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Neurogenic Differentiation of Bone Marrow-derived Mesenchymal Stem Cells Using Neural Induction Medium: A Morphological and Histochemical Study

Ahmedy E.[1], Kandel S.[1], Gabr H.[2, *], Rizk S.[2], Khalifa K.[1], Kamal S.[1]

[1]Clinical pathology department, faculty of medicine, El Menoufia universit, Menofia, Egypt
[2]Clinical pathology department, faculty of medicine, Kasr El Eini University, Cairo, Egypt

Email address:
halagabr@yahoo.com (Gabr H.)

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Abstract: Background: Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in all tissues. They are present in bone marrow, and can differentiate *in vitro* into neurons, glial cells and myofibroblasts . MSCs have been proposed as sources of stem cells for regeneration of the CNS. Thus, one of the goals of regenerative medicine is to regenerate damaged brain tissue and spinal cord by harnessing the power of stem cells to initiate neurogenesis in damaged areas of the brain. Objective: The aim of this work is to study in-vitro induced neurogenesis using MSCs as model of stem cells. Methodology : Bone marrow-MSCs were isolated, expanded and passaged. MSCs were identified using morphology and flowcytometric analysis. Co-expression of Oct ¾ was done. MSCs were induced to neural lineage using Neural Induction Media (NIM) : a cocktail of-retinoic acid dissolved in DEMSO, recombinant human Fibroblast Growth Factor (FGF) basic, recombinant human Epidermal Growth Factor (EGF) and Insulin-like Growth Factor I (IGF-I) . Neural induction was verified morphologically, and immunologically using GFAP positivity and nestin expression. Results: BM-MSCs express CD44 and OCT ¾ which decrease with age. MSCs induced with NIM show morphological changes consistent with neurogenesis, positive GFAP and nestin expression as compared to the uninduced cells. Conclusion: MSCs isolated from bone marrow aspirate and can be differentiated into GFAP positive neural cells.

Keywords: Mesenchymal Stem Cells, GFAP, Retinoic Acid, Neurogenesis

1. Introduction

Stem cells (SCs) are multipotent, self-renewing cells found in all body tissues. The most appealing characteristic of stem cells is their ability to differentiate into a variable number of cell lineages[1, 2], a property referred to as "Plasticity" or "transdifferentiation". Adult SCs can be isolated from a number of sources, namely, umbilical cord blood and tissue, bone marrow and adipose tissue[3].

Mesenchymal stem cells (MSCs) represent an important type of multipotent adult stem cells. MSCs are plastic adherent stromal cells which have the ability to differentiate into bone, cartilage and adipose tissue. They can also transdifferentiate into various mesodermal lineages[4] including neural lineage, cardiac tissue and my ofibroblasts[5].

Neural stem cells (NSCs) are adult stem cells responsible for endogenous neural regeneration.[6] However, due to the limited proliferative capacity of NSCs, they differentiate to replace lost or injured neurons [7].

Regenerative medicine is a new evolving branch of medicine addressing the replacement of damaged tissues or cells. MSCs have been proposed as an important potential sources of neural regeneration[8, 9].

MSC can induce tissue regeneration and repair through a number of mechanisms: secreting soluble factors that enhance tissue regeneration, stimulating proliferation, migration, and differentiation of endogenous stem cells, anti-inflammatory and immunomodulatory actions [10]. Moreover, MSCs can actually transdifferentiate into functioning neural cells[10].

2. Materials and Methods

2.1. Subjects

The present study involved 25 patients aged between 18 years to 50 years, undergoing diagnostic bone marrow aspiration. Only samples diagnosed as normal bone marrow were used. Ethical committee approval and informed written consent were taken.

2.2. MSC Isolation and Expansion

Bone marrow aspirate (10 ml) was diluted with sterile PBS in the ratio of 1:1 and layered on top of ficoll-hypaque. The MNC fraction was collected and seeded at a concentration of (million cells/ cm2) and allowed to adhere to tissue culture plastic flasks 25 cm2 (cell star), incubated at 37 C and 5% CO2 in 5 ml of the fresh complete nutrient medium (F10) which was constituted of the following: Low-glucose DMEM (DMEM-LG) with L-glutamine (2mmol/L),10% FBS, Penicillin- streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin and Fungizone (50 µl/ml) (0.25 µg/mL). Half medium was changed every 4 days for removal of non-adherent cells.

At day 9, when fibroblast-like cells reach 80-90% confluence, these cells were harvested by trypsinization.

