Induction of mesenchymal stem cells into neuronal cells via two formulas

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Abstract
Mesenchymal stem cells (MSCs) are appropriate source of multipotent stem cells that are valuble source for cell-based therapies. They can be differentiated into neuron cells under appropriate conditions. We aimed to conduct a comparative study to evaluate the best differentiation method of MSCs induction into neural cells. We tested two types of neural differentiation formulas. The first formula was butylated hydroxyanisole (BHA), and the second formula was β-mercaptoethanol (BME). This study was done by using three different neural markers: nestin (NES) as immaturation stage marker, neurofilament light- chain (NF-L) as an early neural marker, and microtubule association protein (MAP-2) as maturation marker. These markers represented the different neurogenesis stages starting from mesenchymal stem cells (as undifferentiated cells), neural stem cells production stages, and neuron cells (as differentiated cells).

The results of immunocytochemistry analysis of both BME and BHA methods showed that both methods were successful in produced neuron cells in culture with a different efficiency. However, the best formula used was BME which induces fast neural differentiation. There was upregulation to NES protein significantly within first hour of exposure compared with BHA method which needs longer time. NF-L upregulated significantly later was compared to undifferentiated MSCs. The neural maturation marker MAP-2 expression increased in BME method with 29h while it needs 144h for BHA method. The results suggest using BME method as preferable neurogenesis induction method in regenerative medicine applications.

Keywords: Comparative study, stem cells, neuron cells, NES, NF-L, MAP-2.

Introduction

Stem cell technologies have attracted many investigators in the past two decades. The progress in embryology, hematology, neurology, skeletal biology and many other disciplines in stem cells research has led to a complete understanding of these cells. It is centered on the isolation, characterization, differentiation of *in vitro* and *in vivo* studies and therefore uses it in the treatment of a variety of diseases[24,30,47]. The stem cells therapies are potential clinical treatment for neural regenerative diseases. Although these therapies are attractive option to reverse neural tissue damage and to recover neurological deficits, it is a limited treatment because the conditions of the nervous system cannot be completely recovered after treatment[12,46].

Also, these therapies are still under development so as not to show significant treatment effects in clinical settings[40]. Therefore, for improving the treatment of neurodegenerative therapy, differentiated cells from mesenchymal stem cells (MSCs) sources have been investigated throughout the past two decades for use as an alternative source than undifferentiated stem cells [5]. Many research demonstrates that the MSCs (as one of the primary sources of stem cells) can be differentiated into different neural cells, and then used in clinical treatments[4,27,33,35,45,46,48,50].

These differentiated cells can be used in treatments and generate cells for transplantation therapies in number of central nervous system (CNS) injuries and neurodegenerative diseases (as cell-based therapies) in Iraq and many others countries, which can replace these lost cells or repair their damaged areas, therefore provide functional recovery[15,20]. Until now, many protocols induced neural differentiation of MSCs *in vitro* [37]. It was about isolation, culture conditions, and molecular characterization, but in general the results of these studies lack consistency and comparability. Defining the best formulas used for neural induction and also defining the exact time of neural expression during the differentiation of MSCs towards mature neuron which will be useful to use in neural disease. For all the above reasons the primary objective of this study is to select the best differentiation formula that induces neural cells from bone marrow MSCs.

Material and Methods

Induction of mesenchymal stem cells in culture: Bone marrow cells were prepared and maintained as described by Freshney[13]. In the animal house, donor male mice were killed by cervical dislocation (3- 6 weeks old, the protocol agreed by the ICCMGR animal care and use committee). The fur sterilized using 70% alcohol and the femurs and tibias were released and transported into a Petri dish containing serum free media MEM (Minimum Essential medium) (US Biological, Massachusetts USA) supplemented with 500µg/ml streptomycin and ampicillin (Capricorn-scientific, Germany)[1]. In the laboratory, under

sterilized conditions, the femurs and tibias were clean off from remaining muscle tissues (with sterile surgical tools) and washed for three times using Phosphate Buffer Saline (PBS), then hold the femur with forceps and cut both ends to flush out the marrow cells.

The marrow was dispersed to a suspension by pipetting. Lastly, the cell suspension was dispensed into 25-cm^2 tissue culture flasks (Thermofisher scientific, USA) and incubated at 37˚c in humidified 95% air and 5% CO_2. The cells were allowed to adhere, and non-adherent cells were washed out (with serum-free medium) after 24h. The nonadherent cells were removed by changing culture medium each 2-3 day until the cultures were getting hold of developing colonies of adherent cells (about 5-7 days) to formed monolayer's cells. After that, cells were recovered after getting monolayer cells using 0.25% trypsin-EDTA (US Biological, Massachusetts USA). The passage one (P1) cells began to proliferate and formed a monolayer after 3-5 days [19].

Protein detection of MSCs in culture: The MSCs were seeded in 8-well tissue culture chamber slide (IWKA, Japan). The cells were allowed to developing a monolayer for phenotyping using immunocytochemistry assay. The cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, washed with PBS and left to dry; the cells were stained with five specific markers immunophenotypic analysis. The markers tested were: CD90 (1:100; Mouse anti-human antibody, US biological, USA, C2441-06), CD105 (1:100; Mouse anti-human antibody, US biological, USA, C2446-50B), CD44 (1:100; Rat anti-mouse antibody, US biological, USA, C2398-01T), CD34 (1:100; Goat anti-human antibody, Santa Cruz Biotechnology, SC-7045), and Nestin (1:50; Mouse anti-rat antibody, Santa Cruz Biotechnology, Europe, SC-58813). Mesenchymal stem cells were blocked in a humidified chamber with 1% hydrogen peroxide (H_2O_2) for 10 minutes and then incubated with 1.5% blocking serum for 30 minutes at room temperature (the kit from ImmunoCruz mouse ABC Staining System, SC-2017, Santa Cruz Biotechnology, Europe).

The primary antibodies were incubated overnight at 4ºc in a humidified chamber. After extensively washed with PBS, the biotinylated secondary antibody (anti-mouse for all CD markers except in CD34 anti-goat) was incubated for 30 minutes, washed 2-3 times with PBS. The avidin was conjugated to horseradish peroxidase (HRP) and added for each slide and incubated in a humidified chamber for another 30 minutes, then washed 2-3 times with PBS. For visualizing the peroxide, liquid DAB chromogen solution mix was added for each slide for 20 minutes at room temperature, washed extensively with PBS and counterstained in Hematoxylin stain for 30-60 second. The slides were mounted with DPX and then were inspected by using a light microscope and photographed by using a digital camera [2].

Viable Cell Counting: The cells were counted to study the differentiation efficiency before and after induction. Viable cell counting on studied cells was accomplished using Trypan blue exclusion. Dead cells, unlike viable cells, took up the dye within a second making them easily distinguishable from viable cells under the microscope. The protocol was done according to Janke et al[22]. One part of the cell suspension (0.2 ml) was added to one part on trypan blue stain (0.2ml) and add eight parts (1.6 ml) PBS to complete the final volume (2 ml). Then 20 µl of sample was transferred to the edge of the cover - slip, running into the counting chamber. Finally, the cells were counted in the four 1mm center squares which included a separated count of viable and non-viable cells, this counting was done by using a light microscope under 40X objective.

The observation was done by using a light microscope under 40X objective. The cell concentration (cell/ml), cell viability (%), and total cell count were calculated from the following equation[11,14]:

C= n x d x l0000

where C= Cell concentration (cell/ml), n= number of counted cells, d= dilution factor diluted by with free serum media = l0). Total Cell Count = C (cell/ml) x the original volume of fluid from which the cell sample was removed.

Neural induction in culture: Subconfluent cultures of P1 MSCs were used to induced neural differentiation by using two different differentiation formulas as follows:

Butylated hydroxyanisole (BHA): Santa Cruz Biotechnology, USA was modified[54] for 24, 25, 27, 29, 48, 96, and 144h. These exposure times were divided as following: pretreated in MEM media with 20% FBS and ten ng/ml bFGF (USbiological, USA) as preinduction media for 24h exposure. Then another exposure was used (of these cells as another formula) to induce neuronal differentiation (as post induction media) by using MEM media without FBS (serum free media), 2% DMSO (Santa Cruz Biotechnology (USA)), and 200 µm BHA (Santa Cruz Biotechnology, USA) for 1, 3, 5, 24, 72, and 120 hrs. as modified from Kalcheva et al[23].

β mercaptoethanol (BME): Santa Cruz Biotechnology, Europe, USA for 1, 6, 12, 18, 24, 25, 27, 29 and 34 hrs. exposure times was used; these exposure times were divided as follows: pretreated in MEM media with 20% FBS and 1 mM BME as preinduction media for different exposure times (1, 6, 12, 18, and reaching to 24 hrs. exposure). Then another exposure was used (of these cells as another formula) to induce neuronal differentiation (as post induction media) for all these exposure times by using MEM media without FBS (serum free media), and 5 nM BME for different exposure time (1, 3, 5, and 10 hrs.) as modified from Li[32].

Morphological Study by H and E Staining: The undifferentiating (MSCs) and differentiating cells (neural

cells) for each differentiation methods BME (for 24, 25, 27, 29 and 34 h exposure time) and BHA (for 25, 27, 29, 48, 96 and 144 h exposure time) were used for morphological study using Hematoxylin as nuclear stain (SYRBIO, Syria) and Eosin as cytoplasmic stain (SYRBIO, Syria) as modified by Lim et al[33] as the follows: The MSCs (P1) were cultured in multi-well tissue culture plates (8-wells), then the differentiation was induced in all different exposure time of each BHA and BME differentiation methods compared with MSCs (as control), the slides were washed with PBS after exposure.

After that, the slides were stained with Hematoxylin for 5 minutes, and washed with distilled water for 2 minutes. After that the slides were stained with Eosin for 2.5 minutes, dehydrated with ethanol 95% for 1 minute and ethanol absolute (100%) for 4 minutes. Finally, cleared with xylene for 2 minutes, washed with ethanol absolute for 2 minutes, and mounted with DPX. All slides were photographed by light microscope camera.

Protein detection of differentiated cells in culture: The cells were stained for NES (1:50; Mouse anti-rat, Santa Cruz Biotechnology, Europe, SC-58813), and NF-L (1: 100; Mouse anti- porcine, US biological, USA, 031403) as immature neuronal cells markers, and for MAP-2 (1:50; Mouse anti-human, Santa Cruz Biotechnology, SC-74421) as mature neuronal marker. Then stained using staining kit (ImmunoCruz mouse ABC Staining System, Santa Cruz Biotechnology, Europe, SC-2017).

Statistical analysis: All data were statistically analyzed using One Way ANOVA and LSD test in IBM SPSS Statistics Software (version 20) and the difference of means was considered significant at $p<0.05$ for ICC assays (as average percentage means).

Results and Discussion

Induction of Mesenchymal stem cells in culture: Mesenchymal stem cells were cultured after isolation method in tissue culture flasks in MEM medium supplemented with 20% FBS (as culturing and maintenance media) and left for 24 h., then maintained for use in this study. After 24 h. culture, the results showed that only a few cells attached to the plastic culture flasks sparsely and formed adherent cells (which represent MSCs) with the rounded or spherical shape. While the non-adherent cells (which represent erythrocyte cells and another cell type) were discarded by the first medium usually changing after 24 or 48 h. (Fig. 1A). After that, the adherent cells began to proliferate. Moreover, 2-3 days after cultivation, the numerous fibroblasts like-cells could be observed and gradually grow to form small individual colonies displaying as fibroblast-like cells morphology.

In addition to, small round cells can also be seen (Fig.1B). Mesenchymal stem cells are categorized by their capacity to form colonies including spindle-shaped cells deriving from a single cell. The number of cellular colonies with different size has increased. After few days, the cells in large colonies appeared more densely distributed and showed a spindle-like shape, as the growth of cells continued, colonies gradually expanded in size with adjacent ones and interconnected with each other.

