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Transdifferentiation of Bone Marrow Mesenchymal Stem Cells into Neural Cells via Cerebrospinal Fluid

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Abstract Research showed that adult bone marrow mesenchymal stem cells (BM-MSCs) differentiate into mesodermal cell types and also reprogram to transdifferentiate into endodermal and ectodermal cell types. The present study induced BM-MSCs to transdifferentiate into neural-like cells (either neurons or glial cells) using Cerebrospinal fluid(CSF)*in vitro*. The harvested BM-MSCs from rabbit femur were cultured and characterized immunocytochemically by CD146 that showed positive reaction for MSCs. Autologous CSF was added daily to the culture media. Dramatic morphological and cytochemical modifications were observed in the cultured cells and identified by different staining methods. Nissl bodies observed in the soma usingcresyl violet stain, and cell bodies' processes (axons and dendrites) usingsilver impregnation, proved that BM-MSCs differentiated into neuronal cells. Periodic Acid-Schiff demonstrated glycogen granules in astrocytes used to verify astrocytes differentiation. Immunocytochemical examination for glial fibrillic acid protein marker showed positive reaction indicating successful astrocytes differentiation. This study showed that induction of BM-MSCs with CSF mimics the strategy done in CNS. CSFcould provide an essential niche for promoting the transdifferentiation of BM-MSCs into neural cells, that hopefully help in treating acute and chronic neurodegenerative diseases.

Keywords: *BM-MSCs, CSF, neuronal cells, astrocytes, cresyl violet, silver impregnation, Periodic Acid-Schiff, CD146, glial fibrillic acid protein*

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1. Introduction

The neurological disorders of the spinal cord,the brain after injury, and the neurodegenerative diseases including Parkinson's and Alzheimer occur as a result of neuron death [1], this is because axons do not usually regenerate noticeably in their native environment [2]. Recent studies have been reported that tissue engineering and regenerative medicine show a great potential exploring a new paradigm for regeneration and treatment of degenerative and autoimmune diseases of the nervous system [3], particularly using bone marrow mesenchymal stem cells (BM-MSCs) [4]. Therefore, controlling the BM-MSCs *in vitro* to differentiate toward the neural lineage becomes an important source of cells used for cell therapy [5]. Under appropriate culture conditions, BM-MSCs, differentiated into chondrocytes [6], osteocytes [7], and adipocytes [8], which considered as mesodermal in origin. In addition, BM-MSCs could be reprogrammed to transdifferentiate into cells expressed endodermal and ectodermal origin [9,10]. *Hermann et al* [10], suggested that BM-MSCs have the ability to differentiate into neuroectodermal cell types *in vitro* and also *in vivo* after transplantation into the human brain and spinal cord**.**BM-MSCs showed to possess a great potential to differentiate into functional neurons because they expressed neuron phenotype and membrane channel protein, and also exhibited functional ion currents [11]. Several *in vitro* studies described conditions affecting on BM-MSCs to transdifferentiate into neural cells, either neurons or glial. These neural differentiation protocols were described by many authors, including chemical inducers [12], such as cytokines and growth factors [13,14,15], co-culture with neurons or glia [13,16,17], chemical inducers plus cytokines [10,18], and special supplements plus cytokines [19].

Cerebrospinal fluid (CSF) is derived from the niche of the nervous system, where circulating in spinal cord and the ventrical regions of the brain. It has a vital role in controlling the development of the central nervous system CNS, and essential for the formation of the neurons' layers in the cerebral cortex [20]. In addition, CSF is arich source of proteins, lipids, vitamins, hormones, cholesterol, glucose, micro RNAs, growth factors and adequate trophic support plus many other molecules and metabolites. These components provide full complement nutrition to the cell types of the nervous system such as the neurons and the glial cells and influence a multitude of CNS functions, including neurogenesis inembryos and adults [21,52]. This fluid help the regulation of neural stem cells (NSCs)

toward proliferation and commitment into neural progenitor cells that self-renew to produce semi-commitment transitional cells (neural precursor cells or neuroblast) which differentiate into mature neurons able for migration, maturation and synapse formation [22,23]. The niche environment in which stem cells reside has been identified as a critical component to the effectiveness of cellular engraftment and differentiation [24]. Therefore, the present study designed to use CSF in vitro to induce the BM-MSCs transdifferentiation into cells with a neural phenotype, imitating that occurred in the ventrical regions in the CNS.

2. Material and Methods

2.1. Sample Collections

Nine healthy 3 months old adult male New-Zealand white rabbits, each of them about 3 Kg weight, were used for isolation of both BM-MSCs and CSF samples. The procedures used were approved to surgically isolate samples in accordance with the protocol of laboratory animal unit of Tissue Engineering Laboratories, Faculty of Dentistry, Alexandria University. Experiments performed in line with the ethical considerations recommended by Alexandria University, Egypt.

a. Surgical Isolation of BM-MSCs

Animals were pre-treated by intramuscular (IM) administration of XylazineHCl 2% (Xyalazect, Adwia, Egypt) in a dose of 5 mg/kg for animal sedation. After upper parts of femurs were shaved, the animals were anesthetized by injection of ketamine HCl (Ketamine, Sigma, Egypt) in a dose of 50 mg/kg. Then, the bone marrow samples were collected and mixed with anticoagulant solution and immediately processed under aseptic condition. The surgical wound was closed in layers [25,26]. Steps of surgical isolation were illustrated in *Figure 1*.

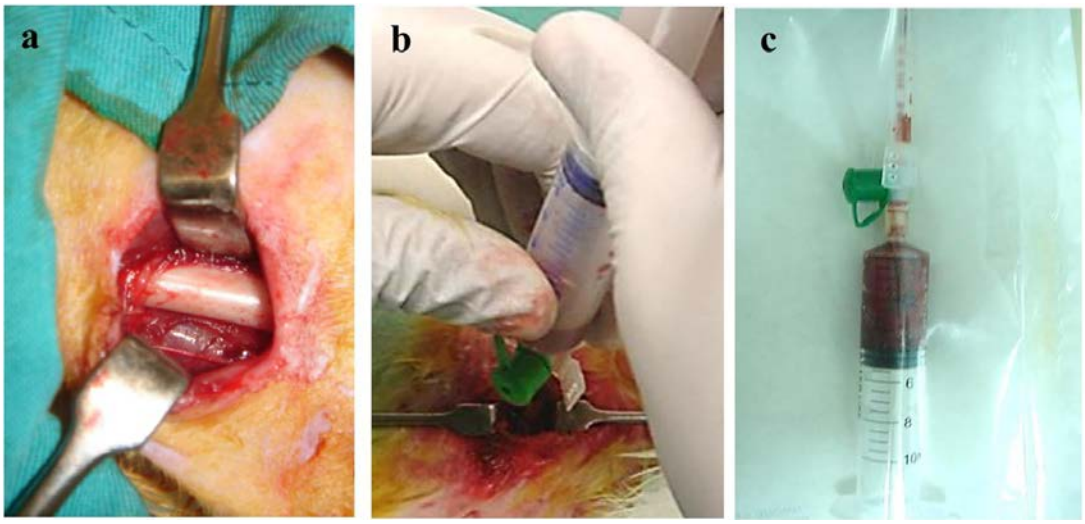

Figure 1. Photomicrographs showing steps of surgical isolation of BM-MSCs: (a) Rabbit femur after exposure. **(b)** Sample collection using a sterile syringe containing anticoagulant under aseptic condition. **(c)** A sterile syringe containing the harvested bone marrow sample

b. Surgical Isolation of Autologous CSF

Pre-operation rabbit's relaxation was done similar to the procedure described for BM-MSCs isolation. Then head of rabbits were bent to the chest with the curve not too much to affect its breath, the skin of the neck was shaved and disinfected. The rabbit was anesthetized by intramuscular (IM) administration of ketamine HCl (Ketamine, Sigma, Egypt) in a dose of 50 mg/kg. Sterile 3 ml syringe was inserted into the cisterna magna (Atlanto-occipital membrane puncture). Finally, the CSF collected in a sterile empty test tube and stored immediately into -20°C freezer [27,28].

2.2. Separation and Cultivation of BM-MSCs

The aspirated bone marrow specimen was mixed with 8 ml of Dulbecco's Modified Eagle Medium (DMEM, Lonza, Belgium) supplemented with 10% Fetal Bovine Serum (FBS, Lonza, Belgium), centrifuged and washed with phosphate buffer saline (PBS, Lonza, Belgium). Then the cells were seeded into T- 25 cm^2 tissue culture flask with vented filter caps and the flask incubated in water jacketed CO_2 incubator 37°C, 5% CO_2 and 95% humidity.Media were changed every 3-4 days, and when fibroblast like-cells at the base of the flask reached confluence, they were ready for the first passage *(Passage 1, P_1)*, then trypsinized (using 0.25% trypsin /EDTA), counted by Trypan blue assay using hemocytometer, finally examined its viability with an Inverted Phase Contrast Microscope. These cultured cells were marked as the *primary generation (Passage 0, P_0)* [26,29].