2.3. MSC Identification

MSCs were identified, according to the criteria of ISCT through: plastic adherence, fibroblastoid morphology, immunophenotyping and trilineage differentiation (Figure 1).

In addition, expression of OCT3/4 on MSCs was measured.

2.4. Neural Induction of Isolated MSCs

MSCs were plated on sterile cover slips in 35-mm suspension tissue culture dishes at a density of 500 -1000 cells per dish in Neuron induction medium (NIM) consisting of : retinoic acid (RA) added to F10 medium at 30-µM final concentration, 10 ng/ml recombinant human Fibroblast Growth Factor (FGF) basic, 10 ng/ml recombinant Human Epidermal Growth Factor (rHEGF), 10-25 ng/ml Insulin-like Growth Factor I (IGF-I). Medium was refreshed after 3 daysfter 3 to 4 days. Evaluation of cell induction was studied using morphology, immunocytochemistry using glial fibrillary protein (GFAP) and flowcytometric nestin expression.

2.5. GFAP Immunofluorescence Staining of Plates

On the 5th day a sterile fibronectin-coated cover slip was inserted in 35-mm suspension tissue culture dishes. Cover slips were seeded with 500 cells in NIM. The cells were fixed with 0.5 ml of 4% par aformaldehyde in PBS for 20 minutes at room temperature then washed and permeabilized and blocked with 0.5 mL of 0.3% Triton X-100, 1% FBS serum in PBS at room temperature for 45 minutes. After blocking, the cells were incubated with 300 uL/plate of 1X mouse anti-human GFAP overnight at 2 - 8° C in the dark then washed and incubated with 300 ul/well of secondary antibody in the dark, at room temperature for 60 minutes. After washing they were immediately examined with fluorescent microscopic .

2.6. Flowcytometric Detection of Nestin Expression

Nestin mononclonal antibody (Abcam) was used to detect nestin expression

3. Results

3.1. Isolation and Growth Curve of MSCs

Age ranged from 18 – 50 years old, with a mean ± standard deviation of 34.16 ± 10.31. The MNCs number ranged from 45 - 80 million with a mean ± standard deviation of 61.04 ± 11.7. The viability ranged between 95% to 98%. The total number of cells in induced and uninduced cultures at day 3 showed non significant statistical correlation (P value > 0.05). The total number of non-induced cells started to be higher than induced starting from day 6 (P value ≤ 0.05 at day 6 and P value ≤ 0.001 at days 9 and 12) (table 1).

Table 1. *Comparison of Proliferative Capacity of induced and non induced groups in different days.*

	Studied groups		**Mann Whitney U test**	**P value**
	Non induced N = 8	**Induced N = 8**		
Day 0 (x 10^3)				
X±SD	10.0 ± 0.0	10.0 ± 0.0	0.0	1.0
Median	10.0	10.0		
Range				
Day 3 (x 10^3)				
X±SD	35.37±12.04	29.62 ±11.07	0.94	0.34
Median	35.50	29.0		
Range	56 – 20	45 – 15		
Day 6 (x 10^3)				
X±SD	85.37±9.86	68.25±11.38	2.52	0.012
Median	86.0	66.50		
Range	98 – 68	87 – 54		
Day 9 (x 10^3)				
X±SD	132.87 ±11.26	78.0 ±13.96	3.36	0.001
Median	134	75		
Range	150 – 117	98 – 61		
Day 12 (x 10^3)				

	Studied groups		Mann Whitney U test	P value
	Non induced N = 8	Induced N = 8		
X±SD	164.62±21.79	87.25±16.42	3.36	0.001
Median	160.50	80		
Range	200 – 135	115 – 70		

P value of > 0.05 was considered statistically non significant.
P value of ≤ 0.05 was considered statistically significant.
P value of ≤ 0.001 was considered statistically highly significant.