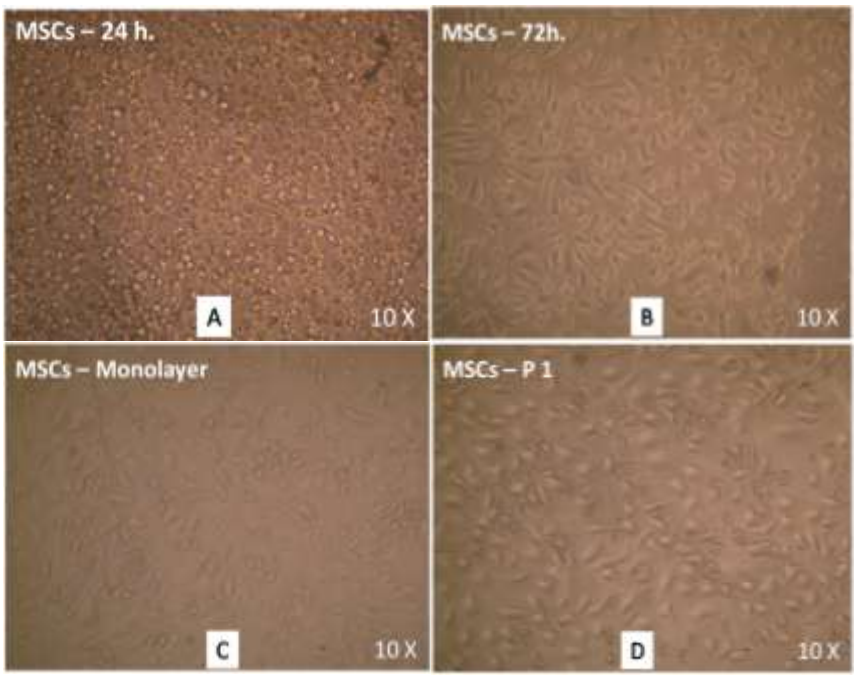


Figure 1: Culture and maintenance of MSCs (A): after 24 h. (B): after 72 h. note that some of cells adherent and began to elongate. (C): after 5-6 days of culturing (monolayer cells as P0), note that the colonies interconnected with each other and reaching a confluent stage. (D): after the first passage (P1), the culture of MSCs as revealed under an inverted microscope for 10 X for each of culture respectively.

Then when the primary cells as passage zero (P0) grow to 70-80% confluence after 5-6 days, they were ready to passage for the first time by sub culturing (Figure 1C). The subculture done was poured out in the media, treated with 0.25% trypsin-EDTA and cell suspension passed into new tissue culture flasks under the same conditions at a split ratio 1:2 for the first passage culture. The MSCs began to grow and formed colonies then expanded, and after few days from P1, homogeneous layer of fibroblastoid-like cells occupied the whole plastic surface (Fig.1D). The cells can be reseeded in same conditions for the second passage culture, and these adherent cells could be readily expanded *in vitro* by successive cycles of trypsinization, seeding, and culture every 5-7 days without visible morphologic alteration.

Viable Cell Counting: To study the differentiation efficiency before and after differentiation (for the different exposure times used in this study), the cell counting and viability were determined in this study. The results showed that the viability of the cells in each primary culture P0, P1, or even P2 was suitable to enter the next experiment with high percentages of viability cells (ranging between 75-90%) and with high cells counting (table 1). Therefore, the MSC number in this study seemed to be enough for *in vitro* study.

Phenotyping detection of MSCs in culture: With the aim of study of the nature of the fibroblast-like cells (MSCs) in culture, the P1 of MSCs was re-cultured in 8-well tissue culture chamber slide and left to grow and expand within 2-3 days to form a homogeneous layer occupying the whole slide surface. Then the culture medium aspirated and the cells were fixed to prepare for labeling against MSCs-related surface antigens which are as follows with their cellular distribution (immunocytochemistry technique) for five markers: CD105, CD90, CD44 as positive markers and CD34 and NES as negative markers.

The results showed that the immunophenotypic characterization of the cultured MSCs stained with CD34 and NES showed negative results through stained whole cells in culture with blue color (from hematoxylin stain) for each cell surface and nucleus, and the identification percentages were 5% and 10.5% for each CD34 and NES respectively (Figure 2 AD). These results indicated that these cells were not from the hematopoietic origin (which represent CD34) or differentiated cells (which represent NES), but they were MSCs (Table 2). Slides that stained positive for CD44, CD90, and CD105 showed brown cytoplasm and with blue nucleus. CD44 expressed in 89% of cells (Figure 3A, B) (Table 2).

Cells stained with CD90 and CD105 antibodies showed dark brown color for each cytoplasm and nucleus in 94% of cells (Figure 3 CF) compared with negative control which takes the blue color only (Figure 2 AD) and table 3. Therefore, these results indicate that they retain the phenotype of MSCs. The results showed that MSCs were negative for CD34 and NES which take the blue color (from Hematoxylin stain), which indicates that these cells are not of hematopoietic origin, but they were MSCs.

Table 1
Cells counting in the experimental study.

Cells type		**Count in 25 cm^3 tissue culture flask**
MSCs (as a primary culture)		8.33×10^6
MSCs (P1)		2.50×10^6
MSCs (P2)		2.54×10^6
Differentiated cells using BME	24 h	1.90×10^6
	25 h	1.40×10^6
	27 h	1.45×10^6
	29 h	0.90×10^6
	34 h	0.73×10^6
Differentiated cells using BHA	24 h	2.05×10^6
	25 h	1.20×10^6
	27 h	1.00×10^6
	29 h	1.00×10^6
	48 h	0.75×10^6
	96 h	0.60×10^6
	144 h	0.35×10^6

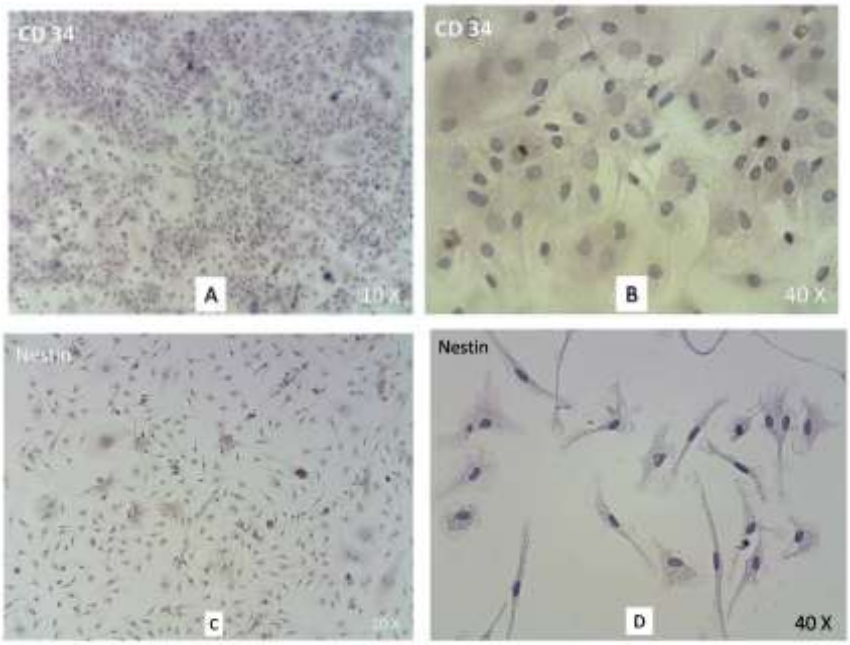


Figure 2: Immunophenotypic analysis of MSCs at the first passage revealed under the light microscope show that the MSCs were negative by cell stained with blue color. (A and B): CD 34. (C and D): Nestin. Note that the two markers showed with 10 and 40X respectively.

Table 2
ICC results for different surface markers of cultured MSCs.

	CD Markers	Identification percentages (%)
Positive markers	CD 44	89.0
	CD 90	95.0
	CD 105	95.0
Negative markers	CD 34	5.0
	NES	10.5

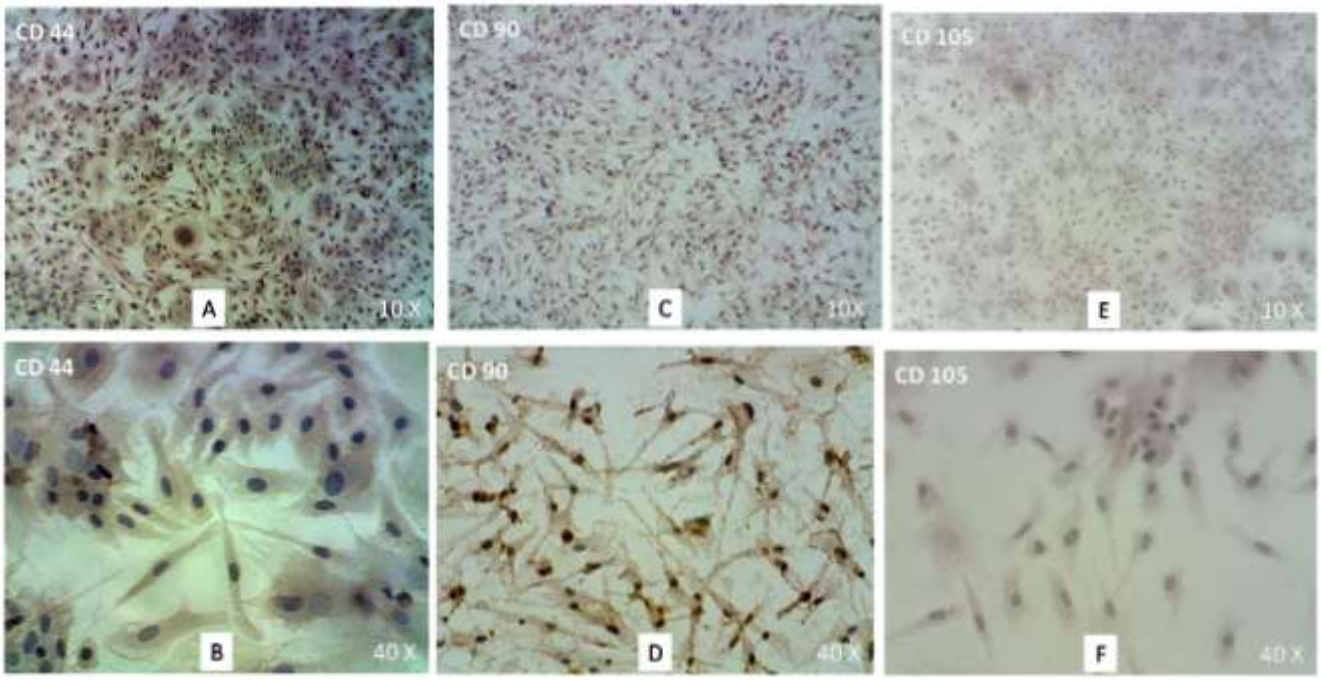


Figure 3: Immunophenotypic analysis of MSCs at the first passage revealed under light microscope show that the MSCs were positive cells which stained with brown color. (A and B): CD44, (C and D): CD90, (E and F) CD105. Note that all CDs showed with 10 and 40X respectively

Neural induction in culture: The differentiation process of stem cells occurred through a tremendous genetic nest by changing their expression levels by up and down of protein regulation starting from stem cells toward neuron cells formation [2]. The results showed that MSCs from mouse bone marrow were successfully induced neural differentiation by using the two different formulas used in this study. As the first differentiation method by BHA, sub confluent cultures of P1 MSCs were pretreated for 24 h in MEM medium with 20% FBS and ten ng/ml bFGF (as preinduction medium). Then neural differentiation (as post induction medium) was induced with MEM medium without FBS (serum-free medium), 2% DMSO, and 200 µm BHA.

The results showed that the MSCs in the induction medium (for 24 h exposure) showed to be more elongated and increased in the proliferation rate (Figure 4 A) compared with undifferentiated MSCs (as negative control); these results proved the proliferation of the stem cells in culture with the present of b-FGF (as proliferation factors) in the preinduction medium. Then, after exposure to BHA with DMSO for different time intervals (as postinduction differentiation medium), the MSCs started to be more elongated and increased in size (including the nucleus and cytoplasmic content) (figure 4 B, C). Then starting from 29 h, the differentiated cells formed cells branched, and then increased the cells branched for each 48 and 96 h exposure. Towards 144 h showed the formation of the cell body, dendrites, and axon as the neuron cells morphology (figure 4 D-G).