2.2.1. Morphological Characterization of Undifferentiated BM-MSCs

Morphological features of undifferentiated BM-MSCs released from the primary passage *(P0)* were plated in poly-D-lysin coverslips (Neuvitro Corporation, Germany)with a cell density $1\times10^4/cm^2$ and cultured in DMEM media, then examined as the following:

a. Inverted Phase Contrast Microscope(IPCM)

Cell growth and morphological features of the undifferentiated cells were examined and photographed daily using IPCM (TE2000; Nikon, Japan) for 9 days at room temperature (25°C).

b. Light Microscope

Cells fixed using a 10% neutral formalin, were stained with hematoxylin and eosin stain(H&E), dehydrated, cleared in xylene, mounted with DPX and examined using light microscope (Nikon, Japan) [30].

c. Scanning Election Microscope (SEM)

The specimens were fixed with 4% paraformaldehyde at room temperature(RT)followed by secondary fixation in 2.5% glutaraldehyde at 5°C. Then, washed twice with PBS, dehydrated by ethyl alcohol series and dried overnight. The cells were gold coated using a sputter-coating system, and examined with SEM (JEOL, JSM-5300) at a high voltage (20 kV) and high vacuum mode with a tilt of 30° [31,32].

2.2.2. Immunocytochemical Characterization of Undifferentiated BM-MSCs

Undifferentiated BM-MSCs were characterized using mouse anti-rabbit CD146 surface antigen. The cells were fixed for 20 min with 4% paraformaldehyde at RT, then washed with buffer (0.1% bovine serum albumin (BSA, Sigma) in phosphate buffer saline, PBS). The cells were incubated in blocking buffer (10% normal goat serum (Sigma) with 1% BSA in PBS) for 45 min. After that mouse anti-rabbit CD146 monoclonal primary antibody IgG1 (ebioscience, USA) (1:200 dilution with 0.1% BSA in PBS) was added and incubated for 60 min. Then incubated in goat anti- rabbit tetramethylrhodamineisothiocyanate (TRITC) secondary antibody IgG (ebioscience, USA) (1:1000 dilution with 0.1% BSA in PBS) for 60 min in dark place. Finally the samples were cleared, mounted with DPX and examined using Confocal Laser Scanning Microscope (CLSM, Bio-Rad, Italy) [33].

2.3. Differentiation of BM-MSCs into Neural-like Cells

The undifferentiated BM-MSCs released from the primary passage *(P0)* were plated in poly-D-lysin coverslips with a cell density 1×10^4 cells/cm^2 in six well plate. When the cells grow to 70% confluence and after 72 h, 10 µl of auto-CSF was added to the culture medium every day and the differentiated cells were examineddaily for 9 days [29], as follows:

2.3.1. Morphological Studies of the Differentiated Cells

Cell growth and morphological features of the differentiated cells examined daily and photographed at room temperature using IPCM [29], light microscope after staining with H&E [30] and SEM [33] with the same procedure used to examine undifferentiated BM-MSCs.

2.3.2. Cytological Studies of the Differentiated Cells

Three special cytological stains were performed to characterize the differentiated cells as described below:

a. Cresyl violet acetate stain: Used for investigating the neurons by demonstrating the Nissl bodies found in their soma. The differentiated cells were fixed and stained in 0.1% cresyl violet acetate (pH3). The stained cells were differentiated in 95% ethyl alcohol, dehydrated in 100% ethyl alcohol, cleared, mounted and examined using light microscope [34].

b. *Bielschowsky's*silver impregnation method: Used for investigating the neurons by depicted the cell bodies' neurites. The fixed differentiated cells were immersed in 20% silver nitrate for 60 min (45°C- 50°C) and incubated in dark place till the cells became light brown color. The samples washed for 10 min in reducer solution (5% formalin and 20% absolute ethyl alcohol in distilled water). Then transferred to 1% ammonium hydroxide (NH_3OH) and incubated in dark place in ammoniacal silver nitrate solution ([Ag(NH3)2]NO3(aq)) till the fibers taken black color. To stop the silver reaction, the samples were just dipped in 1% NH_3OH, then transferred to 1% oxalic acid for 5 min, and retransferred to 5% sodium thiosulfate for 5 min. Finally, dehydrated, cleared, mounted and examined using light microscope [35].

c. Periodic Acid-Schiff's reagent (PAS): Used for glycogen demonstration localized mainly in astrocytes. Glycogen is considered a sensitive marker to assess the degree of neuronal and astrocytic differentiation. The fixed differentiated cells were immersed in 5% periodic acid followed by staining in Schiff's reagent, and rinsed in sulfurous solution. After that, the cells were dehydrated, cleared, mounted and examined using light microscope [36].

2.3.3. Immunocytochemical Characterization of Differentiated Neural-like Cells

The differentiated neural-like cells were characterized using mouse anti-rabbit glial fibrillary acidic protein (GFAP). The cultured cells on the cover slips were fixed in 4% paraformaldehyde at RT, washed with buffer (0.1% BSA in PBS), thenincubated in blocking buffer (10% normal goat serum with 1% BSA in PBS) for 45 min. Mouse anti-rabbit GFAP monoclonal primary antibody IgG1 (ebioscience, USA, 1:100 dilution with 0.1% BSA in PBS) was added and incubated for 60 min, followed by washing twice. Then incubated in goat anti- rabbit Fluorescein isothiocyanate (FITC) secondary antibody IgG (ebioscience, USA) (1:500 dilution with 0.1% BSA in PBS) for 60 min in dark place. The incubated cells washed, cleared, mounted with DPX and examined by CLSM [29,37].

3. Results

3.1. Characterization of Undifferentiated BM-MSCs

After 24hrs of cultivation, the true MSCs adhered to the plastic culture flask and exhibited a fibroblastic-like morphology. Within 4 days of cultivation, the adherent cells clearly appeared as individual or clusters of few spindle shaped cells *(Figure 2 a, b).* These clustered cells replicated rapidly and expanded mitotically to form a small discrete colony of 50 or more fibroblast-like cells within 7 days. Those colonies grew quickly in size and number within 14 days *(Figure 2c), and* fibroblast cells reached confluence of 70–80% within 21-27 days *(Figure 2d).*

The undifferentiated BM-MSCs stained with H&E and examined with light microscope after 24hrs, appeared as a spindle or bipolar- fibroblastic morphology with central round nuclei in their centers *(Figure 3 a).* The undifferentiated BM-MSCs had static morphological exhibition after 4, 7, and 9 days of cultivation.

Examination of the undifferentiated BM-MSCs using SEM showed the surface topography, edge details, and the outer structure displayed as spindle shape or flat morphology with very short processes *(Figure 3 b).*

Undifferentiated BM-MSCs elucidated by immunocytochemical techniques to examine the expression of surface antigens CD146, and examined with CLSM. The true BM-MSCs showed positive CD146 immunoreactivity after 24 hrsof incubation in the cultured media and exhibited a flat, spindle or fibroblastic-like structure *(Figure 3 c, d).*

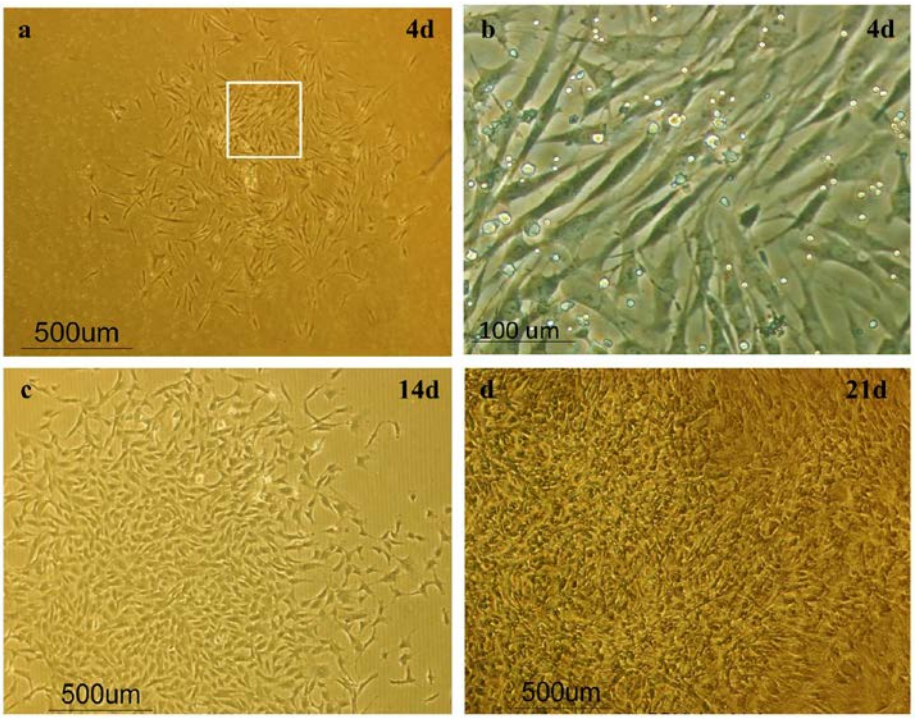

Figure 2. Photomicrographs by IPCM showing themorphological features and proliferation of cultured undifferentiated BM-MSCs: (a) Undifferentiated BM-MSCs appeared as few clusters of spindle shaped cells after 4 days of cultivation (Scale bar = 500µm).(b) Higher magnification of the white square shows spindle shaped cells at the same day (Scale bar = 100µm).(c) Colony size expanded within 14 days of cultivation, and the cells arranged in a swirling pattern (Scale bar = 500µm).(d) Highly crowded bipolar fibroblast BM-MSCs at 21 days of primary culture (Scale bar = 500µm)