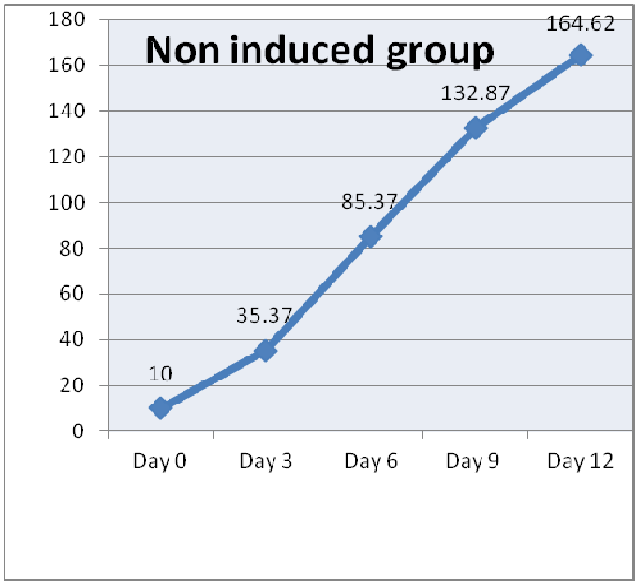

Fig. 1a. *Growth curve of uninduced group.*

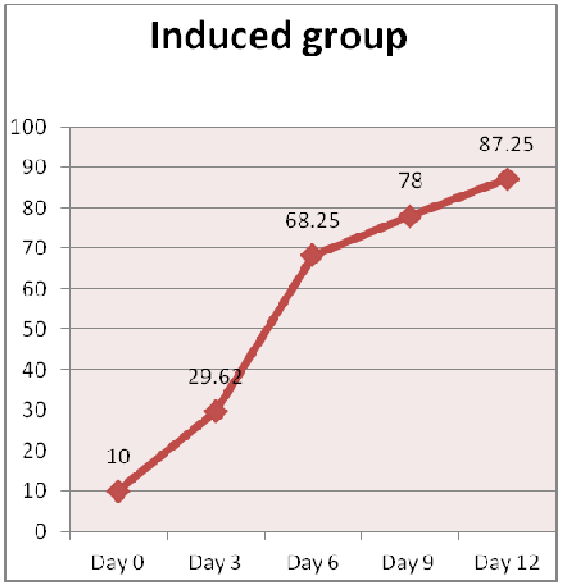

Fig. 1b. *Growth curve of induced group.*

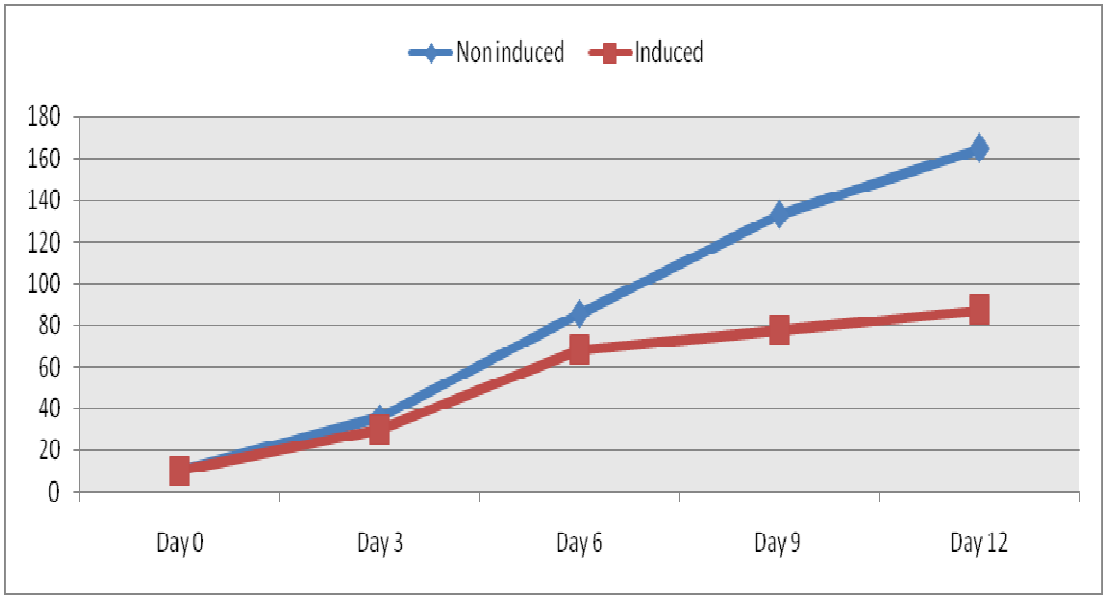

Figure 1c。 *Growth curve analysis of Induced and non-induced MSCs.*

3.2. Morphological Examination of MSCs

At day 1 and 2 after the initial plating, the adherent cells show cytoplasmic projections and a tendency to form small clusters . The onset of fibroblast like cell formation could be observed approximately at day 3 & 4. MSCs grew as a monolayer of large, flat cells . At day 5, cells tend to assume a spindle shaped morphology. At day 6 spindle shaped cells increase in number and tend to form colony forming unit fibroblast (CFU-F) which were cell clusters of more than 30 cells originating from one clonal cell 11 .At day 7, cells showed multi-polar fibroblastoid cells and a 60 % confluence which gradually increased to reach 80% - 90% confluence at about 9 days (Figure 1).

