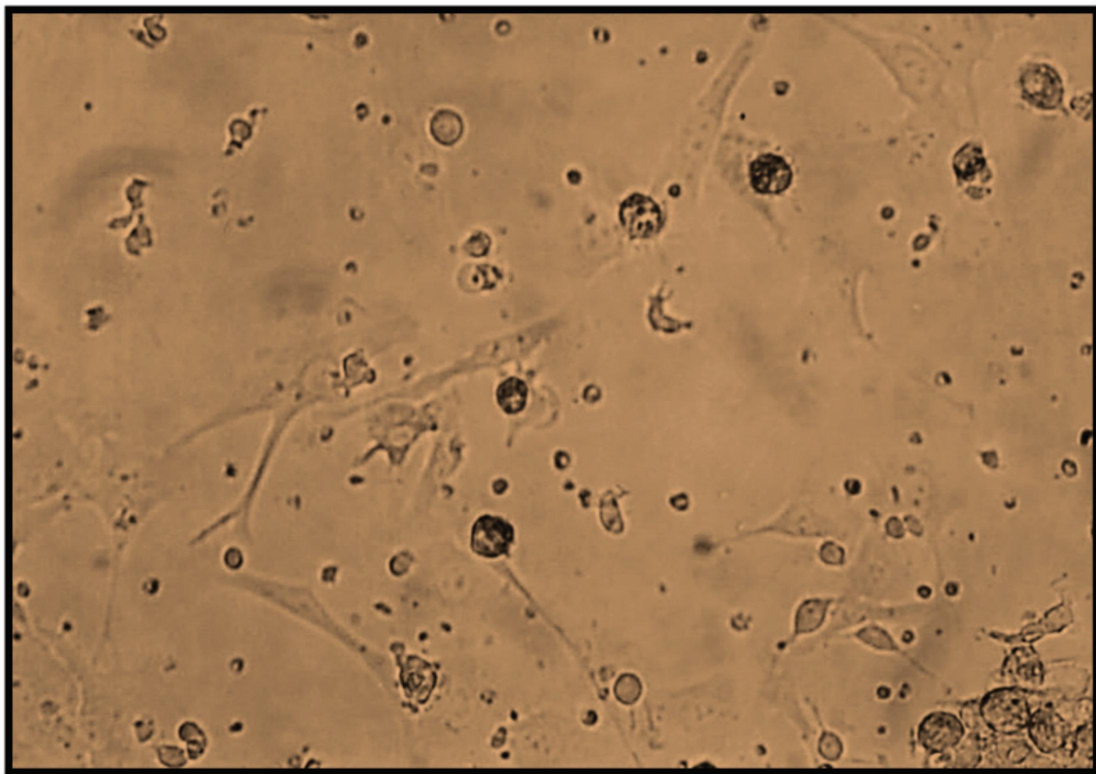
Figure(9):The morphology of differentiated MSCs in vitro after two days for treatment with pre-induction medium, the cells acquired the morphology of neuron cells (X100.8)



Figure(10):The morphology of differentiated cells after 3 days from treated with neural induction medium, the differentiated cells were arranged into network like structures(arrows) (X100.8).



Figure(11):The morphology of differentiated cells after 5 days from treated with neural induction medium , the cells exhibiting neural –like morphology (X100.8).



Figure(12): The morphology of differentiated cells after 7 days from treated with neural induction medium , the cells exhibiting neural –like morphology (X100.8).