As the second differentiation formula, the sub confluent cultures of P1 MSCs were using to induced neural cells differentiation by using BME for different exposure time (1, 6, 12, 18, 24, 25, 27, 29 and 34 h). This method was divided into two-step, pre-treated step in MEM medium with 20% FBS and 1 mM BME for different exposure times (1, 6, 12, 18, and 24 h), then transport these cells in to post-treated step by using MEM medium without FBS and 5 mM BME for different exposure time (1, 3, 5, and 10 h).

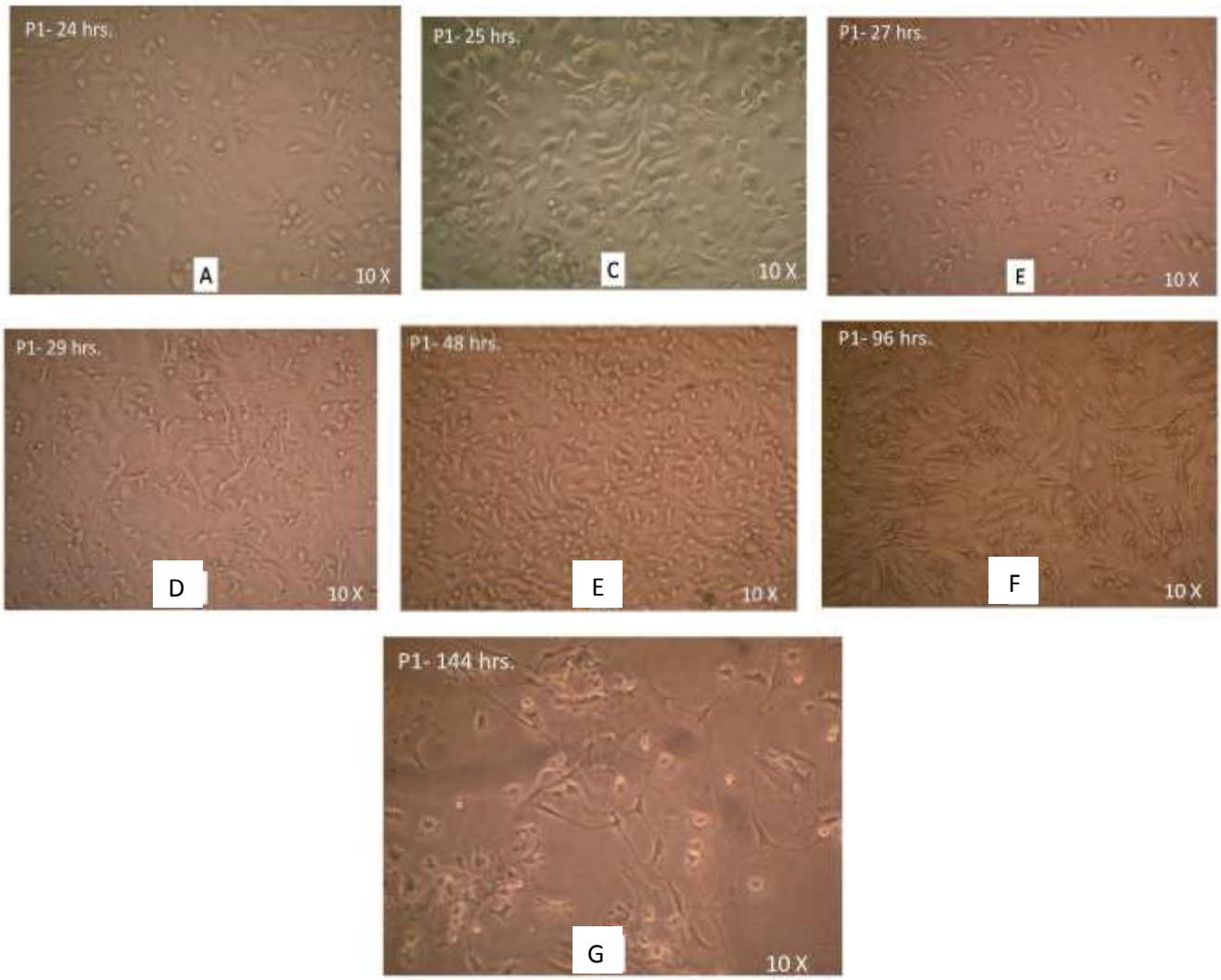


Figure 4: MSCs after induced differentiation by BHA, the pointer arrows were showed the elongation of cells and branched form towards neural cells as revealed under an inverted microscope, all figures showed in 10X, the figures (A-G) presented (24-144 h) exposure time to differentiation medium.

The results of preinduction medium (1, 6, 12, 18, and 24 h exposure time) indicated that the differentiated cells were shown to be more spherical than MSCs, these changes were started from 24 h (Figure 5 A) reaching to the post-induction medium (25, 27, 29, and 34 h). These changes occurred by increase in cells size, forming cells branches, then formed cell body and dendrites towards neuron cells morphology (Figure 5 B-E). The BME as antioxidants reagents is known to influence metabolism and promote cell survival in some cell culture systems. Nevertheless, their effects are on the modulation of bone cell differentiation *in vitro* [18]. Also, it was a successful differentiation method as neural inducer within a few hours, throw their expression of many neural markers through differentiation process[11,19,27,35,43,44,54]. Other studies used BME with other neural inducers as pri-induction reagents such as with retinoic acid [38], with retinoic acid and creatine [9], with b-FGF [54], or with different supplemental reagents [28]. Therefore, the BME was successful in induced neuron cells in this study compared with other formula used in this study.

Morphological Study by H and E Staining: Mesenchymal stem cells and induced neuron cells morphology were studied for BHA differentiation method at specified intervals (24, 25, 27, 29, 48, 96, and 144 h exposure time) by staining with Hematoxylin (as a nuclear stain) and Eosin (as a cytoplasmic stain). The results showed that the MSCs after differentiation started to be more spherical in cell shape with the dark red color of the cells (from Eosin stain) and the nucleus seems to be bigger with dark blue color (from Hematoxylin stain) through the differentiation process (figure 6). Then many cells formed branched as neural cells, and these branched increased in length and formed a nest of cells branched connecting with each other through the different exposure times (figure 6 C-P) compared with the classical shape of MSCs (figure 6 A and B). The undifferentiating (MSCs) and differentiating cells (neural cells by using BME for 24, 25, 27, 29, and 34 h exposure time) were used to study the morphological changing through using Hematoxylin (as a nuclear stain) and Eosin (as a cytoplasmic stain).

The results showed that the MSCs after differentiation started to be more spherical with the dark red color of the cells (from Eosin stain) and the nucleus seems to be bigger with dark blue color (from Hematoxylin stain) through the differentiation process (figure 7). Then many cells formed a branched as neural cells, and these branched increased in length and formed a nest of cells branched that connect with each other through the different exposure time (figure 7 C-L) compared with the classic shape of MSCs (figure 7 A and B).

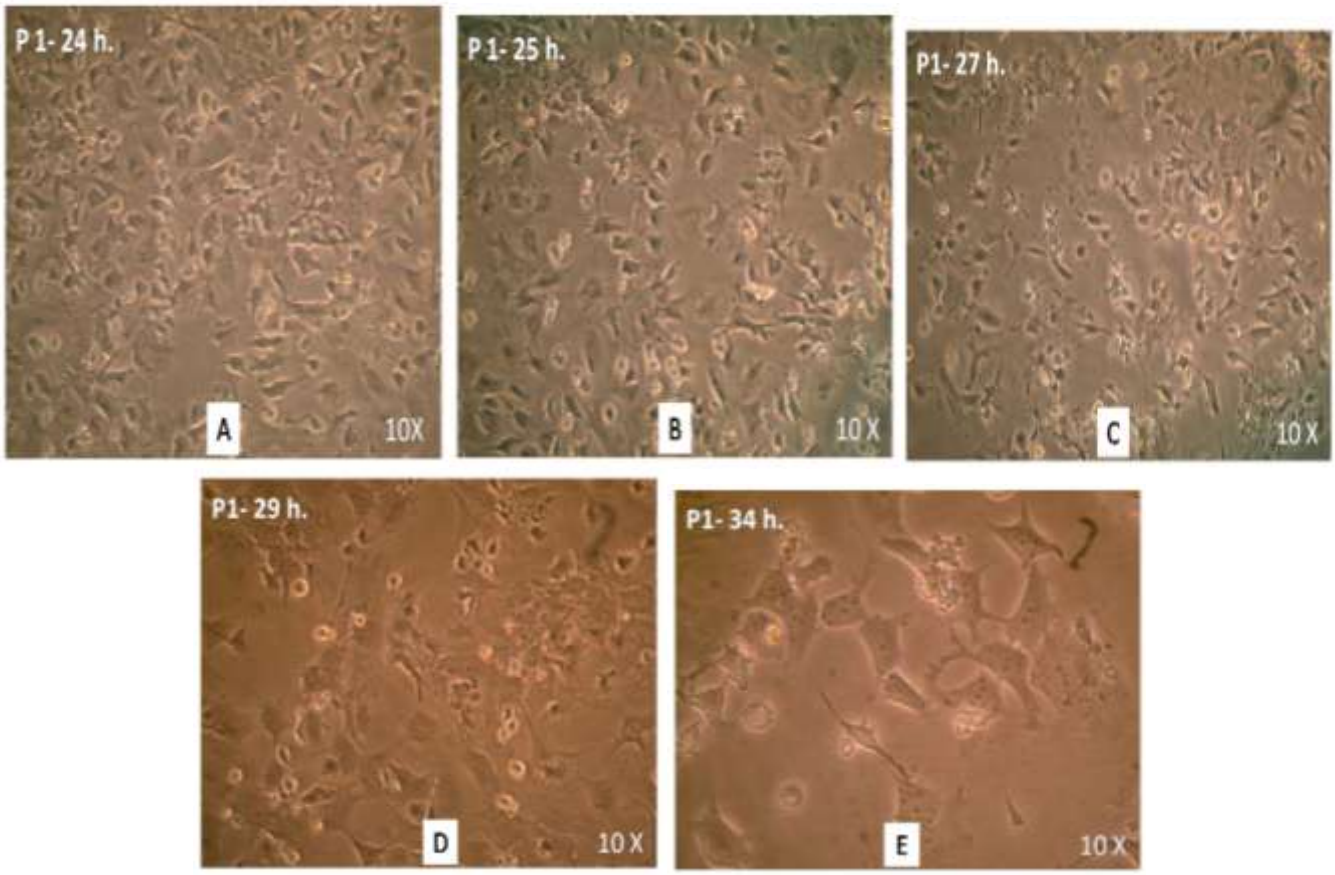


Figure 5: MSCs after induced differentiation by BME, the pointer arrows were showed the spherical shape of cells and branched form towards neural cells as revealed under an inverted microscope; all figures showed in 10X. The figures (A-E) presented (24-34h) exposure time to differentiation medium.