Morphology of Non-induced MSCs

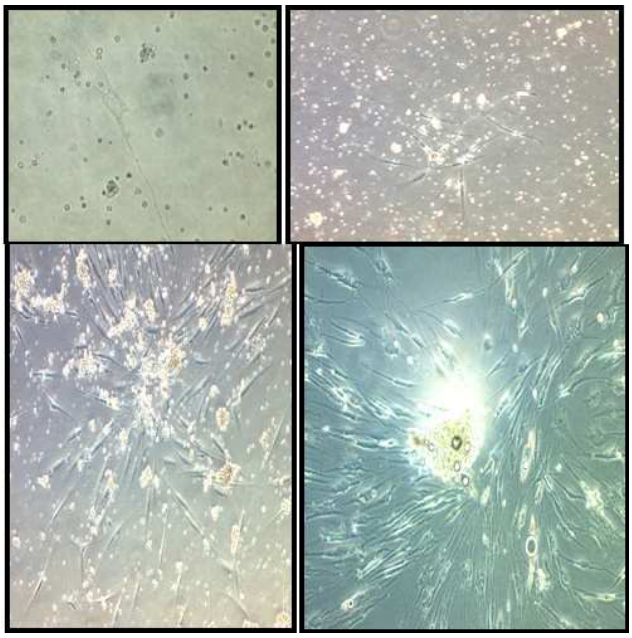

Figure 2. *Inverted microscope image (100X) of adherent MSCs .*

3.3. Flowcytrometric Identification of MSCs

MSCs showed positive expression for CD 44 (ranging between 60.30 to 98.9 with a mean ± standard deviation of 81.54±11.58) . MSCs showed positive expression for CD Oct 3/4 (ranging between 29.5 to 89.8 ,with a mean ± standard deviation of 56.12±17.37) . MSCs showed positive expression for double expression of CD44-OCT3/4 (ranging between 27 to 88.3 with a mean ± standard deviation of 54.03 ± 17.42) . Highly significant statistical correlation (P value <0.001) was found between age and double expression of CD44-OCT3/4 . No significant statistical correlation (P value > 0.05) was found between MNCs and double expression of CD44-OCT3/4 (Figure 3).

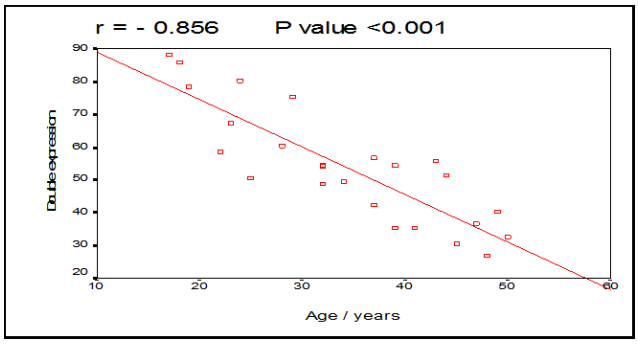

Figure 3. *Correlation between age and double expression of CD44-OCT3/4.*

3.4. Evaluation of Neural Induction of MSC

Post-induction morphological changes:

At the 8th day post induction the cells became contracted and smaller, and formed bipolar or multipolar prominences extending from the cell body, single long axon-like process also developed. Some neurons exhibited pyramidal cell morphologies. Neural morphology was distinguished from the normal cell morphology by a number of criteria, including the refractile appearance of the cells, the presence of either bipolar or multipolar neurite-like projections (with the projection at least equal to or exceeding the diameter of the soma) and the occasional presence of secondary extensions (Figures 4- 11).