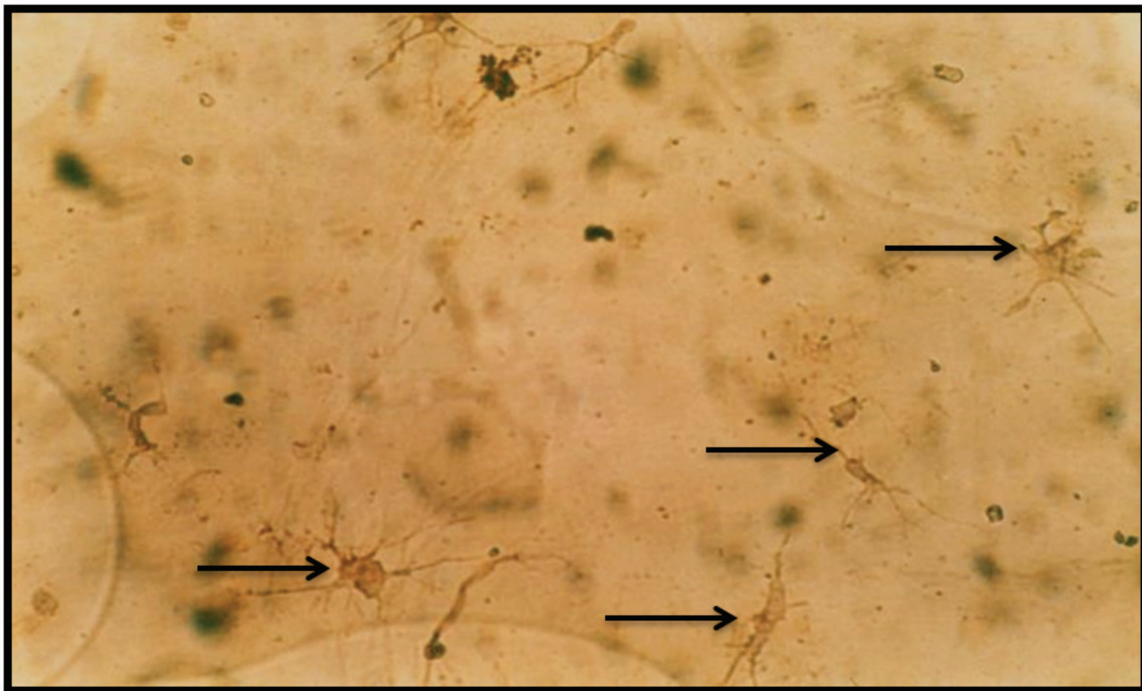
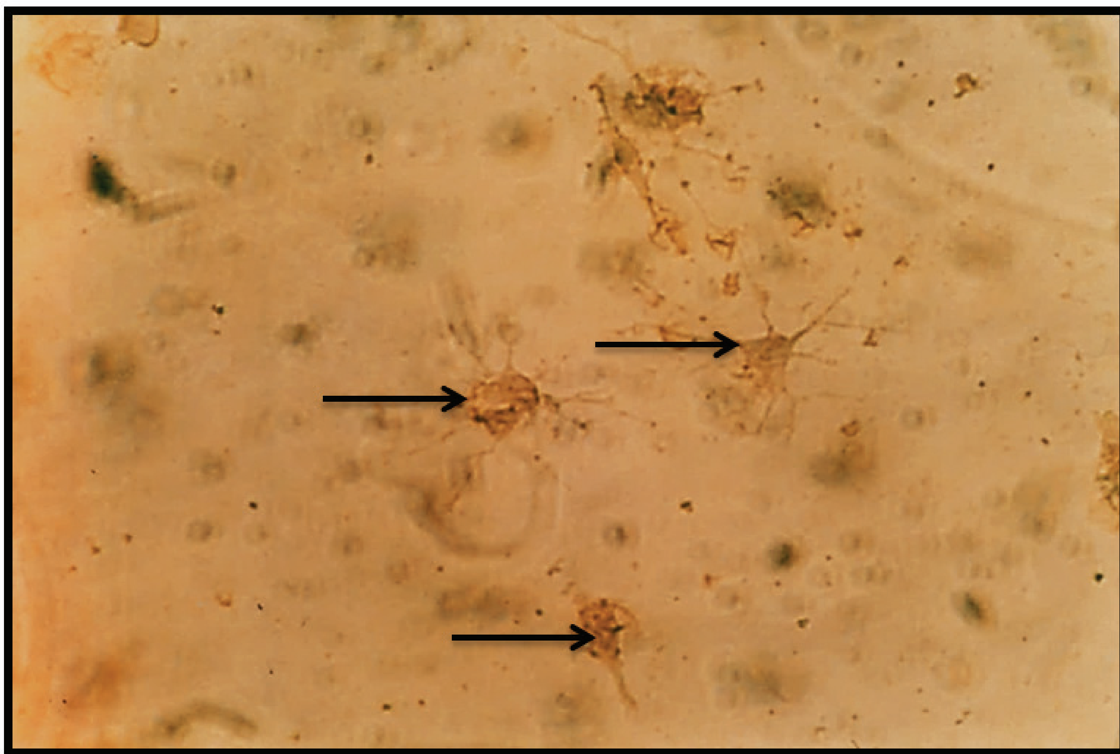


Figure (13):Detection of nestin marker in vitro neurogenic differentiation of UCB-MSCs showed neural – like cells were positive result(brown stain)(arrows).