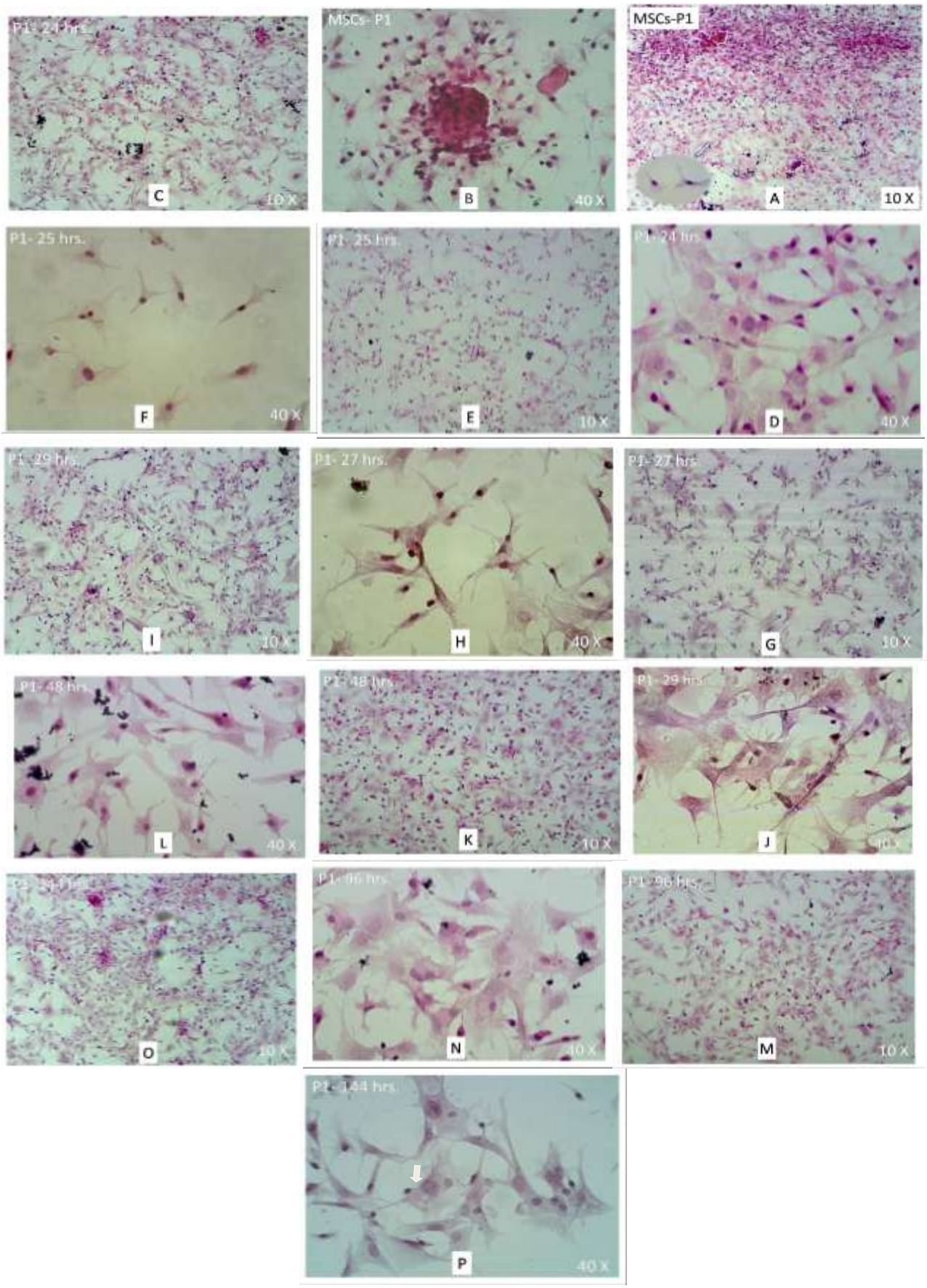


Figure 6: H and E study, the results were showed the differentiation of MSCs by BHA towards neural cells through different exposure times as revealed under light microscope, all figures showed in 10X and 40X respectively. The figures (A-P) presented (MSCs- 144 h) exposure time to differentiation medium.

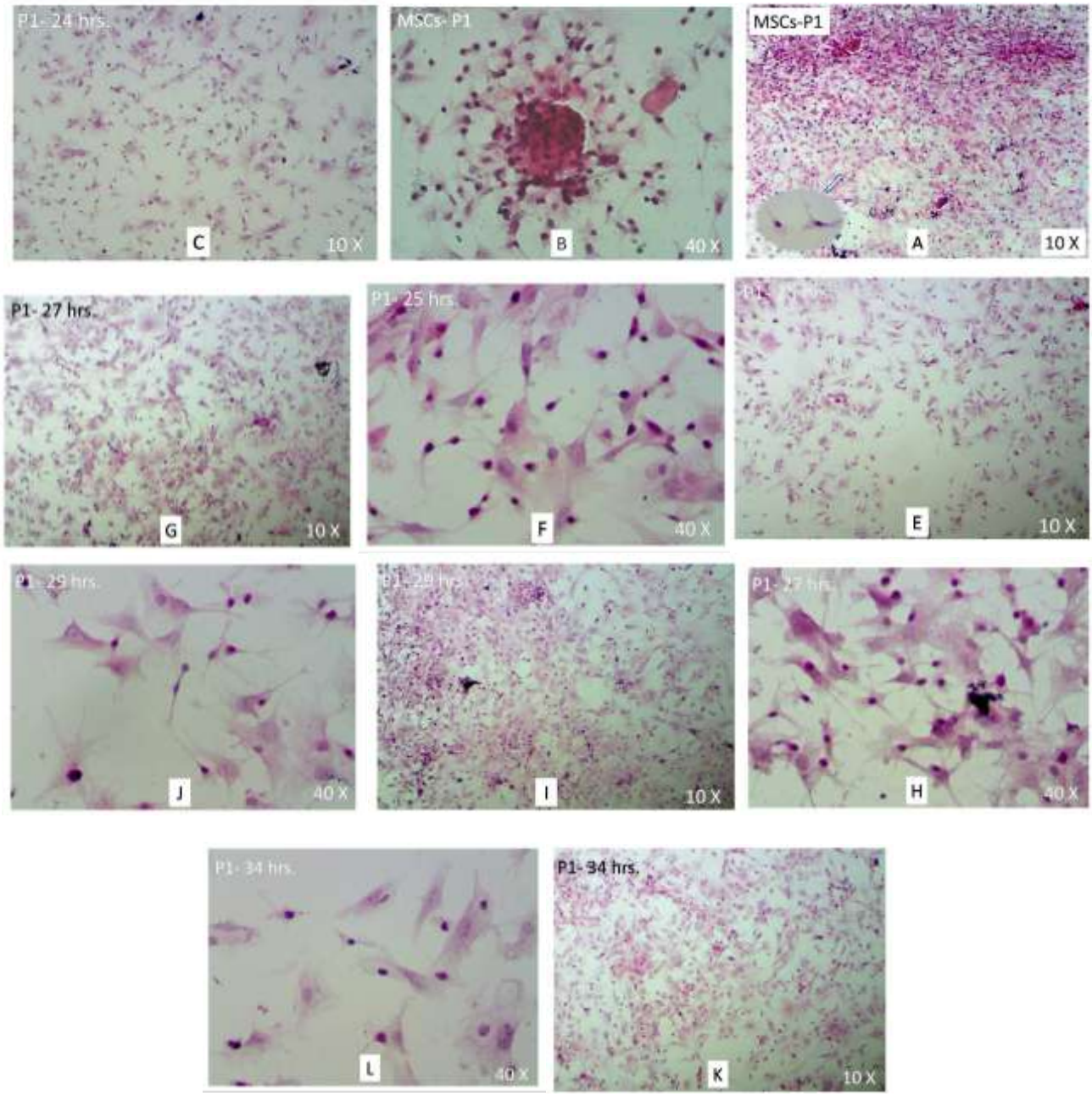


Figure 7: H and E study, the results showed differentiation of MSCs by BME towards neural cells through different exposure time as revealed under light microscope, all figures showed in 10X and 40X respectively. The figures (A-L) presented MSCs-34 h exposure time to differentiation media.

Protein detection of differentiated cells in culture: The MSCs were processed for immunocytochemistry assay which represents undifferentiating MSCs (as negative group) and the neural cells after using BHA for 24, 25, 27, 29, 48, 96 and 144 h exposure time by using three differentiation markers NES as NSCs and immaturation marker, NF-L as an early neural marker, and MAP-2 as maturation neuron marker as showed in figures 8, 9,10 and 11. The ICC results showed that the NES protein founded in the MSCs in deficient levels (10.5%), at the beginning. NES expression was increased significantly ($p<0.05$) through neural differentiation intervals. It started from low (12.3%) at 24h exposure time (as preinduction medium) and continued to increase significantly ($p<0.05$) through different exposure time (25, 27, 29, and 48 h) as postinduction differentiation medium with 45.1, 50.1, 49, and 61.2% respectively. The highest proteins level was at 48h of exposure. Then, NES protein expression was decreased through the next 96 and 144h (with 39.9 and 43.8 % respectively) compared with the undifferentiating MSCs as a negative control as shown in figures 8 and 11.

Nestin is an intermediate filament protein (IFPs) used in this study for evaluation of the two differentiation formulas was expressed in dividing cells during the early stages of development in the CNS, peripheral nervous system, adult brain and in myogenic and other tissues[3,28]. Also, it is expressed by many types of cells during development,

although its expression was usually transient and does not persist into adulthood during differentiation. This gene was down-regulated and replaced by tissue-specific intermediate filament proteins such as the NES protein of NSCs in the adult mammalian forebrain replaced by neurofilament and glial fibrillary acidic protein (GFAP) in each neuron and glial cells[39]. Also during neuro- and gliogenesis processes it was replaced by cell type-specific intermediate filaments neurofilaments NFs and glial fibrillary acidic protein (GFAP) [36].

In the generation of neuron cells from neurosphere culture, nestin expression was down regulated after differentiation compared with neurospheres cells which were expressed in high levels [55]. This research proved the expression of NES protein during the neurogenesis process which matched with this study. Correspondingly, the results showed that NF-L protein levels was shown to be increased significantly ($p<0.05$) started from 25 h exposure time (27.5%) reaching to different exposure time 27, 29, 48, 96, and 144 h (with 31.4, 39.6, 69.8, 73.1, and 86.7% respectively), with higher protein levels at 144 h taking dark brown color compared with MSCs (as negative control) which recorded low protein levels (15.1%) as shown in figure 9 and 11.

Neurofilament protein was one of the essential cytoskeletal proteins of neurons, and it was a biomarker for neurodegeneration [14], the subtype light-chain of the neurofilament group (NF-L) represented the most abundant and also most soluble neurofilaments subunit compared with other subunits [44].

The NF-L subunit was found in significant amount in blood and serum compared with other two type (M and H-chain) and also used as a biofluid source which was easily accessible for longitudinal studies of neurodegenerative neurological diseases [14]. Also, the expression of each class of neurofilaments was changing during different developmental stages. For instance, NF-M and NF-L subunits were expressed early during embryonic neurogenesis while NF-H appears later during the postnatal period in rats[7,29]. Therefore, it was indicated that NF-L was expressed in the earlier stage of neurogenesis and it is a good choice for use in the study of the neurogenesis stages during *in vitro* differentiation.

The results of this study showed very low levels of MAP-2 protein through the differentiation process compared with both NES and NF-L used in this study, which started to increase with significant difference level ($p< 0.05$) starting from 25, 27, 29, 48, 96 and 144 h exposure time (with 13.9, 9, 6.2, 16.4, 21.8 and 23.9% , respectively) with higher protein levels in 144 h compared with no protein levels in MSCs (as negative control) with 0.5% which takes the blue color as shown in figure 10 and 11.