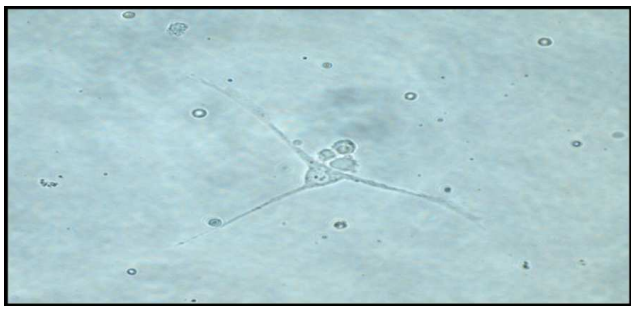

Figure 4. *Inverted microscope image x 200 of BM derived MSCs cultured in neurogenic medium. Cells assumed a multipolar neuron-like morphology.*

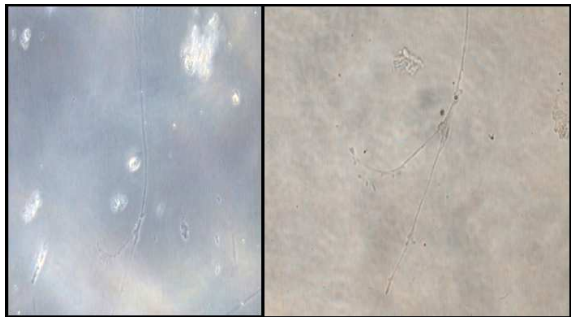

Figure 5. *Inverted microscope image BM derived MSCs cultured in neurogenic medium, the cells showed bifurcation of the processes.*

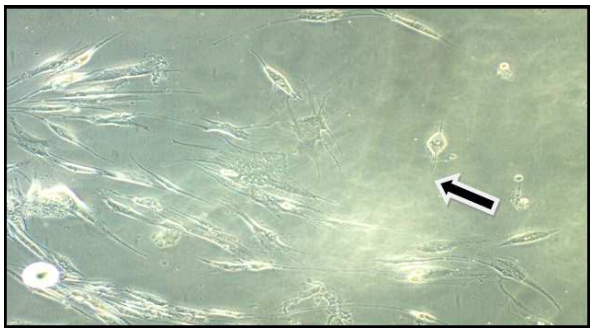

Figure 6. *Inverted microscope image x200 of BM derived MSCs cultured in neurogenic medium showed a typical oligodendrocytes.*

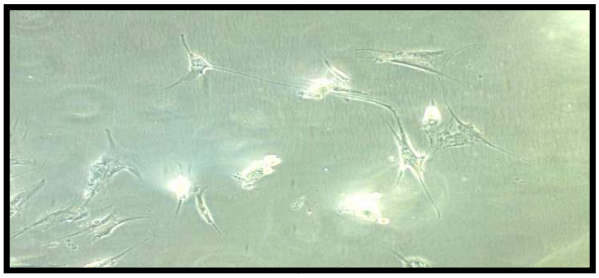

Figure 7. *Inverted microscope image x400 up & 200 down of BM derived MSCs cultured in neurogenic medium, showed connection between 2 astrocytes.*

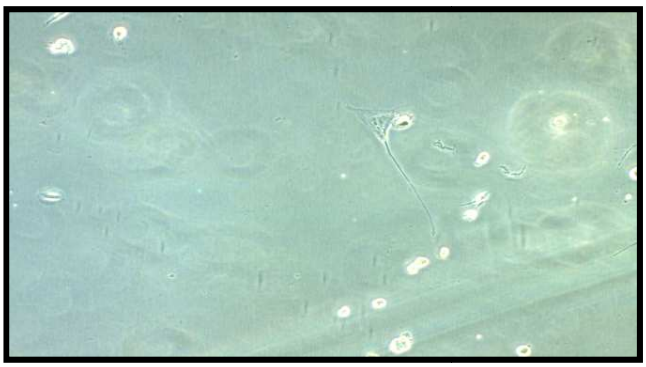

Figure 8. *Inverted microscope image x100 of BM derived MSCs cultured in neurogenic medium, showed pyramidal like cells.*

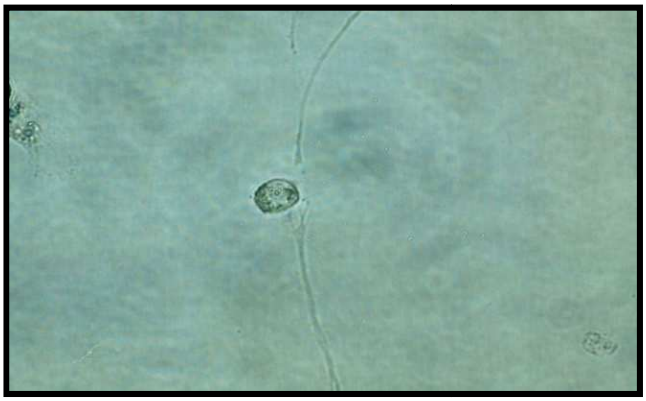

Figure 9. *Inverted microscope image X400 . Nerve cells showed synaptic connections.*