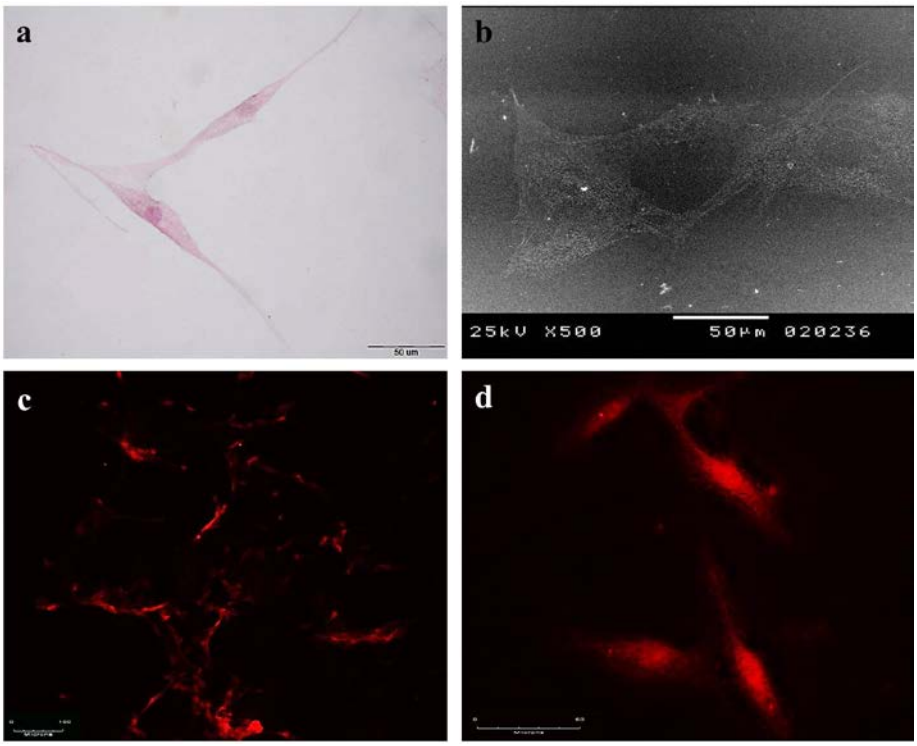

Figure 3. Photomicrographs showing the morphological features of the undifferentiated BM-MSCs: (a) The undifferentiated BM-MSCs stained with H&E and examined with light microscope, shows static morphology as bipolar fibroblastic structure (Scale bar = 50µm).**(b)** The undifferentiated BM-MSCs examined with SEM shows flatten or spindle-shape cells (Scale bar = 50µm).**(c)** Clusters of undifferentiated BM-MSCs examined with CLSM, expressed positive CD146 immunoreactivity and exhibited as spindle or fibroblastic-like morphology (Scale bar = 100µm).**(d)** Higher magnification of undifferentiated BM-MSCs examined with CLSM, and having static morphology as bipolar fibroblastic structure with positive CD146 (Scale bar = 50µm)

3.2. Characterization of the Differentiated Neural-like Cells:

3.2.1. CSF Promotes Proliferation and Differentiation of Progenitor Cells

Since the CSF is a complex fluid containing many components provides essential growth and survival factors for the developing neurons in the nervous system, we tested it in the BM-MSCs. The morphology of the tested cells showed dramatic changes after 2-4 days of incubation in the CSF. The differentiated cells were morphologically characterized by inverted phase-contrast microscopy (TE2000; Nikon, Japan- Egypt), and analyzed by Motic Images Advanced (V. 3.2) acquisition software (Motic, Seneco, Milan, Italy). Figure 4 shows Phase-contrast microscopy images demonstrating the morphologic features of neural progenitor-like cells – multipolar and bipolar neuronal progenitor cells mixed with oligodendrocytes progenitor cells – and neural elongated-like stem cells (NSCs).In addition these images demonstrated the early formation of connections between branched oligodendrocyte progenitor cells (OPC) attached with oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells that may restricted to astrocyte development and can generate oligodendrocytes. The neural progenitor-like cells were characterized using silver impregnation method, which multipolar and bipolar neuronal progenitor cells strongly depicted with silver stain after 3 days of incubation in CSF *(Figure 5 a, b, c).* Confocal immunofluorescence images showed differentiated neuronal progenitor cells attached with pro-oligodendrocyte progenetor cell expressing positive GFAP immunoreactivity *(Figure 5 d, e).*

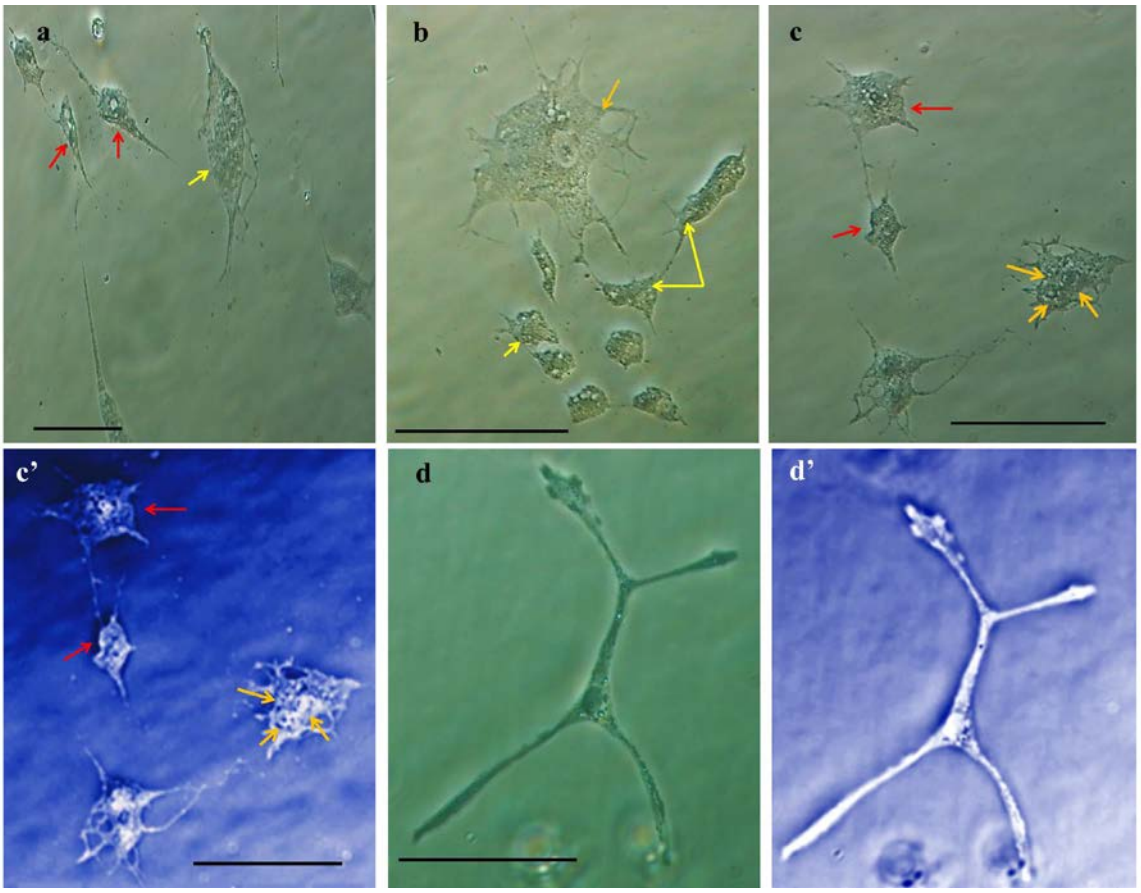

Figure 4. Photomicrographs shows proliferation and differentiation of neural progenitor-like cells from BM-MSCs after incubation in culture media supplemented with CSF: (a) Phase-contrast microscopy picture showing differentiated bipolar neuronal progenitor cells (red arrows) after 4 days, yellow arrow indicate pro-oligodendrocyte precursor cell (Scale bar = 100µm). (b) Phase-contrast microscopy picture showing formation of connections between branched oligodendrocyte progenitor cell (yellow arrow) and attached with them oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells (Orang arrow) (Scale bar = 100µm). (c, c') Phase-contrast microscopy picture showing differentiated multipolar neuronal progenitor cells (red arrow) after 4 days, orang arrows indicate multinucleated neural progenitors (stem cells) differentiated into oligodendrocyte-type-2 astrocyte (O-2A)-like progenitor cells (Scale bar = 100µm). (d, d') Phase-contrast microscopy pictures showing neural elongated-like stem cell after 7 days (Scale bar = 100µm)