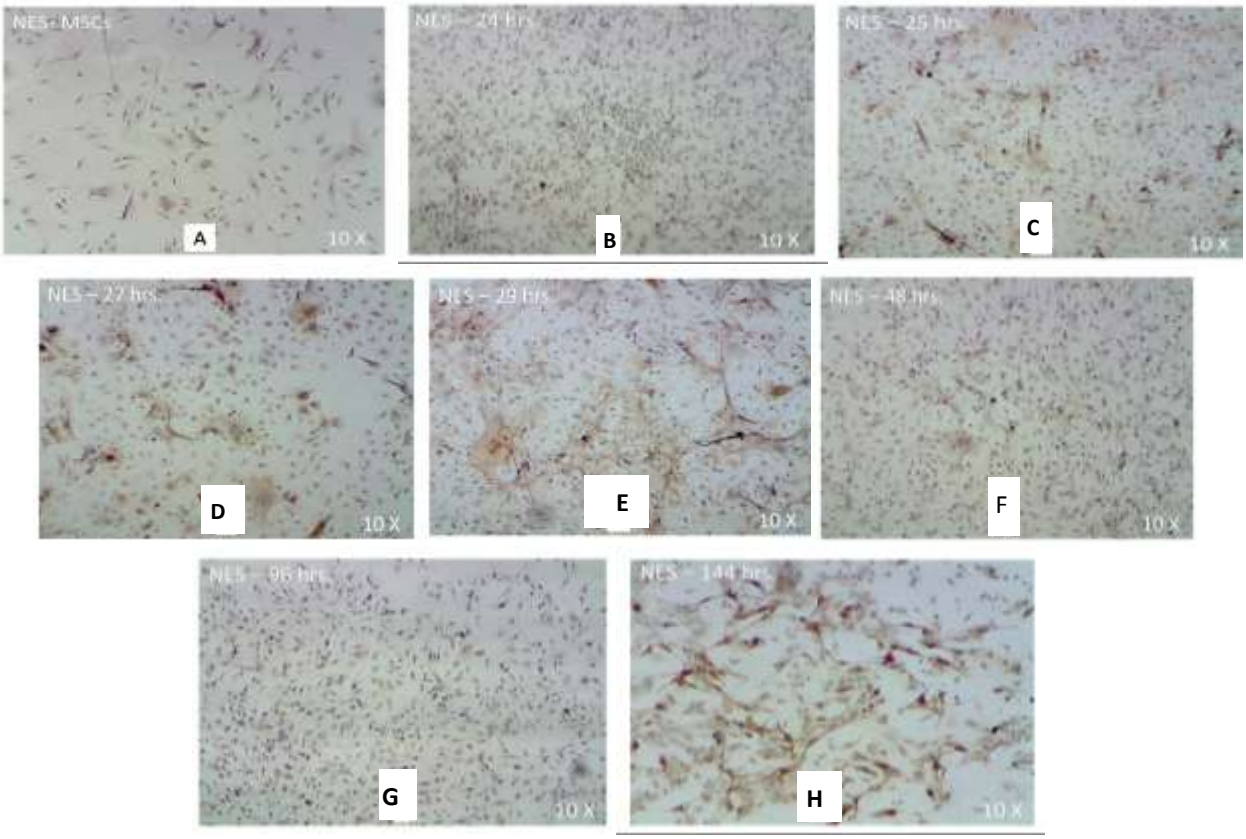


Figure 8: ICC detection results of MSCs after induced differentiation by BHA, the results showed increased of NES protein through different exposure times as revealed under a light microscope, the figures (A-H) represented (MSCs-144 h) exposure time.

Also, the analysis of protein expression of MAP-2 has been used for neural confirmation [23,25]. These mature specific markers played a role in the stability of axons, and neuronal cell bodies throw differentiation process [22]. Therefore maturation markers were absent or with low expression in the early stage of neural differentiation in our study. In general, the MAP-2 gene expression is weak in neural precursors, but it increases during neuron development process. Also, its expression is confined to neurons and reactive astrocytes [6,49].

Therefore, MAP-2 was expressed in the early and immature neural stage but at very low levels, therefore it matched with the results of this study. In general, the ICC results for BHA showed increase in the protein expression levels of each NES and NF-L protein but in low levels started significantly from 25 h and increased through the 27, 29, 48, h with the higher proteins expressions in 48 and 144 h for each NES and NF-L respectively compared with MAP-2 protein which showed less protein expression levels. The ICC results of using BME as the second differentiation formula showed that the NES protein was found in the MSCs (ac control group) in very low levels (10.5%) (in positive results, the cytoplasm of the cell took brown color from the DAP stain). Then its level increased significantly at $p<0.05$ through neural differentiation starting from 24 h exposure time with 61% which displayed the brown color. After that, NES was upregulated significantly ($p<0.05$) through 25 and 27 h exposure time (with 68.5 and 86.3%) with morphological changing of cells towards neural cells, and with the highest protein levels in 27 h, then NES protein was decreased its levels in 29 h with 61.8% significantly ($p<0.05$) compared with control with highly morphological changing towards neural cells as showed in figure 12 and 15.

The results of this study showed that the NF-L protein expression increased significantly ($p<0.05$) through the differentiation process (24, 25, 27, and 29 h) exposure time which recorded 32.2, 65.9, and 80.8% through increase of the dark brown color in the differentiated cells reaching to the highest protein levels in 27 h exposure time which take the dark brown color.

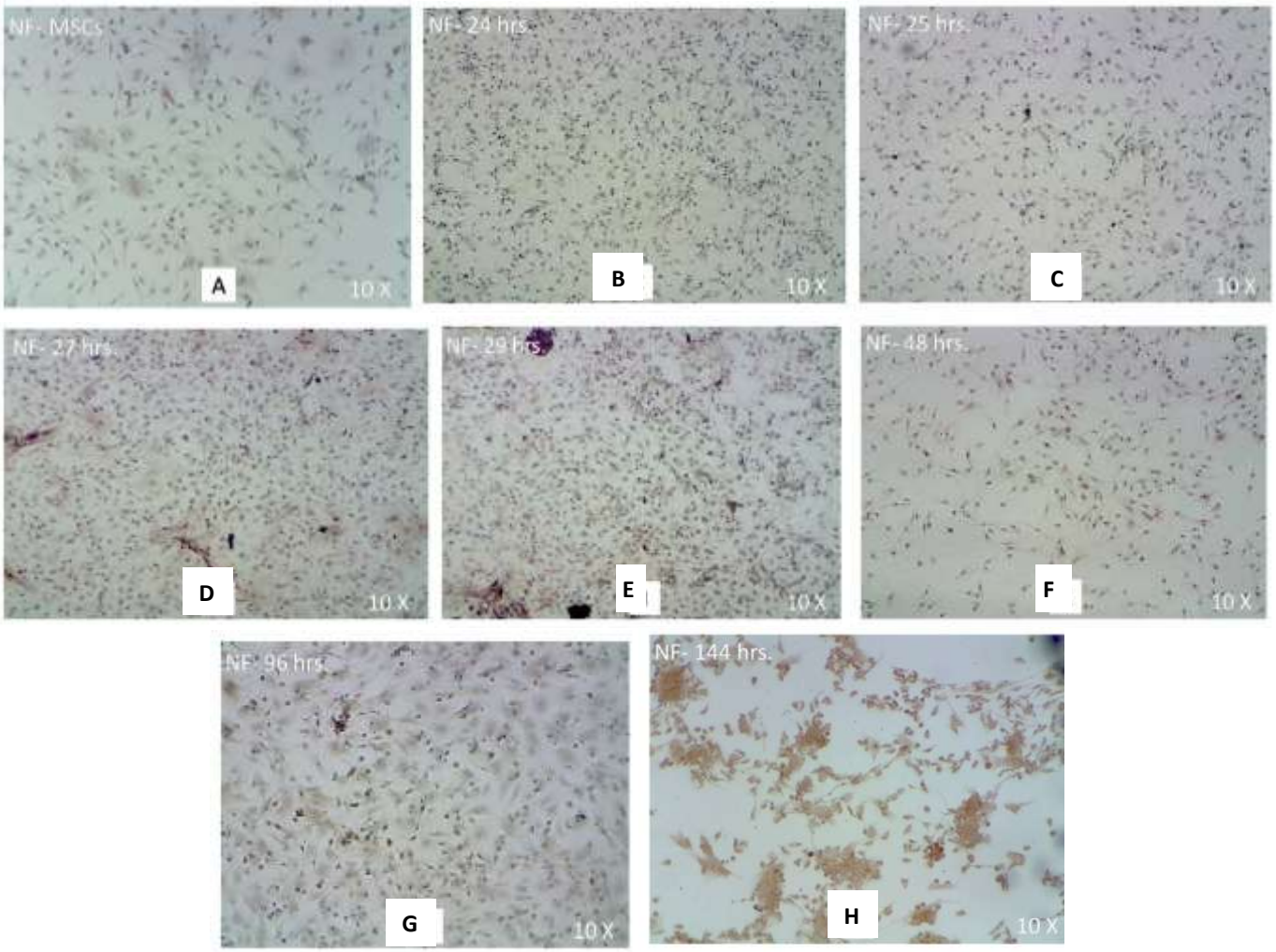


Figure 9: ICC detection results of MSCs after induced differentiation by BHA, the results showed increase of NF-L protein through different exposure times as revealed under a light microscope, the figures (A-H) represented (MSCs-144 h) exposure time.

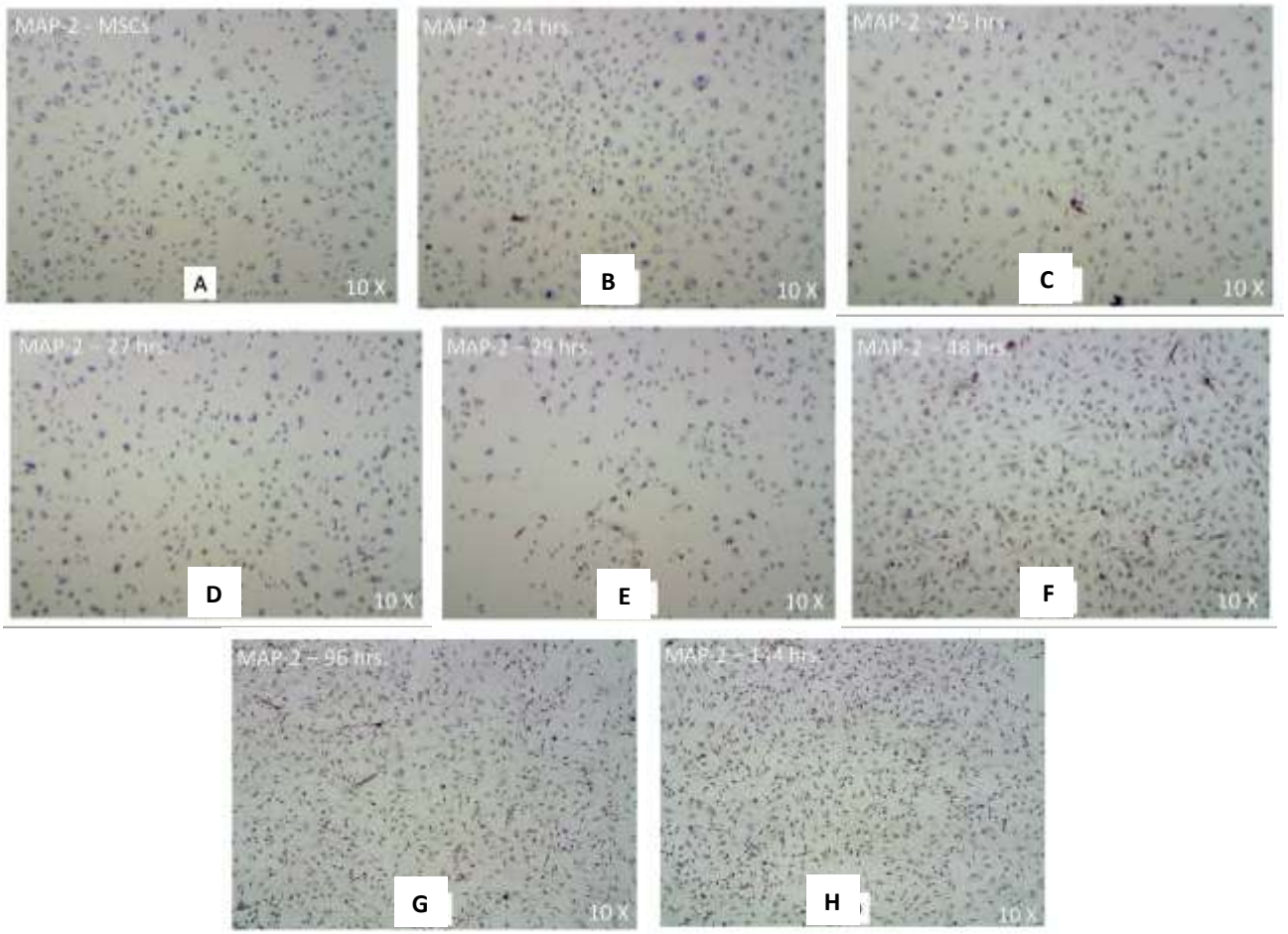
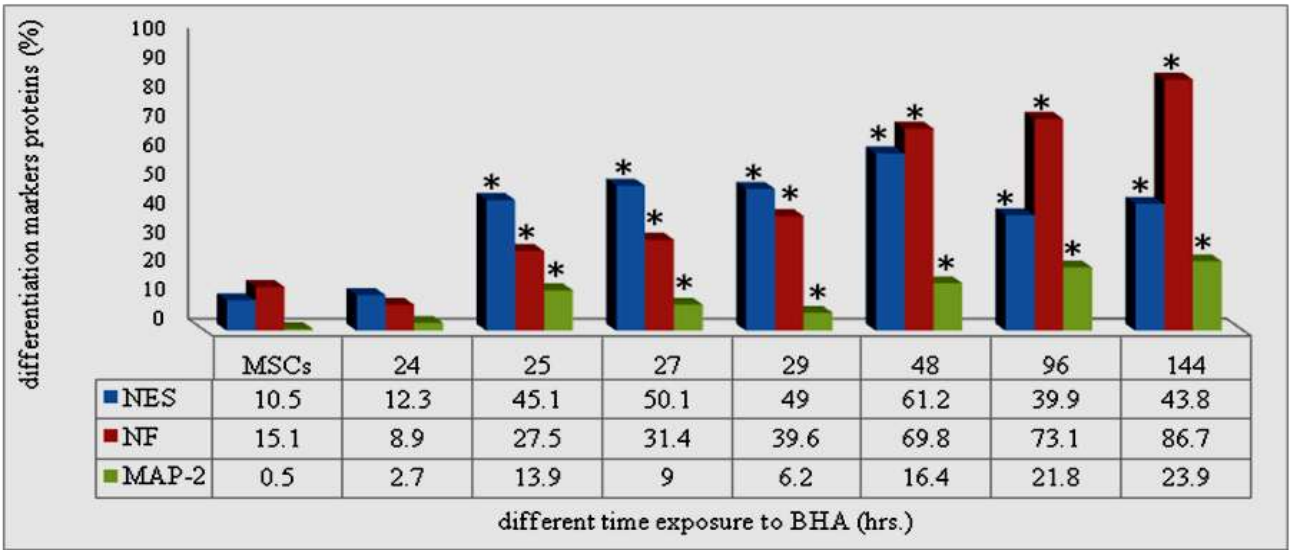


