

# Two-Step Induction of Dopaminergic Neurons Differentiation of Leukapheresis-Derived Mesenchymal Stem Cells

**Zeinab M. Ismail<sup>1</sup>, Menna M. Abdel-Dayem<sup>1</sup>, Nagla M. Salama<sup>1</sup>, Hala Gabr<sup>2</sup>, Dalia I. Ismail<sup>1</sup>,  
Ahmed S. Abdelhafiz<sup>3</sup>, Shaimaa I. El-Jaafary<sup>4</sup>**

<sup>1</sup>Histology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

<sup>2</sup>Clinical Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

<sup>3</sup>Department of Clinical Pathology, National Cancer Institute, Cairo University, Cairo, Egypt

<sup>4</sup>Neurology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

## **Email address:**

halagabr@yahoo.com (H. Gabr)

## **To cite this article:**

Zeinab M. Ismail, Menna M. Abdel-Dayem, Nagla M. Salama, Hala Gabr, Dalia I. Ismail, Ahmed S. Abdelhafiz, Shaimaa I. El-Jaafary. Two-Step Induction of Dopaminergic Neurone Differentiation of Leukapheresis-Derived Mesenchymal Stem Cells. *American Journal of Bioscience and Bioengineering*. Special Issue: Stem Cells for Neuro-regeneration: Where Do We Stand. Vol. 3, No. 4-1, 2015, pp. 7-17. doi: 10.11648/j.bio.s.2015030401.12

**Abstract:** *Background:* Neurodegenerative diseases represent a great medical challenge with marked consequences on quality of life of the patients and their families. Parkinson's disease (PD) constitutes a relatively common neurodegenerative disorder characterized by defect in dopaminergic neurons. The regenerative effect of mesenchymal stem cells (MSCs) stimulated research into their effect in treatment of PD. *Subjects and Methods:* Leukapheresis-derived MSC were isolated from 10 leukapheresis products using plastic adherence. Isolated MSCs were passaged, and passage III cells were induced to dopaminergic neurones using two-step protocol applying sequentially nerve growth factor and addition of ascorbic acid. Neuronal induction was evaluated by immunostaining against neurofilament protein, while dopaminergic neurons induction was evaluated using immunostaining against tyrosine hydroxylase. *Results and Conclusions:* MSCs were isolated in a rate of 0.12%-0.15% of leukapheresis cells, with viability ranging from 70-96%. NF positivity was  $15.38 \pm 3.84$ , while the percentage of cells stained for TH was  $5.94 \pm 0.65$ . MSCs could be successfully induced to dopaminergic neuron-like cells in vitro.

**Keywords:** Mesenchymal Stem Cells, Dopaminergic Neurons, Parkinson's Disease

## **1. Introduction**

Parkinson's disease (PD) is the most common neurodegenerative disorder next only to Alzheimer's disease (1). The disease is caused by the loss of midbrain dopamine neurons resulting in decrease in striatal dopamine (2). The worldwide prevalence of PD ranges from 57 to 230 per 100,000 persons (3).

Stem cells represent a good promise as an inexhaustible source for novel cell-based therapies for (PD), with excessive recent work exploring stem cells properties and differentiation into different cell types (4). They have been heavily investigated for their potential to differentiate into dopaminergic neurons either spontaneously or through certain induction protocols (5). Mesenchymal stem cells

(MSCs) represent an easily accessible source of stem cells that have few ethical problems and can be efficiently expanded in vitro to provide strong opportunities for clinical application(6). They showed promising results especially in delivering trophic factors for treating PD and other neurodegenerative diseases (7).

Mesenchymal stem cells (MSCs) represent a heterogeneous population of cells with multipotent capabilities. MSCs possess a number of characteristics which make them ideal candidates for regenerative therapy. They can be easily obtained from a number of sources, can be expanded in vitro and possess angiogenic and paracrine activity stimulating regeneration(8).

MSCs can be harvested from many sources, namely, bone marrow, adipose tissue and peripheral blood after stem cell mobilization (leukapheresis)(9). Leukapheresis is the least

invasive method of MSCs collection, with documented mobilization of bone marrow-MSCs using G-CSF(10).

It is now proven that Bone marrow MSCs (BMSCs) do not spontaneously differentiate in vivo after transplantation, and even if they differentiate, the differentiated cells would be extremely low. Therefore, in clinical practice, establishing a specific protocol for inducing BMSCs to produce dopamine neurons prior to transplantation is essential (11). Based on the fact that BMSCs proliferate rapidly in culture as an adherent population and differentiate into several lineages, they can provide cells useful for tissue engineering and gene therapy (12).