Figure(14): Detection of nestin marker in vitro neurogenic differentiation of UCB-MSCs showed neural – like cells were positive result(brown stain)(arrows).

Discussion

There are many hematopoietic stem cells and mesenchymal stem cells in the umbilical cord blood, there are many methods to isolated MSCs including: flow cytometry, gradient density centrifugation and immunomagnetic selection. The Ficool- paque (1.077 g/ ml) were used to isolate MSCs from UCB combined the gradient density centrifugation with plastic adherence and changed the medium many times to purify MSCs after gradient centrifugation. The isolated cells expressed the characteristics of MSCs suggesting that the method is an ideal way to isolate UCB derived MSCs. UCB-MSCs possess versatile differentiation potential ranging from mesenchyme- related multipotency to neuroectodermal and endodermal competency, such as osteoblasts, adipoblasts, neuron- like cells and hepatocyte- like cells(6; 5). In the present study, cells were successfully isolated from UCB using the attachment method and this method is relatively simple and can easily give homogenous MSCs, these findings are in agreement with previous studies.

Mesenchymal stem cells are capable of self renewal and possess a high proliferative capacity and the potential from multilinage differentiation (9).

Previous studies have reported that HCB tissue demonstrated the advantages of providing an abundant supply of cells from donors via a non invasive procedure with low risk of infection and can therefore be considered an attractive source of MSCs (1, 19).

The results of present study showed that many of the adherent cells isolated displayed a typical fibroblast- like morphology, these results correspond to other studies results, which reported the morphology of isolated MSCs from UCB and Whoton,s jelly of human umbilical cord tissue [1,9,15]

The Immunophenotypic analysis of cultured MSCs from UCB suggest that these cells closely resemble cultured MSCs obtained from bone marrow [13,16,18].As reported by Pittenger *et al.* (1999) ,UCB derived MSCs have a characteristic set of surface markers that include cluster of differentiation (CD) markers, for example MSCs are positive for: CD24,CD44, CD71, CD90, HLA-A,B,C and SH2, SH3 and negative for: CD10, CD11b,CD14, CD34, CD45 and HIA-DR, DQ. Our results indicated that the MSCs-UCB are positive for CD71, CD90 and negative for CD34. This observation are in agreement with other studies as in (Hou .,2003; Lee *et al.,*2004;Mnati *et al.,*2012 and Li *et al.*,2013).

Many literatures demonstrated the potential of HUCB-MSCs to trans differentiation into neurons by inducing the cells to cross the germ line barrier and generate an ectodermal specified lineage and can be used for treating neurodegenerative disease (Lee et al.,2004).

Jang and colleagues,(2004) have demonstrated that treating MSCs with B-mercaptoethanol and retinoic acid resulted in a vary drastic difference in cellular morphology of MSCs from a fibroblastic to a spindle-shaped elongated process resembling the neuronal phenotype. The results of current study is agreement with results of previous report.

Other studies have shown that the HUCB-MSCs need exposure to four- step treatment with a combination of growth factors for trans differentiation into neurons (Lee *et al.*, 2004).

Retinoic acid (RA) ,a derivative of vitamin A , is essential in maintaining normal cellular growth and development. In fact, RA is present in various tissues of both embryonic and adult animals, in particular in the nervous system (Mc.Caffery and Drager, 1994), where it promotes neuronal differentiation. Previous studies have demonstrated that RA induces both a greater number of neuritis as well as increased nitrite length in cultured neurons(Maden, 2001).Retinoic acid has been used in combination with other factors to induce differentiation of bone marrow stem cells (BMSCs) into neural cells(Sanchez-Romos *et al.*, 2001; Kim *et al.*, 2002).

It has been suggested that (BME) is capable of supporting the viability and differentiation of fetal mice brain neurons, when they used low concentration of this factor in combination with RA(Ishii *et al.*, 1993), with this treatment ,BMSCs slowly differentiated into neuron- like cells and after seven days they expressed neurofilaments(NF).

According to the results of the immunocytochemistry of the present study, the cultures treated with RA and BME were stained for neural marker (nestine) and this marker give positive response for differentiated cells

(Kruminis-Kaszkiel *et al.,*2020)

In conclusion, UCB contains MSCs and should not be regarded as medical waste. It can serve as an alternative source of MSCs to bone marrow and can be a new used as a new source for cell transplantation and cell therapy for neurodegenerative disorders.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the Department of Biology and all experiments were carried out in accordance with approved guidelines.

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