Figure 10: ICC detection results of MSCs after induced differentiation by BHA, the results showed increased of MAP-2 protein through different exposure times as revealed under a light microscope, the figures (A-H) were represented (MSCs-144h) exposure time.



	MSCs	24	25	27	29	48	96	144
NES	10.5	12.3	45.1	50.1	49	61.2	39.9	43.8
NF	15.1	8.9	27.5	31.4	39.6	69.8	73.1	86.7
MAP-2	0.5	2.7	13.9	9	6.2	16.4	21.8	23.9

Figure 11: The proteins expression levels of ICC assay for NES, NF-L, and MAP-2 antibodies of neural cells after induced by BHA.
***refer to significant effect at $p<0.05$.**

Then its level decreased significantly at 29 h exposure at $p<0.05$ with 66.4% compared with low protein levels in MSCs with 15.1% (as negative control) as shown in figures 13 and 15. The results of this study showed very low levels of MAP-2 protein compared with the other two types (NES and NF-L) through the differentiation process, no protein expression with the pre-induction medium and less protein expression levels in post-induction medium were observed.

Then started to be expressed from 27 and 29 h (12 and 29.7%) with significant difference level at $p<0.05$ through the dark brown color compared with no protein levels in MSCs with 0.5% (as negative control) which take the blue color as shown in figures 14 and 15.

In general, the ICC results for BME showed increase in the protein expression levels for each NES and NF-L protein started at 24 hrs. (61% for NES and 32.2% for NF-L). NES was 86.3%, and NF-L was 80.8% compared with undifferentiating MSCs (as a negative control) with 10.5% for NES and 15.1% for NF-L. The MAP-2 proteins showed no protein expression in the pre-induction media. Less protein expression levels were noted in post-induction media which increased starting from 27 and 29h with 12% and 29.7% of protein levels percentages respectively with a significant difference at level $p< 0.05$ compared with undifferentiating MSCs (as a negative control) with 0.5% (as shown in figure 15).

Results indicated the activity of BME in the neurogenesis stages of MSCs towards the neural differentiation cells (by induced the immaturation stage of neural differentiation using BME), throw increase of the nestin protein levels combined with neurofilament levels compared with low protein expression in the MAP-2 protein (as maturation marker) in all exposure time.

The results of this study proved that both NES and NF-L markers used in this study were good choice for evaluating the differentiation formula. Because both markers were involved in the neural induction and fully neuronal differentiation, it threw increase in the protein expression levels of each NES and NF-L protein (as an early neural marker) compared with MAP-2 protein (as late neural markers) which showed less protein expression levels for each BHA and BME which indicated (according to the results above) that these markers worked together respectively and therefore it was a good choice to study the neural differentiation.

Many studies matched with the results of this study through increasing of many neural markers such as NES, NF-L, and in the late stage increasing of MAP-2 after induced neural differentiation by using BHA differentiation formula[11,27,49]. Also, other studies indicated that differentiated and undifferentiated of MSCs also revealed the expression of NES, NF-L, MAP- 2 and many other neural markers, but in low expression levels[21,41,55]. Flow cytometrys, Western blots, and real time-PCR techniques were used in evaluating the plasticity. Multipotency of MSCs in vitro showed that MSCs in culture consistently express native immature neural proteins such as NES and Tuj-1. Then after induced neural differentiation, an increase in the expression was occurring of more mature neural/glial proteins TH, MAP-2, and GFAP and confirming the ability of MSCs to differentiation into neuron and astrocyte cells[51].

Another study demonstrated that NES, MAP-2, and Tub3 were used as markers to investigate neural differentiation in the hippocampus of mice after dental pulp-derived stem/ mesenchymal cells implantation which expressed of NES marker first, then MAP-2 and Tub3 after five days of differentiation[16].

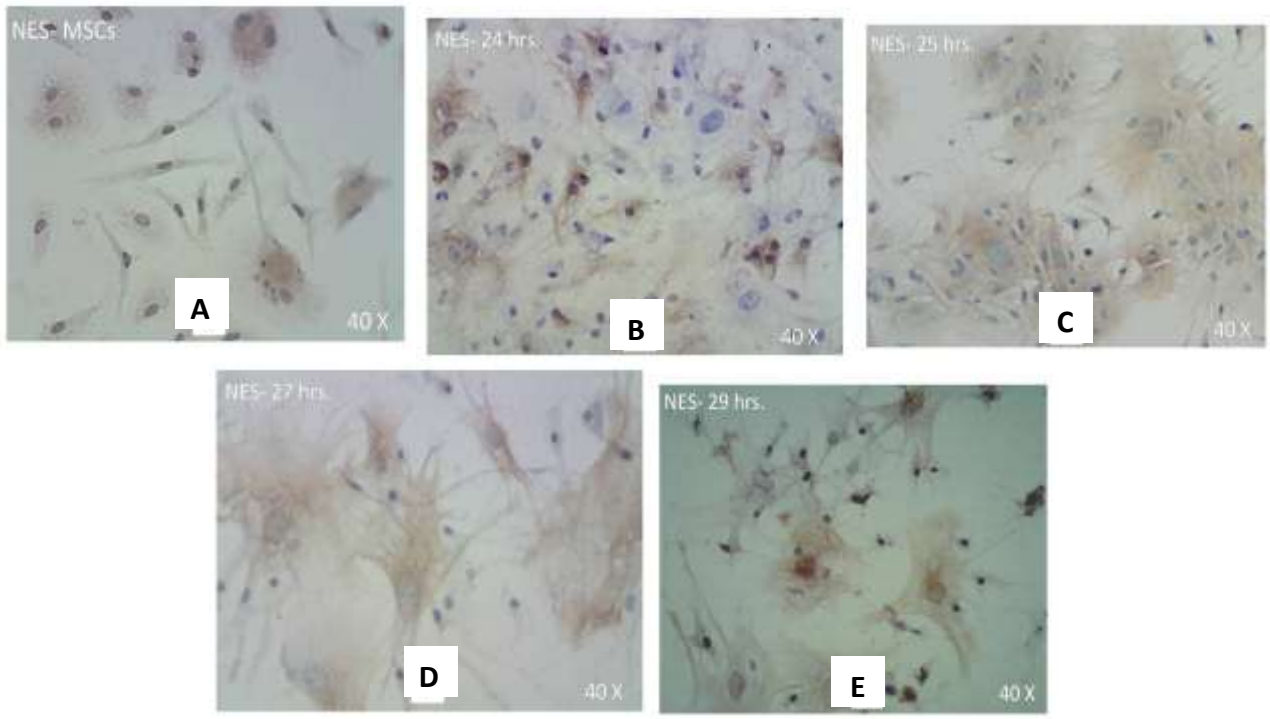


Figure 12: ICC detection results of MSCs after induced differentiation by BME, the results showed increase of NES protein through different exposure times as revealed under a light microscope, the figures (A-E) represented (MSCs-29 h) exposure time.

Also, there were many other studies which demonstrated the expression of NES markers first, then other maturation markers after that, and therefore it was the basic marker in the detection and determined the early stage of neural differentiation of MSCs in culture[8,52,56], with expression in both undifferentiated and differentiated MSCs but with different expression levels.

Assessment efficiency of the current study: The results showed that the BME was successful to induce neuron cells formation with higher protein expression through the differentiation process for the NES (with 61.0, 68.5, 86.3, 61.8% for all exposure time respectively), and NF-L (with 32.2, 65.9, 80.8, 66.4 % for all exposure time respectively) markers with significant difference level ($p< 0.05$). Compared with lower protein expression for MAP-2 marker (with 4.8, 5.8, 12, 29.7% for all exposure time respectively), were significant difference levels ($p< 0.05$) for all exposure time used in this study. Compared with BHA formula (as the second differentiation formula) used in this study, it gives less protein expression with significant difference level at $p< 0.05$ for all exposure time used in this study as shown in figure 16. Therefore, the BME was the best differentiation formula used in the next experiment of regenerative medicine.

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References

1. AL-Qaisy B.A., Yaseen N.Y., Alwachi S.N. and AL-Shammari A.M., Comparison between three different protocols for isolation and culture of mouse bone marrow derived mesenchymal stem cells, *Iraqi Journal of Cancer and Medical Genetics*, **7**, 26-35 **(2014)**

2. Al-Shammari A.M. et al, Establishment and characterization of a receptor-negative, hormone-nonresponsive breast cancer cell line from an Iraqi patient, *Breast Cancer*, (Dove Med Press), **7**, 223-230, doi:10.2147/bctt.s74509 **(2015)**

3. Al-Shammari A.M. et al, In Abstract presented at the 105th Annual Meeting of the American Association for Cancer Research **(2014)**

4. AlShammari A.M., Al-Joboury A.A. and Yaseen N.Y., Isolation and Culture of Neuronal Stem Cells Which Directed into Purkinje Cells To Be Used for Brain Damage and Epilepsy Treatment in mice, *Molecular Therapy*, DOI: 10.1038/mt.2012.86, S201-S202 **(2012)**

5. Alshammari A.M., Salman M.I., Umran M.A. and Yaseen N.Y., Chondrogenic Differentiation of Mouse Bone Marrow Mesenchymal Stem Cells, First Successes in Iraq, XX International Congress ofMediterranean Federation of Health and Production of Ruminants, Egypt, 19-22 **(2013)**

6. Campbell K. and Götz M., Radial glia: multi-purpose cells for vertebrate brain development, *Trends in Neurosciences*, **25**, 235-238 **(2002)**

7. Carden M.J., Trojanowski J.Q., Schlaepfer W.W. and Lee V., Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns, *Journal of Neuroscience*, **7**, 3489-3504 **(1987)**

8. Chen P. et al, Treatment with retinoic acid and lens epithelial cell-conditioned medium in vitro directed the differentiation of pluripotent stem cells towards corneal endothelial cell-like cells, *Experimental and Therapeutic Medicine*, **9**, 351-360 **(2015)**