The aim of the present study is to establish a reproducible in-vitro protocol to induce the human MSCs derived from peripheral blood, after mobilization from bone marrow, to differentiate into DA neurons; as a potential cellular therapy for PD.

## 2. Subjects and Methods

### 2.1. Subjects

The study included 10 healthy donors after confirmed verbal consent. The age of the donors ranged from 25 to 35 years with a mean age of  $29.60 \pm 3.20$  years old. Their mean weight was  $75.00 \pm 7.29$  Kg They were eight males and two females. The donors were subcutaneously injected with G-CSF (Filgrastim) in a single daily dose of  $10 \mu\text{g/kg}$  for 5 days. Then collection of peripheral blood mononuclear cells (MNCs) by aphaeresis was done on the fifth day one hour after injection of the last dose according to Waller et al, 2010(13).

### 2.2. Methods

#### 2.2.1. Mesenchymal Stem Cell Isolation

Total leucocytic counts were determined before and after mobilization. MNCs were collected leukapheresis.

MSCs were plated in a density of  $5 \times 10^5$  cells in T25 flasks in complete DMEM low glucose medium containing 10% fetal bovine serum, 1% penicillin-streptomycin and 10% L-glutamine. Flasks were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  humidified incubator. After 24 hours, non-adherent cells were removed and medium was replenished. Medium was changed every 3 days and flasks examined under inverted microscope until cells 85% confluence Sotiropoulou et al 2006 (14).

Harvest of MSCs was one using 0.05% trypsin-EDTA, harvested cells were counted, and tested for viability using trypan blue exclusion test.

Cells were passaged as before and MSCs harvested from passage III were used for induction of neuronal differentiation.

Identification of MSCs was done using

immunophenotyping (CD90,CD105, CD73, CD34, CD45), and trilineage differentiation (R&D SC006).

#### 2.2.2. Induction of Neuronal Differentiation

Passage III MSCs were induced using 20ng/ml nerve growth factor in complete high glucose DMEM in laminin coated tissue culture plates. Cells were followed morphologically and medium changed every 3 days for 2 weeks. Identification of neuronal induction was done using immunostaining for neurofilament protein.

#### 2.2.3. Induction of Dopaminergic Neuron Differentiation

Induction of dopaminergic differentiation in neural-induced MSCs was done by the addition of 30ul/ml ascorbic acid to the nerve growth factor-containing media described above in laminin coated plates (Yan et al., 2001(15, 16). Cultures were incubated for 7-10 days in 5%  $\text{CO}_2$  incubator. Evaluation of dopaminergic differentiation was done using immunohistochemical staining against tyrosine hydroxylase.

Immunohistochemical staining was done according to the method used by Bancroft and Cook, 1994 (17). Immunohistochemical staining was done for detection of neurofilament (NF) and for tyrosine hydroxylase (TH). Positive tissue control and negative tissue controls were used. Detection of both antibodies was done using Histostain SP kit according to manufacturer instructions. Positive cells for both NF and TH showed brown cytoplasmic deposits while Immunoreactivity was absent in negative controls in which the primary antibody was omitted. Morphometric study was done using Leica Qwin 500 image analyzer computer system Ltd. (Cambridge, England)

Number of NF and TH immunopositive cells (i.e. cells with brown cytoplasmic deposits) was counted in the specimens under 400x magnification. And their percentage in relation to the total number of cells was calculated. The data was expressed as mean + SD and comparison between the NF and TH immunoexpression was done using student T-test