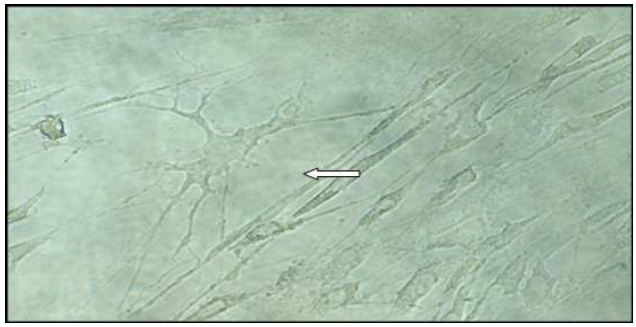

Figure 10. *Inverted microscope image x400 of BM derived MSCs cultured in neurogenic medium .The cells gave rise to a typical oligodendrocyte.*

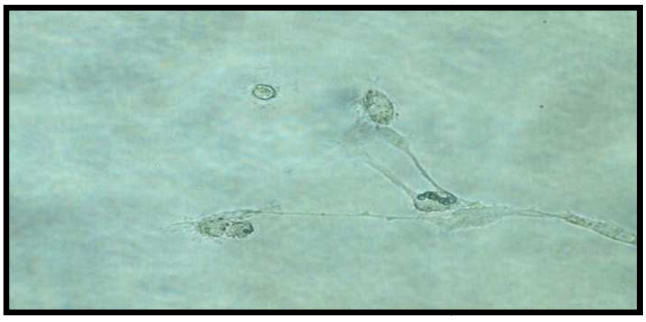

Figure 11. *Inverted microscope image x400 of BMA derived MSCs cultured in neurogenic medium showed vesicles.*

Figure 12. *Inverted microscope image x100 of BMA derived MSCs cultured in neurogenic medium. The nerve cells showed synaptic connections.*

GFAP detection by immunofluorescence

Induced cells showed positive staining with GFAP while uninduced cells shows negative staining .

3.5. Nestin Expression in Induced and Non-induced BMA-MSCs

Non-induced MSCs showed nestin expression ranging from 3.53 to 15.80 % with a mean ± standard deviation of 8.28 ± 3.62%.; while induced MSCs showed high positive nestin expression ranging from 15.8 to 65.4 %, with a mean ± standard deviation of 33.41±15.58. This difference showed high statistical significance, (P value <0.001). A highly significant statistical correlation, (P value <0.001) was found between induced group and double positive CD 44 & oct ¾.

Table 2. *Pearson linear correlation between double positive expression(CD 44& Oct ¾) and induced group.*

	Double expression	
	Correlation coefficient (r)	**P value**
Induction	+ 0.885	<0.001 HS

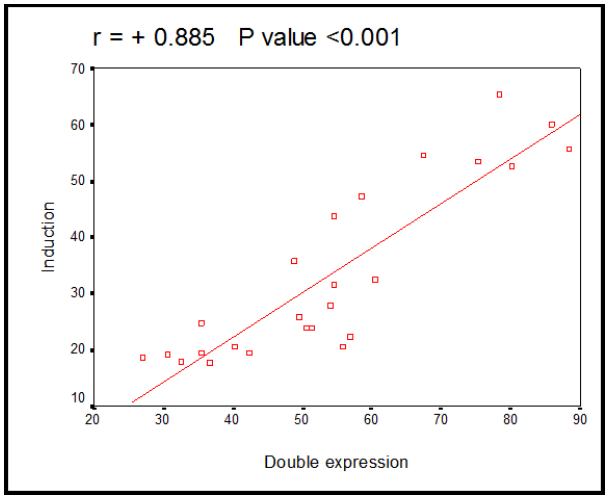

Figure 13. *Statistical correlation between induced group and double positive CD 44 & OCT ¾ MSCs.*

3.6. GFAP Detection by Immunofluorescence

Induced cells showed positive staining with GFAP while uninduced cells shows negative staining.