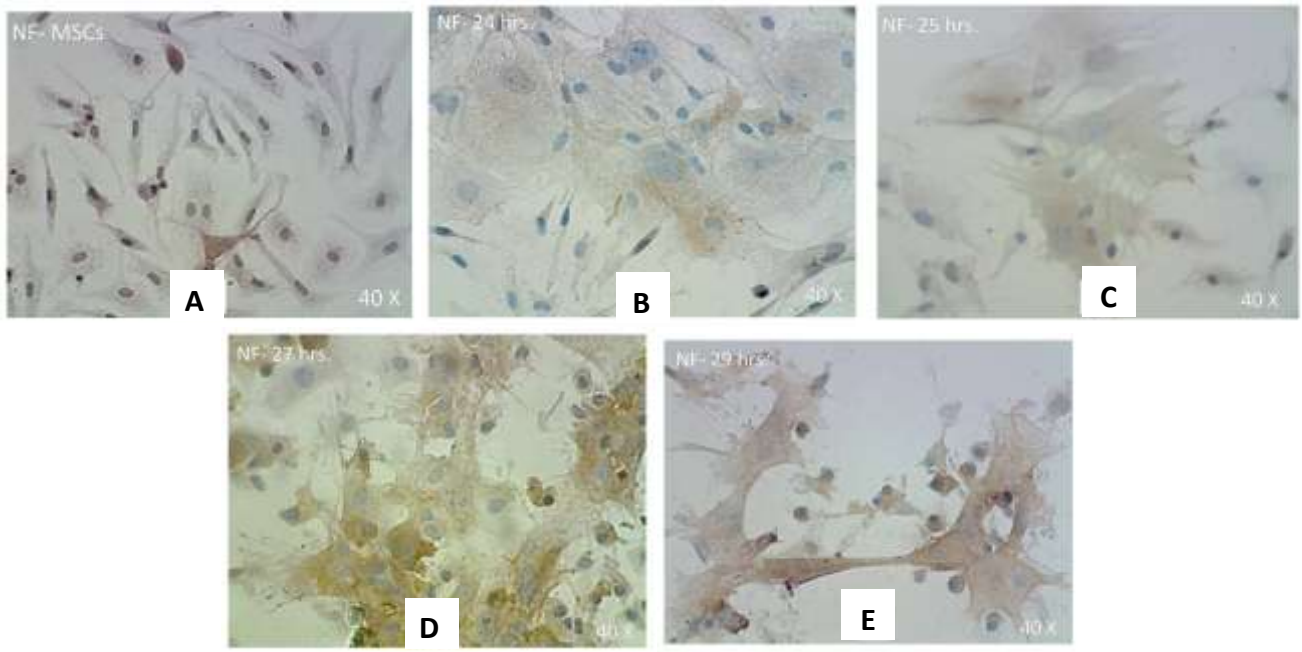


Figure 13: Immunocytochemistry detection results of MSCs after induced differentiation by BME, which showed the increased of NF-L protein throw the different exposure times as revealed under a Light microscope, all figure showed in 40X, the figure (A-E) presented (MSc - 29 hrs.) exposure time.

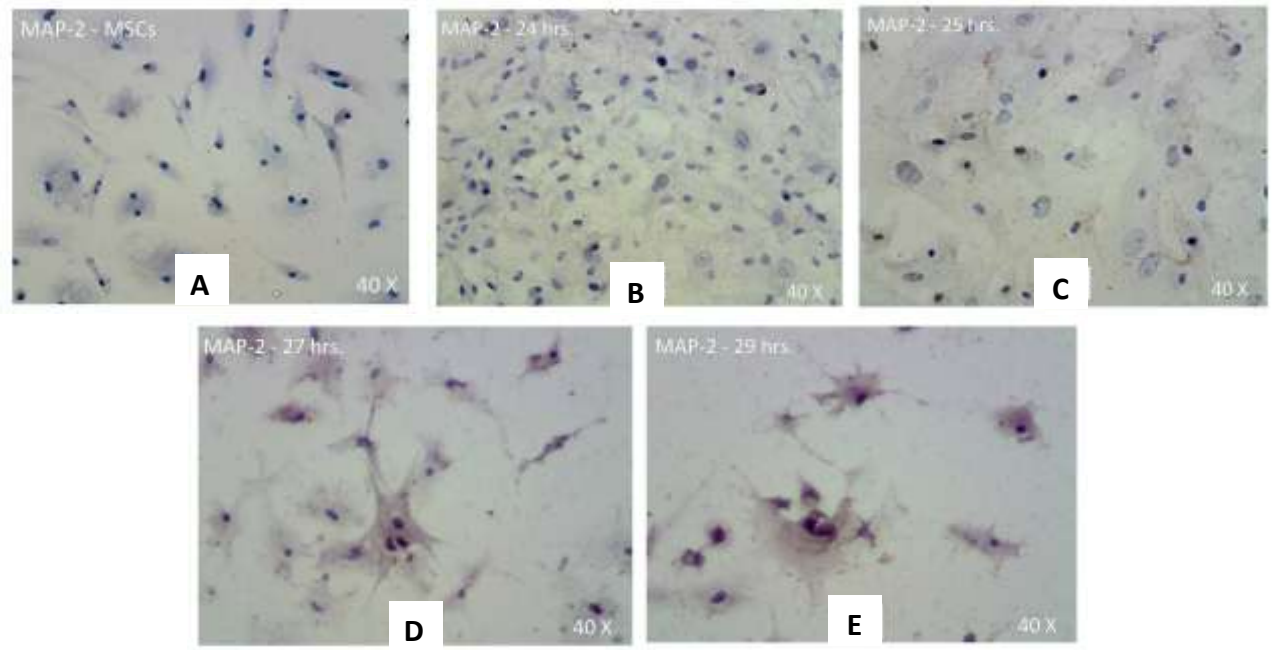
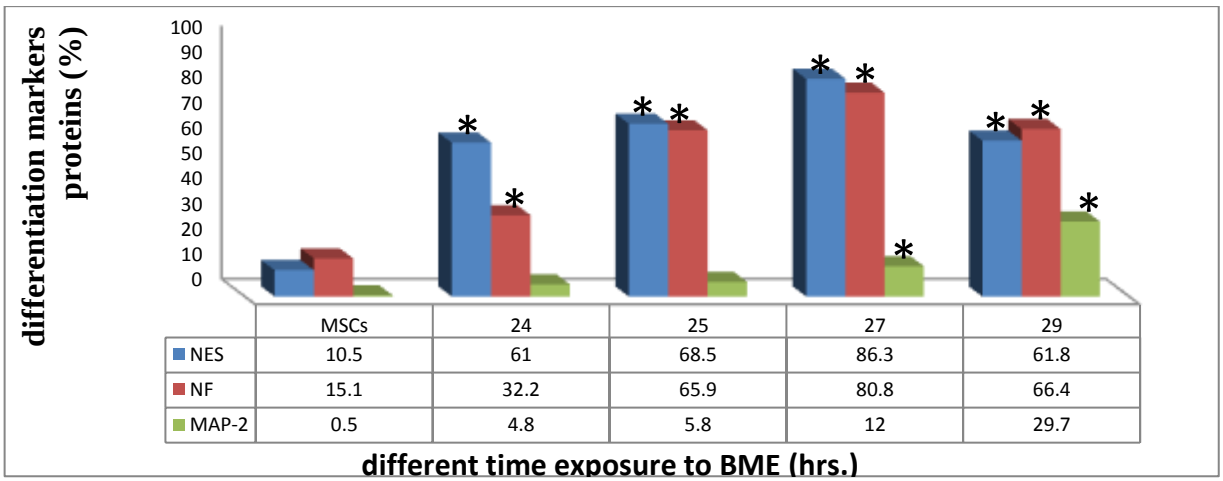
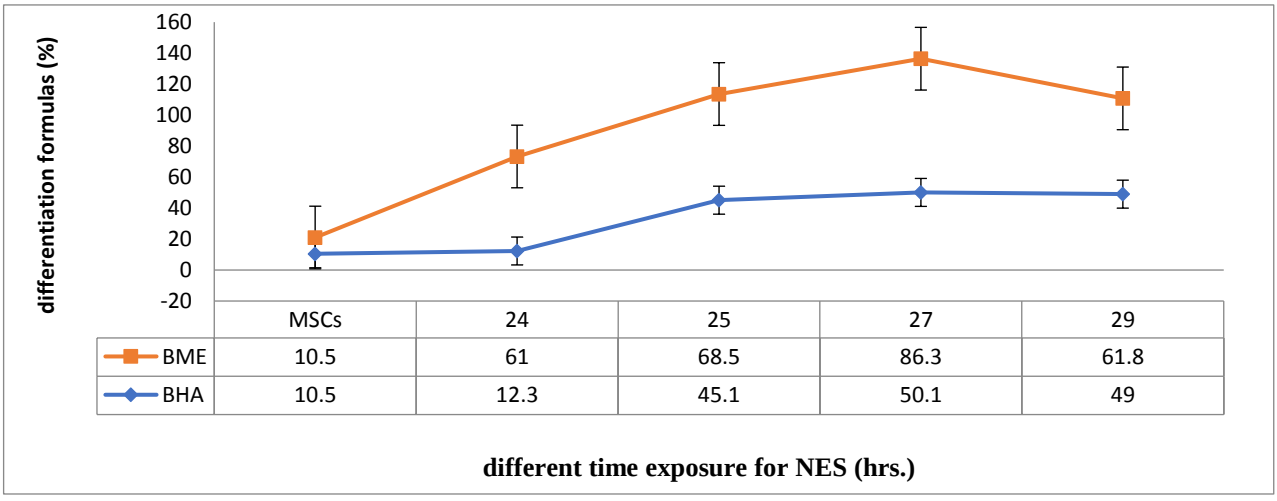


Figure 14: Immunocytochemistry detection results of MSCs after induced differentiation by BME, which showed the increased of MAP-2 protein throw the different exposure times as revealed under a Light microscope, all figure showed in 40X, the figure (A-E) presented (MSc - 29 hrs.) exposure time.

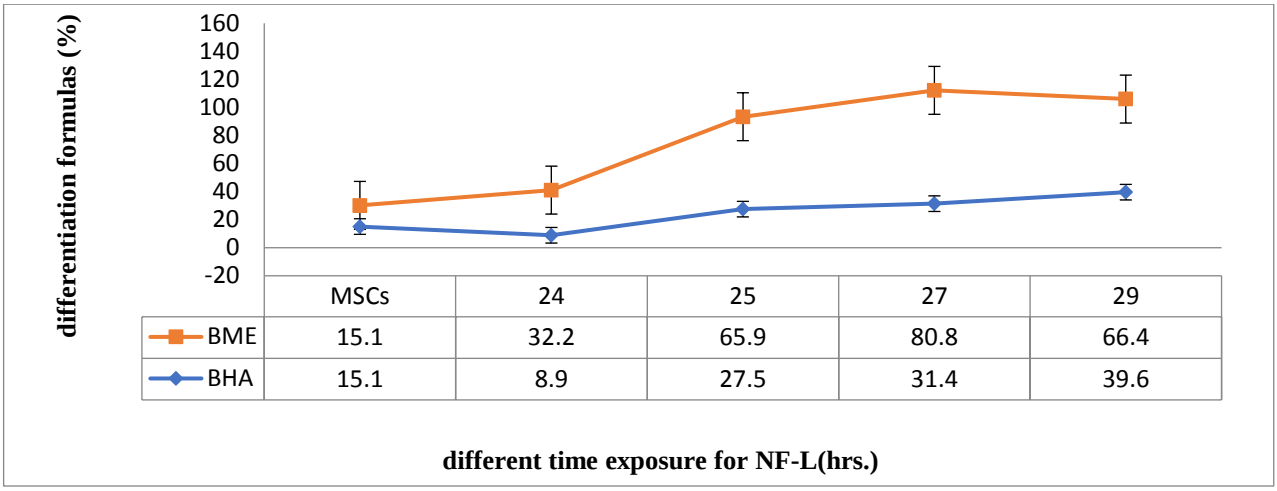


	MSCs	24	25	27	29
NES	10.5	61	68.5	86.3	61.8
NF	15.1	32.2	65.9	80.8	66.4
MAP-2	0.5	4.8	5.8	12	29.7

Figure 15: The proteins expression levels of Immunocytochemistry assay for NES, NF-L, and MAP-2 antibodies of neuronal cells after induced by BME.
*** refer to significant effect at $p<0.05$.**