3.2.2. CSF Promotes Proliferation and Differentiation of Mature Neural Cells

3.2.2.1. Neuronal Cells

At day 0 (before the induction process by auto-CSF), the adherent monolayer cells appeared as fibroblastic or flattened cells which were characteristically to the morphological feature of MSCs *(Figure 2).* After 24 hours of induction in auto-CSF, by IPCM we demonstrated that neuronal precursor-like cells were developed as a pyramidal cell body having phase bright with a neurite elongated-like morphology as shown in Figure 6a. within 3 days of induction, the neuronal precursor-like cells appeared more developed and its neurite appeared as long process on one side (axon like structure), while on the other side the soma were more branched into two to three processes (dendrites like structure) *(Figure 6 b).* The

proliferation and differentiation of neural-like cells were increased and clearly observed after 5 to 9 days of cultivation. Mature neurons (multipolar-like structure) were fully developed and dendrites like structure clearly appeared after 5 days of incubation *(Figure 6c)* & *(Figure 8c)*. Also, bipolar neurons-like structure were shown within 9 days *(Figure 6*d*)*.

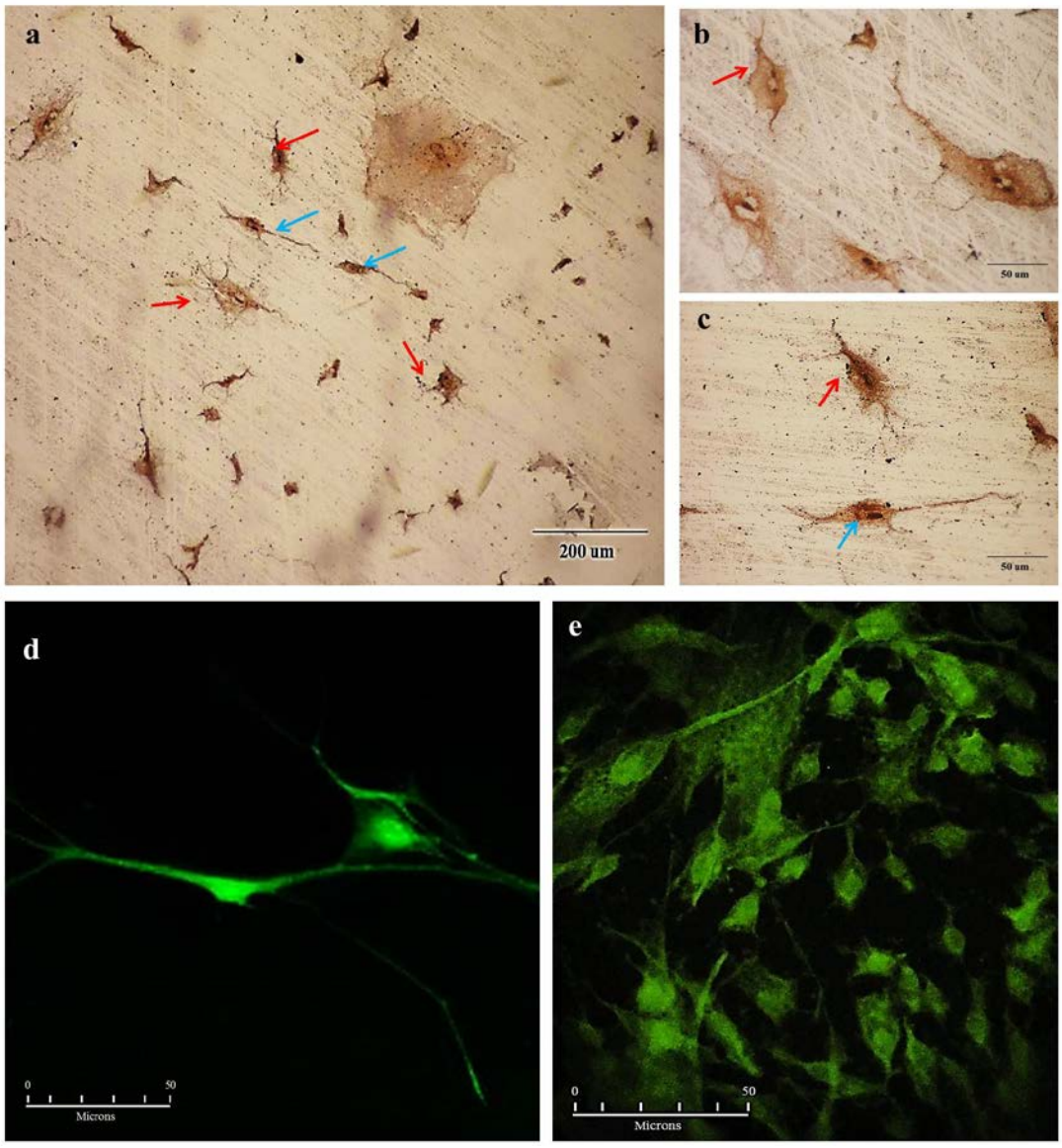

Figure 5. Photomicrographs showing the characterization of neural progenitor-like cells differentiated from BM-MSCs after 4 days ofincubation in culture media supplemented with CSF: (a) Differentiated neural progenitor cells stained strongly with silver impregnation stain (Scale bar = 200µm). (b, c) Higher magnification to the previous showing differentiated multipolar (red arrows) and bipolar (white arrow) neuronal progenitor cells (Scale bar = 50µm).(d) Immunofluorescence image showing differentiated neuronal progenitor cells expressed positive GFAP immunoreactivity culturing (Scale bar = 50µm).(e) Immunofluorescence image showing differentiated neuronal progenitor cells attached with pro-oligodendrocyte precursor cell expressed positive GFAP immunoreactivity (Scale bar = 50µm)

The morphological features of the neural precursor-like cells and the mature neuronal-like cells were demonstrated by using H&E stain. Neuronal precursor-like cells developed as a cone-like cell body with elongated structure like neurites emerged from it *(Figure 9 a, b)*. These cells differentiated into mature neurons after 5 days, with emerging longaxon-like structure from the soma from one side and dendrites-like structure from the other side.

Examining the surface topography using SEM, shows the edge's details and the outer structure of developmental neural-like cells with high resolution *(Figure 9 d, e, f)*. By using a special program attached to the SEM's software, it was possible to measure the progress of the neuronal-precursor cell's length during the different days of cultivation. It was observed that the length of neuronal precursor cells at the first day of induction was the smallest, approximately 44.90 µm. After 5 days the length of the immature cell reachedto be about 69.40 µm. and after 9 days, the neuronal cell represented the longest. The length of the multipolar neuronal cell ranged from 102.67 to 135.34 µm, while bipolar neuronal cell's length was approximately 398.4 µm which is considered to be the longest one.

Special stains methods used in our study to characterize the cyto-morphological features of the mature neuronal-like cells and mature astrocyte-like cells. Before induction process, the undifferentiated BM-MSCs demonstrated negative results for cresyl violet, silver impregnation and PAS stains *(Figure 10a, h)*.