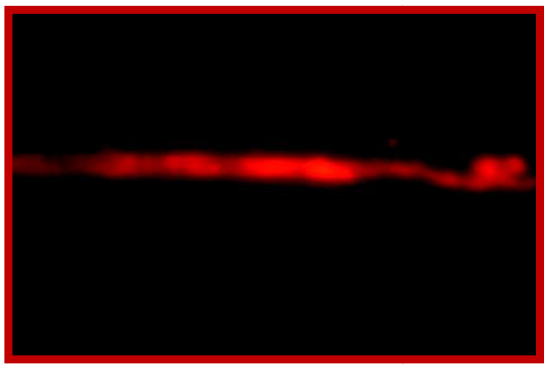

Figure 14. *Inverted microscope image x 400 of BM derived MSCs cultured in neurogenic medium stained by GFAP .*

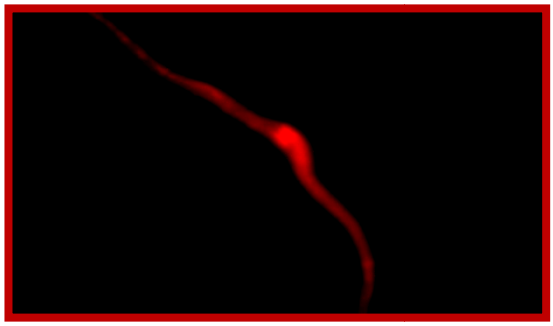

Figure 15. *Inverted microscope image x 200 of BM derived MSCs cultured in neurogenic medium stained by GFAP .*

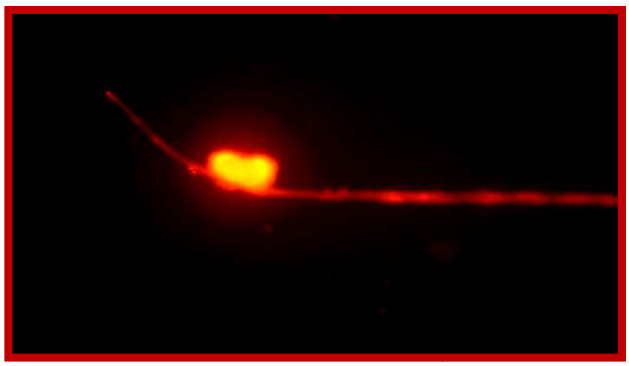

Figure 16. *Inverted microscope image x 200 of BMA derived MSCs cultured in neurogenic medium stained by GFAP .*

4. Discussion

Mesenchymal stem cells (MSCs) are found in different tissues such as bone marrow, fat, umbilical cord blood, amniotic fluid, placenta, dental pulp, tendons, synovial membrane and skeletal muscle. [12]

Neural stem cell (NSC) transplantation has been proposed as a future therapy for neurodegenerative disorders. However, NSC transplantation will be hampered by the limited number of brain donors and the toxicity of immunosuppressive regimens that might be needed with allogeneic transplantation. These limitations may be avoided if NSCs can be generated from clinically accessible sources, such as bone marrow (BM) and peripheral blood samples, that are suitable for autologous transplantation. [13]

Recently it had been proved that MSCs help in stimulation of angiogenesis, neurogenesis and regeneration of damaged tissue .[12]

The bone marrow MSCs had also shown promise in in vitro neuronal differentiation and in cellular therapy for neurodegenerative disorders, including Parkinson' disease .[14]

Although bone marrow cellularity is expected to decrease with age due to a decrease in the hematopoietic compartment as a result of variation in the composition of the stromal cell microenvironment [15]; no significant statistical correlation was observed between age and mononuclear cell number in the bone marrow, in the present study. This might be attributed to the nature of the bone marrow samples used donated mainly by patients with hyperactive marrow conditions with increased cellularity e.g. ITP, hypersplenism etc. [15']

While, hematopoietic stem cells constitute about 1% of the bone marrow population, MSCs constitute only 1/10000 to 1/100000 of the bone marrow nuclear cells[15], a fact which necessitates their expansion in vitro before use. Low glucose DMEM (1000mg/L) was used in this study because MSCs utilize energy more efficiently under restricted glucose treatment and exhibit greater self-renewal and anti-senescence abilities, while their differentiation potentials remain unaffected .[16]