	MSCs	24	25	27	29
BME	10.5	61	68.5	86.3	61.8
BHA	10.5	12.3	45.1	50.1	49



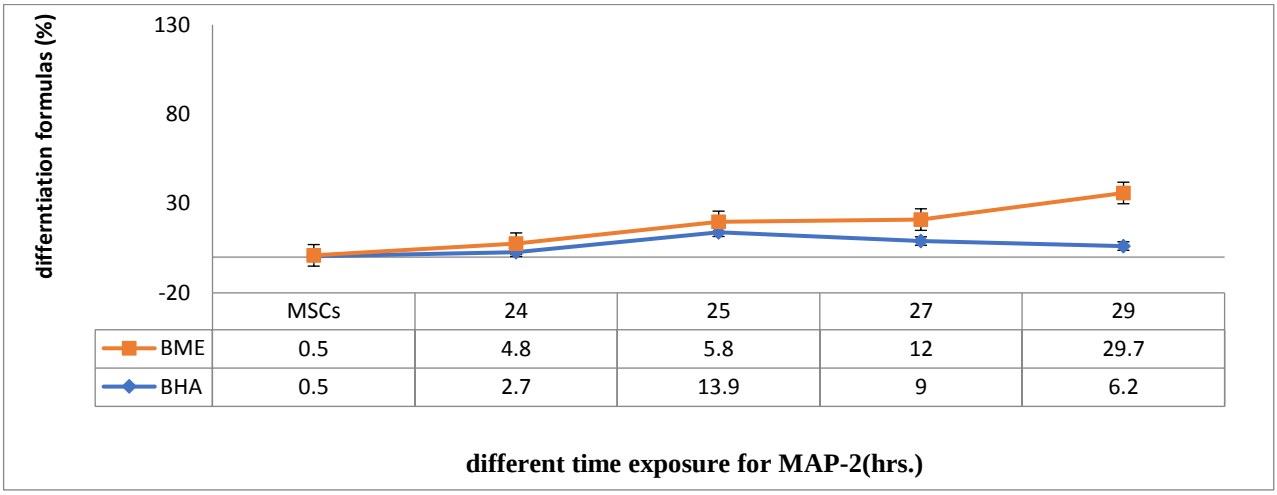


Figure 16: Assessment efficiency of the tow formulas used in this study which showed the protein expression levels of Immunocytochemistry assay for NES, NF-L, and MAP-2 antibodies throw the differentiation process compared with control (MSCs).

9. Darabi S., Tiraihi T., Delshad A. and Sadeghizadeh M., A new multistep induction protocol for the transdifferentiation of bone marrow stromal stem cells into GABAergic neuron-like cells, *Iranian Biomedical Journal*, **17**, 8 **(2013)**

10. Darling D.C. and Morgan S.J., Animal cells: culture and media, John Wiley & Sons **(1994)**

11. Duan P. et al, miR-29a modulates neuronal differentiation through targeting REST in mesenchymal stem cells, *PloS ONE*, **9**, e97684 **(2014)**

12. Esposito E. et al, Melatonin reduces stress-activated/mitogen-activated protein kinases in spinal cord injury, *Journal of Pineal Research*, **46**, 79-86 **(2009)**

13. Freshney R.I., Culture of animal cells: a manual of basic technique and specialized applications, John Wiley & Sons **(2015)**

14. Gaiottino J. et al, Increased neurofilament light chain blood levels in neurodegenerative neurological diseases, *PloS ONE*, **8**, e75091 **(2013)**

15. Hammadi A.M.A., Marino A. and Farhan S., Clinical outcome of 50 progressive multiple sclerosis patients treated with cellular therapy in Iraq, *International Journal of Stem Cells*, **4**, 113 **(2011)**

16. Huang A.H.C., Snyder B.R., Cheng P.H. and Chan A.W., Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice, *Stem Cells*, **26**, 2654-2663 **(2008)**

17. Inui K., Oreffo R.O. and Triffitt J.T., Effects of beta mercaptoethanol on the proliferation and differentiation of human osteoprogenitor cells, *Cell Biology International*, **21**, 419-425 **(1997)**

18. Ishii K., Katayama M., Hori K., Yodoi J. and Nakanishi T., Effects of 2-mercaptoethanol on survival and differentiation of fetal mouse brain neurons cultured in vitro, *Neuroscience Letters*, **163**, 159-162 **(1993)**

19. Jabur A.R. et al, Evaluation of Stem Cells' Growth on Electrospun Polycaprolactone (PCL) Scaffolds Used for Soft Tissue Applications, *Energy Procedia*, **119**, 61-71 **(2017)**

20. Jackson J.S., Golding J.P., Chapon C., Jones W.A. and Bhakoo K.K., Homing of stem cells to sites of inflammatory brain injury after intracerebral and intravenous administration: a longitudinal imaging study, *Stem Cell Research & Therapy*, **1**, 17 **(2010)**

21. Jang S., Cho H.H., Cho Y.B., Park J.S. and Jeong H.S., Functional neural differentiation of human adipose tissue-derived

stem cells using bFGF and forskolin, *BMC Cell Biology*, **11**, 25 **(2010)**

22. Janke C. and Kneussel M., Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton, *Trends in Neurosciences*, **33**, 362-372 **(2010)**

23. Kalcheva N. et al, Genomic structure of human microtubule-associated protein 2 (MAP-2) and characterization of additional MAP-2 isoforms, Proceedings of the National Academy of Sciences, **92**, 10894-10898 **(1995)**

24. Kassem M., Al-Nbaheen M., Ali D. and Aldahmash A., Human Mesenchymal (skeletal) stem cells and clinical applications for bone tissue regeneration, Stem Cells: From Bench to Bedside, 457-475 **(2010)**

25. Kermani S. et al, Effect of lead on proliferation and neural differentiation of mouse bone marrow-mesenchymal stem cells, *Toxicology in Vitro*, **22**, 995-1001 **(2008)**

26. Kim B.J., Seo J.H., Bubien J.K. and Oh Y.S., Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro, *Neuroreport*, **13**, 1185-1188 **(2002)**

27. Kim E.Y. et al, Neuronal cell differentiation of mesenchymal stem cells originating from canine amniotic fluid, *Human Cell*, **27**, 51-58 **(2014)**

28. Kim H. et al, Dual function of Wnt signaling during neuronal differentiation of mouse embryonic stem cells, *Stem Cells International*, http://dx.doi.org/10.1155/2015/459301 **(2015)**

29. Lazarides E., Intermediate filaments: a chemically heterogeneous, developmentally regulated class of proteins, *Annual Review of Biochemistry*, **51**, 219-250 **(1982)**

30. Lee S., Choi E., Cha M.J. and Hwang K.C., Cell adhesion and long-term survival of transplanted mesenchymal stem cells: a prerequisite for cell therapy, *Oxidative Medicine and Cellular Longevity*, doi: 10.1155/2015/632902 **(2015)**

31. Lei Z. et al, Culture and neural differentiation of rat bone marrow mesenchymal stem cells in vitro, *Cell Biology International*, **31**, 916-923 **(2007)**

32. Li P., Animal Models of Acute Neurological Injuries II, Springer, 7-17 **(2012)**

33. Lim J.H. et al, Transplantation of canine umbilical cord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs, *Journal of Veterinary Science*, **8**, 275-282 **(2007)**

34. Lu P., Blesch A. and Tuszynski M.H., Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation or artifact?, *Journal of Neuroscience Research*, **77**, 174-191 **(2004)**

35. Mareschi K. et al, Multipotent mesenchymal stromal stem cell expansion by plating whole bone marrow at a low cellular density: a more advantageous method for clinical use, *Stem Cells International*, doi: 10.1155/2012/920581 **(2012)**

36. Michalczyk K. and Ziman M., Nestin structure and predicted function in cellular cytoskeletal organisation, *Histology and Histopathology*, **20**, 665-671 **(2005)**

37. Mohammad M.H., Al-Shammari A.M., Al-Juboory A.A. and Yaseen N.Y., Characterization of neural stemness status through the neurogenesis process for bone marrow mesenchymal stem cells, *Stem Cells Cloning*, **9**, 1-15, doi:10.2147/sccaa.s94545 **(2016)**

38. Mohammad-Gharibani P., Tiraihi T. and Arabkheradmand J., In vitro transdifferentiation of bone marrow stromal cells into GABAergic-like neurons, *Iranian Biomedical Journal*, **13**, 137-143 **(2009)**

39. Morshead C.M. et al, Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells, *Neuron*, **13**, 1071-1082 **(1994)**

40. Nam H., Lee K.H., Nam D.H. and Joo K.M., Adult human neural stem cell therapeutics: Current developmental status and prospect, *World journal of Stem Cells*, **7**, 126 **(2015)**

41. Nandy S.B., Mohanty S., Singh M., Behari M. and Airan B., Fibroblast Growth Factor-2 alone as an efficient inducer for differentiation of human bone marrow mesenchymal stem cells into dopaminergic neurons, *Journal of Biomedical Science*, **21**, 83 **(2014)**

42. Ni L., Wen Y., Peng X. and Jonakait G.M., Antioxidants N-acetylcysteine (NAC) and 2-mercaptoethanol (2-ME) affect the survival and differentiative potential of cholinergic precursors from the embryonic septal nuclei and basal forebrain: involvement of ras signaling, *Developmental Brain Research*, **130**, 207-216 **(2001)**

43. Park S. et al, Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors, *Neuroscience Letters*, **359**, 99-103 **(2004)**

44. Petzold A., Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss, *Journal of the Neurological Sciences*, **233**, 183-198 **(2005)**

45. Quintiliano K. et al, Neural Differentiation of Mesenchymal Stem Cells on Scaffolds for Nerve Tissue Engineering Applications, *Cellular Reprogramming (Formerly "Cloning and Stem Cells")*, **18**, 369-381 **(2016)**

46. Ryu H.H. et al, Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury, *Journal of Veterinary Science*, **10**, 273-284 **(2009)**

47. Salti A. et al, Expression of early developmental markers predicts the efficiency of embryonic stem cell differentiation into midbrain dopaminergic neurons, *Stem Cells and Development*, **22**, 397-411 **(2012)**

48. Song X.Q. et al, The effect of Id1 gene silencing on the neural differentiation of MSCs, *Biotechnology & Biotechnological Equipment*, **31**, 554-562 **(2017)**

49. Steindler D.A., In Astrocytes, Springer, 9-22 **(2012)**

50. Tamagawa T., Ishiwata I., Ishikawa H. and Nakamura Y., Induced in vitro differentiation of neural-like cells from human amnion-derived fibroblast-like cells, *Human Cell*, **21**, 38-45 **(2008)**

51. Tondreau T. et al, Bone marrow–derived mesenchymal stem cells already express specific neural proteins before any differentiation, *Differentiation*, **72**, 319-326 **(2004)**

52. Wislet-Gendebien S. et al, Plasticity of cultured mesenchymal stem cells: Switch from nestin-positive to excitable neuron-like phenotype, *Stem Cells*, **23**, 392-402 **(2005)**

53. Woodbury D., Schwarz E.J., Prockop D.J. and Black I.B., Adult rat and human bone marrow stromal cells differentiate into neurons, *Journal of Neuroscience Research*, **61**, 364-370 **(2000)**

54. Yan Y. et al, Efficient and rapid derivation of primitive neural stem cells and generation of brain subtype neurons from human pluripotent stem cells, *Stem Cells Translational Medicine*, **2**, 862-870 **(2013)**

55. Yang E. et al, Generation of neurospheres from human adipose-derived stem cells, *BioMed Research International*, http://dx.doi.org/10.1155/2015/743714 **(2015)**

56. Zainal Ariffin S.H. et al, Differentiation of dental pulp stem cells into neuron-like cells in serum-free medium, *Stem Cells International*, doi: 10.1155/2013/250740 **(2013)**.