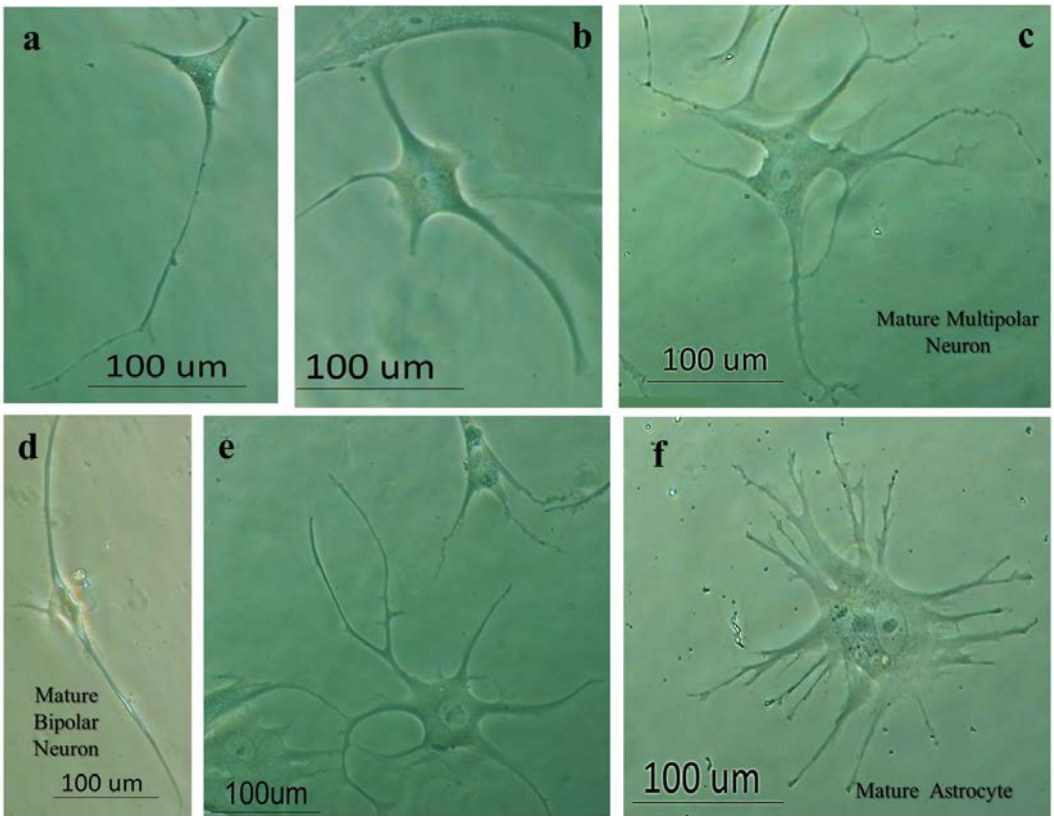

Figure 6. Photomicrographs by IPCM showing the morphological features of the differentiated neural-like cells: (a) Neuronal precursor cell has pyramidal soma with rounded nucleus and elongated neurite (24 hrs after induction)(Scale bar = 100µm).**(b)** More developed immature neuronal cells possess two to three short neurites after 3 days of inductionin CSF(Scale bar = 100µm). **(c)** Completely developed maturemultipolarneuron with distinct soma and distinct axon and distinguished multi-branched dendrites emanated from it after 5 days of induction.**(d)**Mature "bipolar" neuron developed within 9 days of induction(Scale bar = 100µm). **(e)** Immature Astrocyte cell has polygonal cell body with rounded nucleus and more processes emerged from it within 3 days of incubation (Scale bar = 100µm). **(f)** Mature astrocyte completely differentiated after 5 days of induction, with distinct star like structure soma and dense plasma to fibrous dendrites(Scale bar = 100µm)

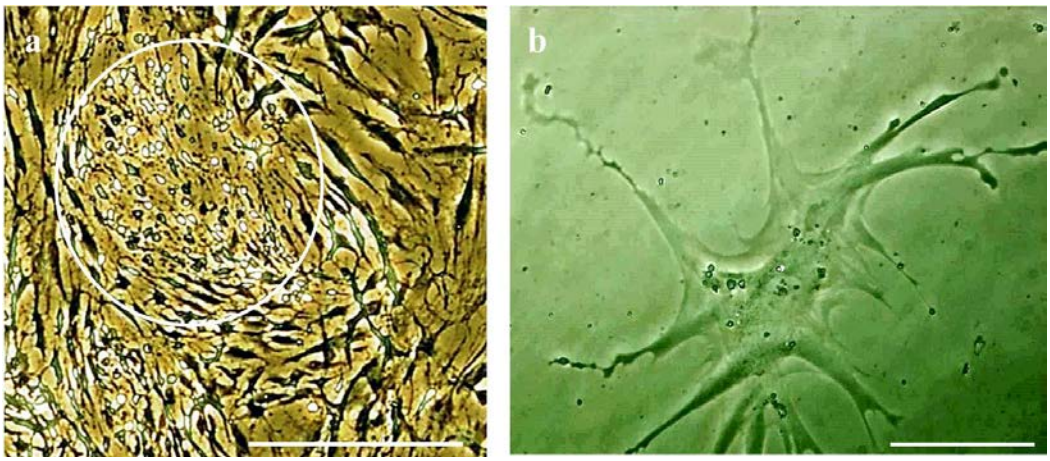

Figure 7. Photomicrographs showing the proliferation and differentiation of astroglial and mature astrocytes from BM-MSCs after incubation in culture media supplemented with CSF: (a)The white circle area in the phase-contrast microscopy picture showing neural stem cells with astroglial morphology describing a neurogenic niches of highly replicating cells (i.e. small neurogenic islands where neurons and glia are continuously generated) (Scale bar = 500µm). (b) Mature star-like structure astrocyte after 5 days (Scale bar = 100µm)

The cytoplasm of neuronal precursor-like cells containing a little bit granules "Nissl bodies" showed weak cresyl violet stained within 24 hrs of incubation. While within 5 days, Nissl bodies clearly appeared in the cytoplasm of neuronal-like cellsas strongly stained granules surrounded the nucleus *(Figure 10*0*b).* In addition, mature neuron-like cells completely developed within 5 to 9 days of incubation which stained with both cresyl violet stain and silver impregnation. Both reactions showed that dense Nissl bodies found in the soma strongly revealed with purple color and the cell bodies' processes depicted with very dark brown or black silver stain *(Figure c, d, e).* Strong silver stain in the neural processes indicates that the neurofilaments have been developed and formedfibrous neuronal-like network. The neurofilaments evolved after 14 days of incubation in CSF, and performed as a fibrous neuronal network and depicted with black color, that also shown with SEM *(Figure 10*0 *c, f, g).* Furthermore, after 24 hrs of incubation in auto-CSF, neural precursor-like cells showed little PAS' granules, exhibiting

weak glycogen reaction. After 5 days of incubation, the neural precursor cells differentiated into mature multipolar neuronal-like cells, showed more intense activity of glycogen *(Figure 10 ki)*. Moreover, mature bipolar neuronal-like cells differentiated within 9 days and appeared with the same strong glycogen activity *(Figure 10 k)*.

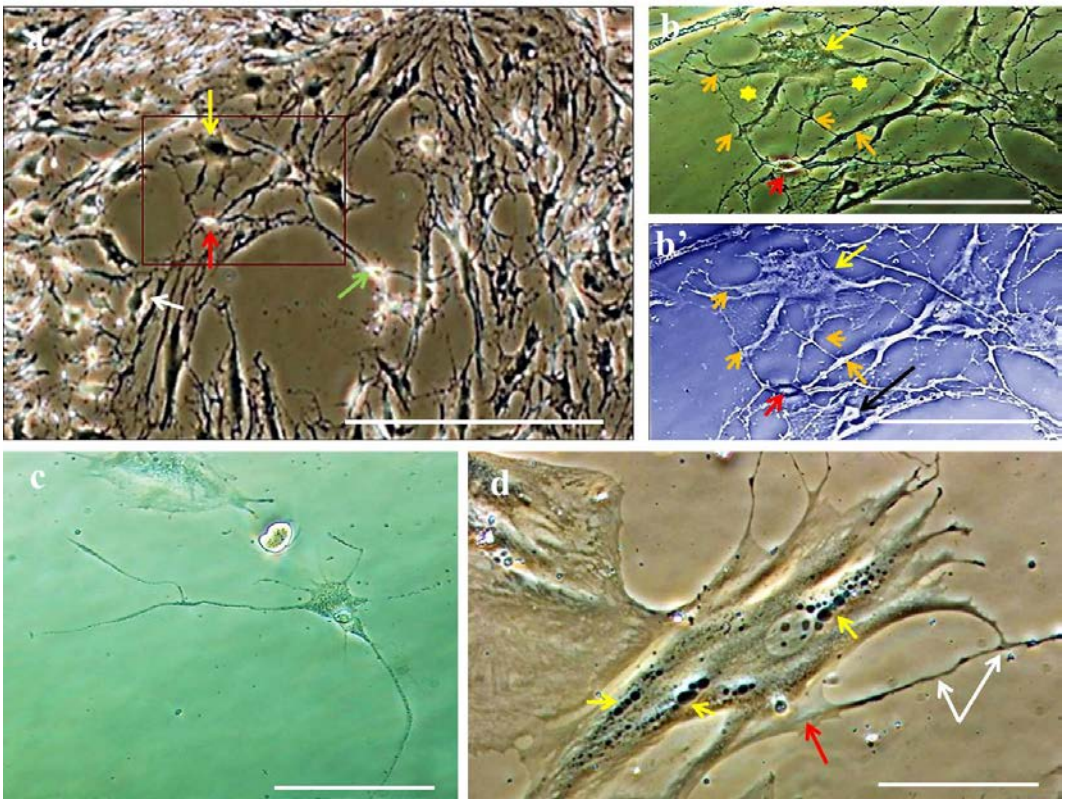

Figure 8. Phase-contrast microscopy picture showing the proliferation and differentiation of mature neural cells from BM-MSCs after incubation in culture media supplemented with CSF:(a)Highly enriched area of differentiated mature bipolar neuronal cell (green arrow) and multipolar neuronal cell (red arrow) with multi-branched neurites contacting with mature oligodendrocyte (yellow arrow) after 5 days, white arrow indicate pro-oligodendrocyte contacting neuritis branches (Scale bar = 500µm). (b, b') Enlargement of the red square area showing mature oligodendrocyte (yellow arrow) with granular cytoplasm which intensively produces myelin component and extending sheaths (yellow stars) contacting neurite branches (orange arrows) of the multipolar neuron (red arrow) for enveloping, black arrow showing cell body of unipolar neuron with coiled proximal axon (Scale bar = 100µm). (c)Mature multipolar neuronal cells after 5 days of culturing (Scale bar = 100µm). (d)Mature oligodendrocyte after 8 days, yellow arrows indicate dense granular cytoplasm area (rough endoplasmic reticulum during myelination), red arrow showing sheaths of membrane enveloping the contacting axon (white arrows) of the neighboring neuron (Scale bar = 100µm)