In the present study MSCs have been detected by their morphology and confirmed by its surface marker as CD44 which was positive on isolated MSCs, ranging from 60.30 – 98.9 %.These data was in accordance with [17,18]. However, Zvaifler et al., reported it as a negative MSC marker [19].Moreover Fu et al. had isolated cells which expressed the characteristic antigens of MSCs, including CD29, CD44, CD73, CD90, CD105, and CD166 ,and did not express hematopoietic markers CD45, CD34, CD14, or CD11[13].LIN et al. had also shown positive CD44 and negative CD 34, working in the same condition of ours[20]. CD44 act as "bone homing receptor", directing migration of human hematopoietic stem cells and mesenchymal stem cells to bone marrow .[21]CD44 glycosylation directly controls its binding capacity to fibrin and immobilized fibrinogen .[22]

There has been a common opinion that CD73, CD105, CD90 and CD44 are highly specific for MSCs, and hence can discriminate multipotential cells from the tissue resident fibroblasts. However, several studies showed that these markers were ubiquitously expressed on stromal cells from many locations as well as on skin fibroblasts [23] and at best they only inform an investigator that the phenotyped cells are non-hematopoietic and stromal in origin.[24]

Oct ¾ in the present study was positive in a range of 29.5–89.8 %. This agreed with Miura et al., who also reported it as a positive MSCs marker.[25]In adult stem cells several studies suggest a role for Oct-4 in sustaining self-renewal capacity of adult somatic stem cells (i.e. stem cells from epithelium, bone marrow, liver, etc.). [26]

In the present study double expression of CD44 and OCT3/4 ranged from 27 to 88.3 % ,indicating that a high percentage of mesenchymal cells show also a preserved stemness character .These findings were in compatible with

those of Orciani et al., who succeeded in isolating MSCs with positive double expressed CD 44 and OCT ¾ surface marker[17]. On the other hand, a highly significant statistical negative correlation between age and double expressed CD44-OCT3/4 was observed, indicating an actual decrease in the number of MSCs with age. These findings–were in accordance with those of Stolzing et al. [27]. On the contrary, Payne et al., reported no correlation of MSC number with donor age this may be attributed to their different protocol of work for example obtaining their bone marrow samples from femur [28] . In the present study , differentiation was achieved by the use of a cocktail of-retinoic acid and growth factors . (Khoo et al.) reported that growth factor-based neural differentiation has yielded promising results [29].In contrast the use of chemical based method in culture had been controversial due to the toxic effect of these chemicals in modifying *cell size* and shape .[30]Retinoic acid A is an active derivative of vitamin A. Its wide expression in the nervous system during development, its role as a potent inducer of cell differentiation and the wide expression of its receptors and binding proteins in the brain suggest an important role in brain development and function .[30]These was in accordance with Montiel-Eulefi et al ., who succeeded in generating neural cells by use of RA and other neural inducing agents.[31]

In the current study, postinduction cells showed positive staining with GFAP in the 5th day post induction while preinduction cells showed no staining .These findings exclude the use of neural committed cells from the bone marrow sample.

These findings were analogous to those of Sanchez-Ramos who had first described neural differentiation by MSCs and detection of nestin and GFAP after differentiation, but importantly failed to report the pre-differentiated phenotype of the cells .[32]

Reyes et al., had succeeded in culturing GFAP positive nerve cells which also expressed tubulin III, NSE, glutamate Gal-C, MAP (other markers of nerve cells)from Human BMSC by plastic adherence cultured in Low glucose, DMEM , FBS ,EGF and PDGF.[33]

Sanchez-Ramos had reported that Co culturing of BMSC with fetal mouse midbrain increased significantly the percentage of BMSC that expressed NeuN and GFAP (markers of neurons and astroglia, respectively). The co-culture experiments support the hypothesis that cell–cell contact, in addition to signaling with trophic factors and cytokines, plays an important role in differentiation of these BMSC.[34]

Fu et al., have observed astrocytes, which were $GFAP^+$ and/or S-$100\beta^+$by using a cocktail of 20 ng/mL of both epidermal growth factor and basic fibroblast growth factor . On the other hand Kohyama et al. (2001) protocols had failed in differentiating MSCs into glial cells, because no GFAP immunoreactive cells were found.[13,35]

5. Conclusion

MSCs can be successfully isolated from bone marrow aspirate samples. Bone marrow should be considered as a very valuable source of MSCs. Bone marrow MSCs can differentiate into GFAP positive neurons under the effect of a cocktail of Retinoic acid (RA) , Recombinant human basic Fibroblast Growth Factor (FGF), recombinant Human Epidermal Growth Factor (rHEGF) and Insulin-like Growth Factor I (IGF-I) .

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