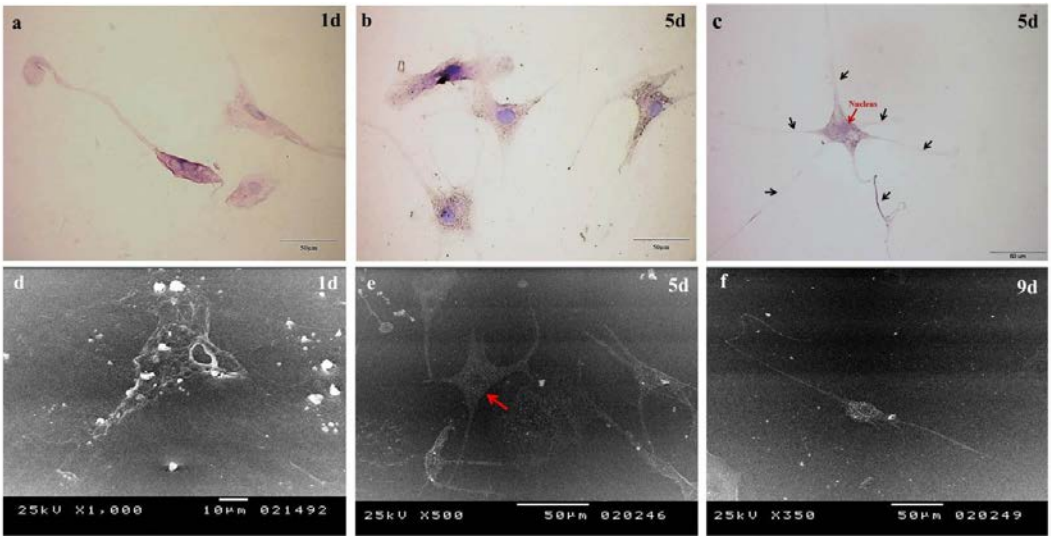

Figure 9. Photomicrographs shows the morphological features of the differentiated neural-like cells: (a) Neural precursor cell stained with H&E, with elongated neuriteemerged from the soma after 24hrs**(**Scale bar = 50µm**). (b)**Semi-differentiated neurons stained with H&E after 5 daysof induction,with distinct soma, axonand distinguished multi-branched dendrites emerge from it(Scale bar = 50µm**).(c)**Fully differentiated mature astrocytes after 5 daysof induction stained with H&E,with distinct soma and multi-branched dendrites (Scale bar = 50µm**).(d)** Neuronal precursor-like cell examined with SEMafter 24 hrsof induction, has pyramidal or cone-like structure soma with central rounded nucleus and extending neurite (Scale bar = 10µm). **(e)**Mature multipolar neuron (red arrow) after 5daysof induction, examined with SEM, has distinct soma with clearly appeared central rounded nucleus, distinct axonand multi-branched dendrites (Scale bar = 50µm). **(f)**After 9 days of induction,a very elongated bipolar neuron cell observed using SEM (Scale bar = 50µm)

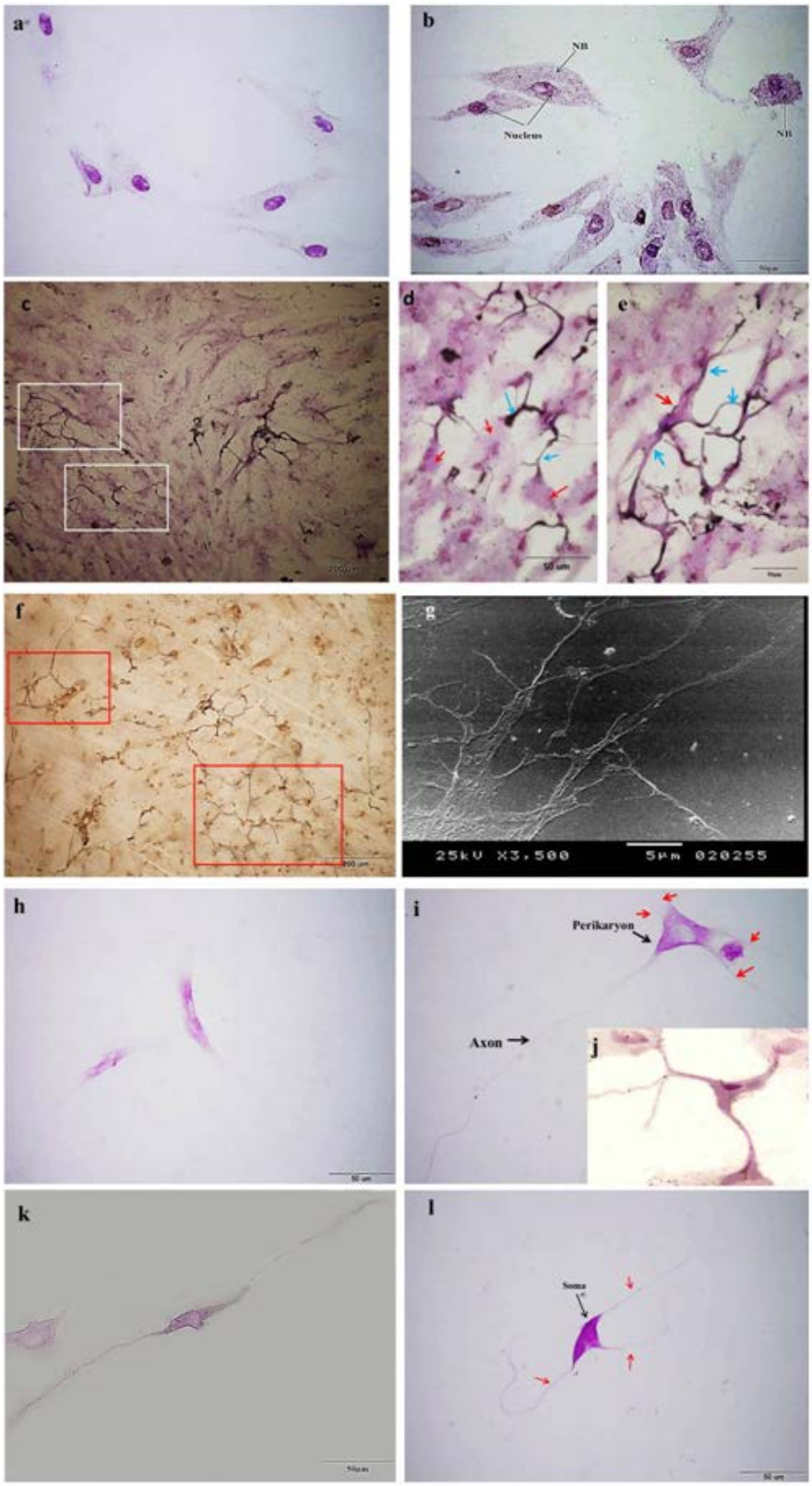

Figure 10. Phase-contrast microscopy picture showing cytological characterization of mature neural-like cells differentiated from BM-MSCs after incubation in culture media supplemented with CSF using special stains: (a) Before the induction process (0 day), the cultured cells appeared normally as spindle shape with weak reaction activity and clearly stained nuclei(Scale bar = 50µm).(b) Differentiated neural-like cells stained with cresyl violet after 5 days of induction showing strong concentration of Nissl bodies "NB" granules surrounded the nucleus "N"(Scale bar = 50µm).(c) Fully differentiated mature neuronal-like cells after 9 days of induction, having soma counterstained with cresyl violet (red arrow)and multi-branched neurits impregnated with black silver stain (blue arrow), forming fibrous network-like structure (red square)(Scale bar = 200µm).(d, e)Higher magnification of the neuron-like cells and fibrous neural-like network in the previous figure (Scale bar = 50µm).(f)Fully differentiated mature neuronal-like cells after 14 days, forming fibrous network-like network (red square) (Scale bar = 200µm).(g)Very dense fiberousneuronal network shown by SEM (Scale bar = 5µm).(h) Undifferentiated BM-MSCs before the induction process (0 day), showing spindle shaped cells with very weak PASstain in their cytoplasm(Scale bar = 50µm).(i, j)Mature multipolar neuronal-like cell stained with PAS showing strong glycogen activity, and stronglystained withcresyl violet, respectively(Scale bar = 50µm).(k)Mature bipolar neuron performed after 7 daysof induction which possess distinct soma with strong glycogen content(Scale bar = 50µm).(l) Mature astrocyte-like cells stained with PAS after 5 days of induction in CSF, revealedintense glycogen content(Scale bar = 50µm)

The immunocytochemical techniques have been applied to observe the expression of neuronal marker GFAP, on the untreated and treated BM-MSCs with auto-CSF, then revealed with FITC conjugated anti-rabbit and examined using CLSM. The results indicated no GFAP immunoreactivity in the undifferentiated BM-MSCs in the absence of auto-CSF, while neural precursor-like cells showed positive immunoreactivity within 2 to 3 days *(Figure 11 a, b)*. The mature neuronal-like cells expressed strong immunoreactivity of GFAP within 5 days of the induction *(Figure 11 c, d)*, as well asstrong silver stain *(Figure 11 d')*.

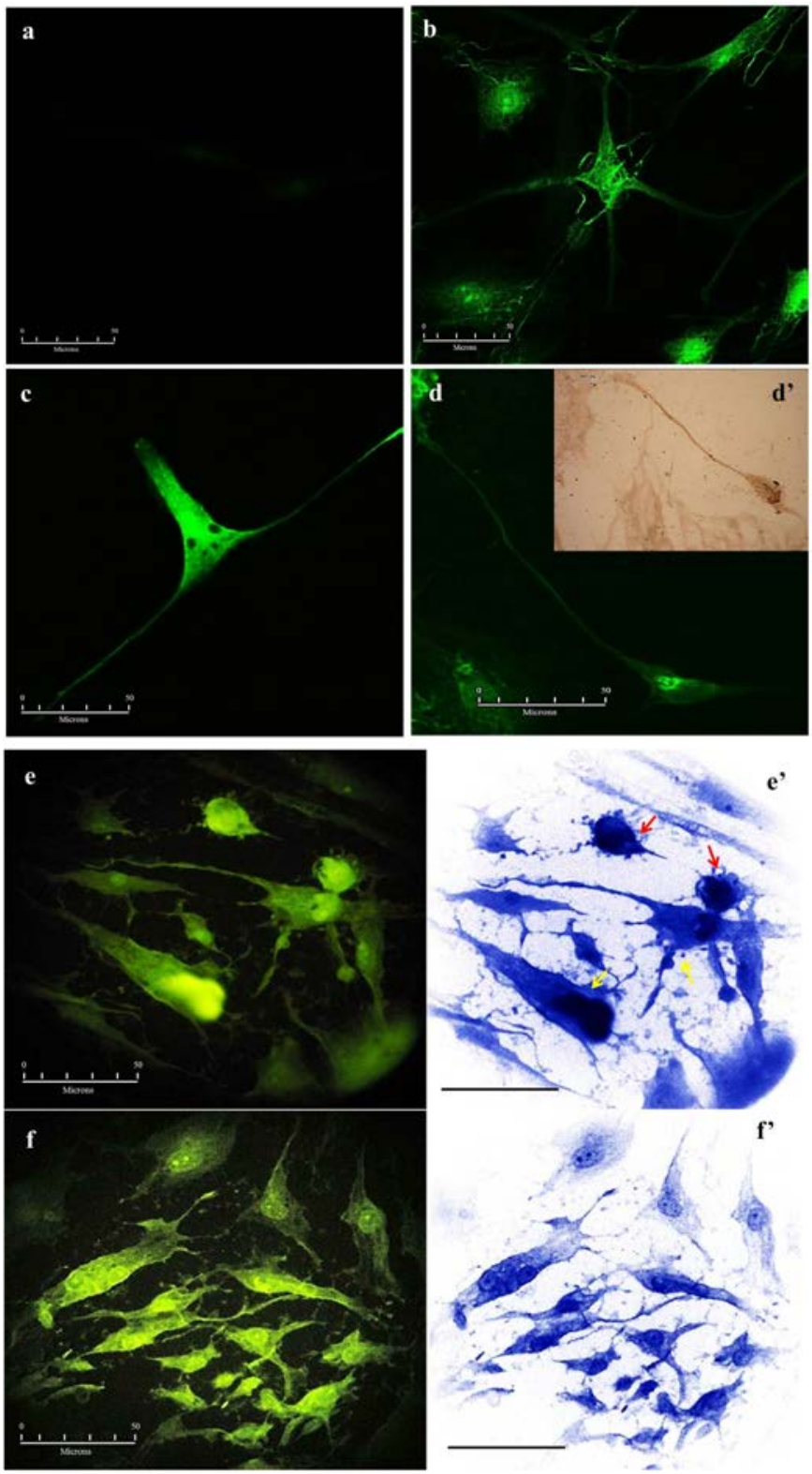

Figure 11. characterization of mature neural-like cells differentiated from BM-MSCs after incubation in culture media supplemented with CSF using GFAP Immunolabelinginvestigated by CLSM: (a)Negative GFAP expression in the undifferentiated BM-MSCs before the differentiation process (0 day)(Scale bar = 50µm).(b)Immunofluorescence image showing positive GFAP immunolabeling for astrocytes within 3 days of incubation,investigated by CLSM(Scale bar = 50µm).(c)Strong GFAP immunolabeling for pseudo-unipolar neuronal-like cell after 5 days, investigated by CLSM(Scale bar = 50µm).(d, d')Mature unipolar neuronal-like cell showing positive GFAP immunoreactive and silver stain, respectively (Scale bar = 50µm).(e, f) Immunofluorescence image showing mature neuronal and oligodendrocyte cells GFAP immunoreactive (Scale bar = 50µm). (e', f') Inverted phase micrograph of e) and f) showing perineural oligodendrocytes; Oligodendrocyte (red arrow), Neuronal cells (yellow arrow). (Scale bar = 50µm)

3.2.2.2. Astroglial Cells

By using IPCM, astrocyte precursor-like cells were developed within 24 hrs of induction in CSF *(Figure 7a)*, having a polygonal cell body with short processes. More differentiated astrocglial showed within 3 days of incubation *(Figure 6e)*, as well as mature astrocyte differentiated within 5 days. Mature astrocyte appeared as star-like structure cell body with branching radiating outward dense fibrous, protoplasmic (plasma to fibrous) dendrite and neurite which were characteristically to the morphological feature of astrocytes *(Figure 6e) & (Figure 7b)*. Also, the morphological feature of mature astrocyte, demonstrated by H&E after 5 days, clearly shows a star-like structure with large polygonal cytoplasm and many small processes *(Figure 9c)*.

The special stains methods used in this study to characterize the cyto-morphological features of astrocyte-like cells. After 24 hrs of incubation, neural precursor-like cells showed little PAS' granules, exhibiting weak glycogen reaction. After 5 days of incubation in auto-CSF, the neural precursor cells were differentiated into astrocytes-like cells and fully mature multipolar neuronal-like cells, and astrocytes showed more intense activity of glycogen reactionthan the neurons developed at the same period as seen in Figure 10 *i, l*.

The GFAP astroglial lineage marker was verified the presence of cultured astrocytes using anti-GFAP antibodies. Star-like structure astrocyte was depicted by the GFAP labelling after 3 days of incubation *(Figure 11 b)* and strong reaction has been shown within 5 days of incubation.

3.2.2.3. Oligidendroglial cells

Within 2-4 days of cultivation, by IPCM we demonstrated the early formation of connections between branched oligodendrocyte progenitor cell attached with oligodendrocyte-type-2 astrocyte (rO-2A) progenitor cells *(Figure 5 a, b, c)* undergoing a morphological transition rapidly to pro-oligodendrocytes cells which is essentially restricted to the oligodendroglial cell lineage. After 8 days of incubation, proliferative pro-oligodendrocyte cells converted into mature oligodendrocytes appeared as flattened cell body with granular cytoplasm intensively producing myelin component and extending sheaths. Mature oligodendrocytes were able to recognize its neighboring neurons and wrap their target axons for myelin production *(Figure 8)*.

Immunofluorescence technique demonstrated positive mature (perineural) oligodendrocyte cells GFAP immunoreactive within 5 days of incubation *(Figure 11 e, f)*.

4. Discussion

BM-MSCs have an important role in tissue engineering and regenerative medicine due to their immunogenicity, for the ability to perform immunomodulatory and anti-inflammatory functions, for the lack of tumorogenicity, and certainly are greatly granted for the ability to secrete multiple bioactive molecules able of stimulating recovery of injured cells [53]. Therefore, many attempts to treat neurological disorders using the strategy of cell transplantation performed mainly using BM-MSCs techniques [38]. *Li et al (2007)* [39] and *Yaghoobi et al (2008)* [40], indicated that transplantation of the undifferentiated MSCs may have better repair capabilities than differentiated cells. On the other hand, *Chen et al (2006)* [41] suggested that the manipulation of the MSCs *in vitro* before transplantation either by pre-differentiation or by fully differentiation, have significant purpose. Pre-differentiation of BM-MSCs directed into more restricted neural cell type, could enhance the survival rate of the transplanted cells and significantly increase the development of mature neurons, and improve its ability to anatomically and functionally integrate into particular part of CNS lesion [29]. In addition, induction of stem cells to differentiate into neural precursor cells may help to regulate the differentiation of transplanted cells in a damaged environment and these cells have lower chance of malignant transformation compared with actively dividing precursors [42]. According to the suggestion declared by *Chen et al (2006)* [41], in the present investigation we manipulated the undifferentiated BM-MSCs *in vitro*, and directed them to differentiate into neural-like cells. In this regard, *in vitro* differentiation of BM-MSCs towards neural cells had been verified with a variety of neural differentiation protocols including chemical inducers [12], cytokines and growth factors [13,14,15], co-culture with neurons or glia [13,16,17], chemical inducers plus cytokines [10,18], and special supplements plus cytokines [19].

Because of CSF directly contacted the stem cell niches of the nervous systems, its proteomic composition suggested to haveall the secretory factors, growth factors, cytokines, extracellular matrixproteins, adhesion molecules and many other materials and nutrients. These components sufficient to maintain neural stem cells survival and regulate proliferation and differentiation of the progenitor cells in to mature cells [52]. So that we hypnotized in our study that adding CSF to the supplemented culture media may provide a better micro-environment for inducingthe transdifferentiate of BM-MSCs and for their into cells with all neural phenotype (neurons, astrocytes, oligodendrocytes) imitating that occurred in the ventrical regions in the CNS.

The present study was assessed firstly by characterizing the undifferentiated BM-MSCs morphologically using IPCM, H&E stained cells examined with light microscope and SEM. In addition using immunocytochemical techniques to investigate the expression of the surface marker CD146 and examined by CLSM. It was observed from this present study that MSCs appeared as population of plastic adherent, highly proliferative cells and able to form colonies according to theory of *Friedenstein et al (1987)* [43]. Also, the resulted BM-MSC colonies had three cells phenotypes: spindle shaped cells were the most abundant, large flattened cells and star shaped cells. The present results were in agreement with many previous studies. *Muraglia et al (2000)* [44] investigated the morphological features of the undifferentiated BM-MSCs isolated from iliac crest of healthy donors by using IPCM andconfirmed by study of *Scintu et al (2006) [33]* by using SEM. Also, the study of *Bahadori et al (2012) [30]* reveled the cellular and microanatomical features of the undifferentiated BM-MSCs harvested from female rats as seen by H&E stains. Numerous investigations were done by *Boxall and Jones (2012)* [47] and *Rasini et al (2013)*

[48] were designed for achieving the immunophenotypic features of the MSCs via identifying their surface markers. In the current approach we used the surface marker CD146 that show positive immunoreactivity for BM-MSCs after 24 hrs of incubation in the supplemented culture media and examined by CLSM. The present results were in agreement with *Sacchetti et al (2007)* [45] who demonstrated high levels of CD146 expression in clonogenic cell population derived from human bone marrow, and *Tormin et al (2011)* [46] who considered that the expression of CD 146 is a classic marker of BM-MSCs**.**

Secondly, the current study was assessed by investigating the morphological features of the differentiated neural progenitor-like cells that will directed into any of the three major neural cell lineages, specifically neurons, astrocytes and oligodendrocytes, using IPCM, H&E stained cells examined with light microscope and finally with SEM. The techniques used in the present study revealed that adding auto-CSF to the culture media promote the proliferation and differentiation of multipolar or bipolar neuronal progenitor-like cells which they restricted into mature multipolar or bipolar neurons. Moreover, neural progenitor-like cells mixed with oligodendrocyte progenitor cells (OPC) and oligodendrocyte-type-2 astrocyte progenitor cells (O2-A) which they restricted into astrocytes and oligodendrocytes. Our findings confirm the *in vivo* study done by *Lehtinen et al (2011)* [52] who demonstrated that CSF having proliferation-inducing factors may be important for promoting proliferation and differentiation of neural progenitor cells in the ventrical zone in the CNS.

Furthermore, CSF initiated significant morphological changes leading to the cells' transformation from flat or spindle-shaped–characteristic for BM MSCs- into semi-commitment transitional cells appeared as pyramidal or cone-like structures with long processes that suggested beingneuronal precursor-like cells. Also, they were transformed into spherical or polygonal shaped with several small branches, and supposed to be astrocyte precursor-like cell. The neuronal precursor cells and the astrocyte precursor cells were completely differentiated into mature neuronal and astrocyte cells within 5 to 7 days of incubation in auto-CSF. Mature oligodendrocytes were differentiated after 8 days of incubation whichappeared as flattened cell bodywith granular cytoplasm intensively producing myelin component could efficiently enwrap multiple axons.Phase contrast analysis gave evidence of axonal myelin shapes, with some loosely wrapped layers and protruding processes. Studies on the mature oligodendrocytes, differentiated from BM-MSCs,which have ability in remyelination might be a promising approach for repairing the injured CNS tissues also in other animals as a new coming strategy for function recovery of damaged nerves.

The current results were similar to the previous results of *Scintu et al (2006)* [33] who used SEM, to investigate the morphological features of the neural-like cells differentiated from cultured BM-MSCs isolated from ten healthy donors and *Ye et al (2011)* [29] who used similar neural differentiation protocols and examined with IPCM. Another study was made by *Bahadori et al (2012)* [30] investigated the neural-like cells differentiated from cultured BM-MSCs harvested from female rats by H&E stains. The cells stained with cresyl violet stain "Nissl stain" and silver impregnation, which used to study detailed neuronal morphology together with cytoarchitecture showed that the undifferentiated cells were colorless, while many neural precursor-like cells were revealed within 2 to 3 days. The intensity of the both reactions were moderate in the semi-differentiated cells within 5 days of induction and the completely differentiated neural-like cells showed the strongest reactions after 7 and 9 days of incubation in auto-CSF. Within 9 to 14 days of incubation the neurofilament will be completely evolved in and performed as a fibrous neuronal network with black silver depiction, also shown by SEM. The Nissl stain directed primarily to the Nissl substances located in the cytoplasm of the cell body, besides Bielschowsky's silver impregnation method had been used alongside the Nissl stain to place labeled both cytoplasm and cytoarchitecture of the cells, where the soma counterstained with violet color and neurites appeared clearly with dark brown or black color. The present results were in agreement with *Pilati et al (2008)* [34] who used both cresyl violet stain and silver impregnation method to reveal the cytoplasm and cytoarchitecture of the neural cells in the nervous tissue of rats' brain. *Spoerri et al (1985)* [49] used the silver impregnation method for visualizing cultured neuronal cells isolated from chicken cerebellum. They observed that the cell bodies and processes of the neurons appeared dark characteristic for neurons while the glial cells appeared light brown, indicating that silver impregnation method was characteristic for neurons. Many previous studies done by *Lovatt et al (2007)* [50] and *Brunet et al (2010)* [51] suggested that the investigation of glycogen metabolism may be a useful marker for identifying mature astrocytes. Moreover, glycogen metabolism seems to represent an exquisite and sensitive marker to assess the degree of astrocytic differentiation. In this context, the present work represented that cultivated cells within 2 to 3 days revealed with faint glycogen content, and this content elevated after 5 and 7 days of incubation to be strong concentration. Furthermore, it was demonstrated from the current result that the astrocyte-like cells revealed more glycogen concentration reaction than neuronal cells because glycogen which is the major energy reserve in brain, where localized almost totally in astrocytes. The present results were in agreement with the study that done by *Brunet et al (2010)* [36], who demonstrated the glycogen by PAS in cultured astrocytes.

From the observations of the current results that confirmed by immunocytochemical techniques and examined by CLSM, it was found that the cells showed positive expression for GFAP which considered being specifically to the neural cells especially the glial cells. The undifferentiated BM-MSCs in 0 day represented negative GFAP expression, thus they showed negative neural phenotype, while represented positive expression to the CD146 marker. The neural precursor-like cells within 2-3 days showed moderately positive immunoreactivity, while immunolabeling of the astrocytes and oligodendrocyte expressed with GFAP within 5 days of incubation showed strong immunoreactivity. These present results were similar to the results of the previous studies illustrated by *Hermann et al (2006)* [10], *Scintu et al (2006)* [33] *and Ye et al (2011)* [29] who used similar

neural marker to confirm their resulted neural-like cells differentiated from BM-MSCs.

5. Conclusion

It was concluded that CSF induced BM-MSCs to transdifferentiate into neuroectodermal cells including neural cells not only neuronal cells but also glial cells (astrocytes and Oligodendrocytes). The induction of BM-MSCs with CSF mimics the natural strategy done in the CNS *in vivo*. For that reason, CSF provided an essential niche for promoting the transdifferentiation of BM-MSCs *in vitro*. Transplantation of these cells could hopefully help in treating acute and chronic neurodegenerative diseases.

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