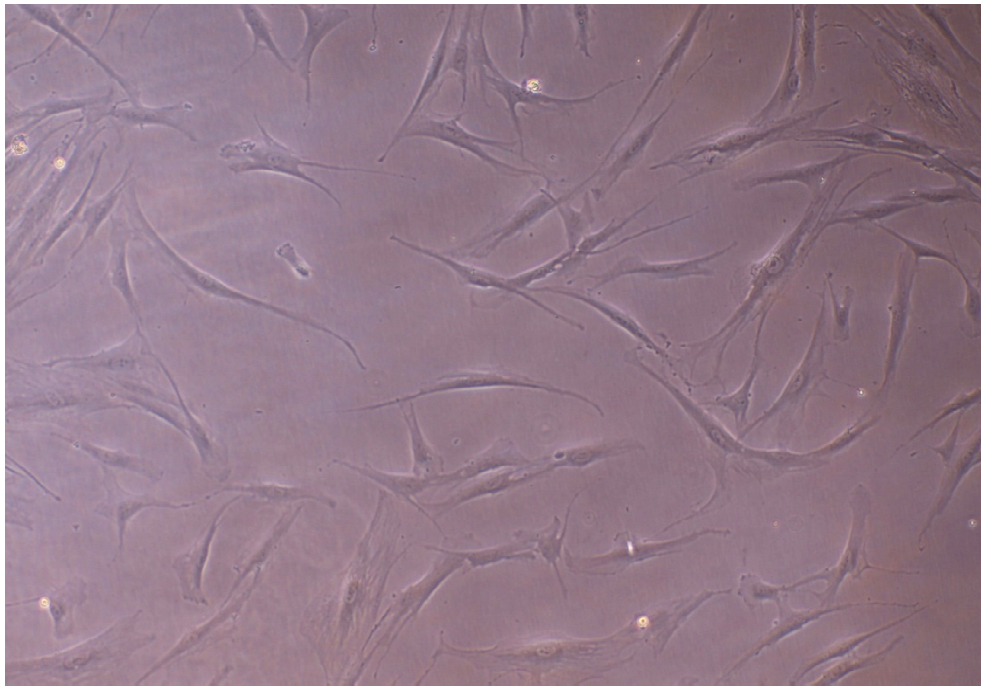
## 3. Results

The total leucocytic count (TLC) before mobilization ranged from  $6 \times 10^3$ - $14 \times 10^3$  /mm<sup>3</sup> while after mobilization it ranged from  $80 \times 10^3$ - $280 \times 10^3$  / mm<sup>3</sup>. Mean MSCs isolated from each flask ( containing an initial cell count of 500,000) was  $60,000 \pm 5,000$  (0.12%). Viability index of MSCs was determined after culture and ranged from 70% -96% with a mean of  $84.10\% \pm 8.57\%$ .

MSCs before neural induction showed fibroblastoid morphology (Figure 1). Surface marker expression shows 80-90% positivity of CD90, CD105 and CD73 with 0.1-3.2% positivity for CD34 and CD45.



**Fig. (1a).** Inverted microscope image x 400 of adherent MSCs with floating non adherent cell at day 5.



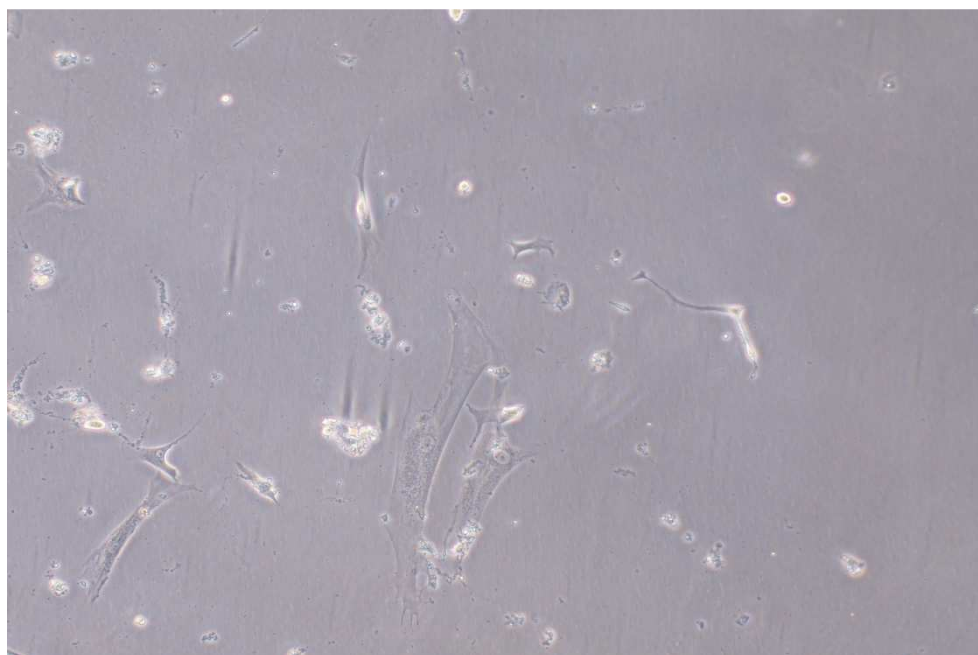
**Fig. (1b).** Inverted microscope image x 400 displayed adherent MSCs that acquired a more defined MSCs morphology after 2 weeks.

## 4. Morphology of Neural-Induced MSCs

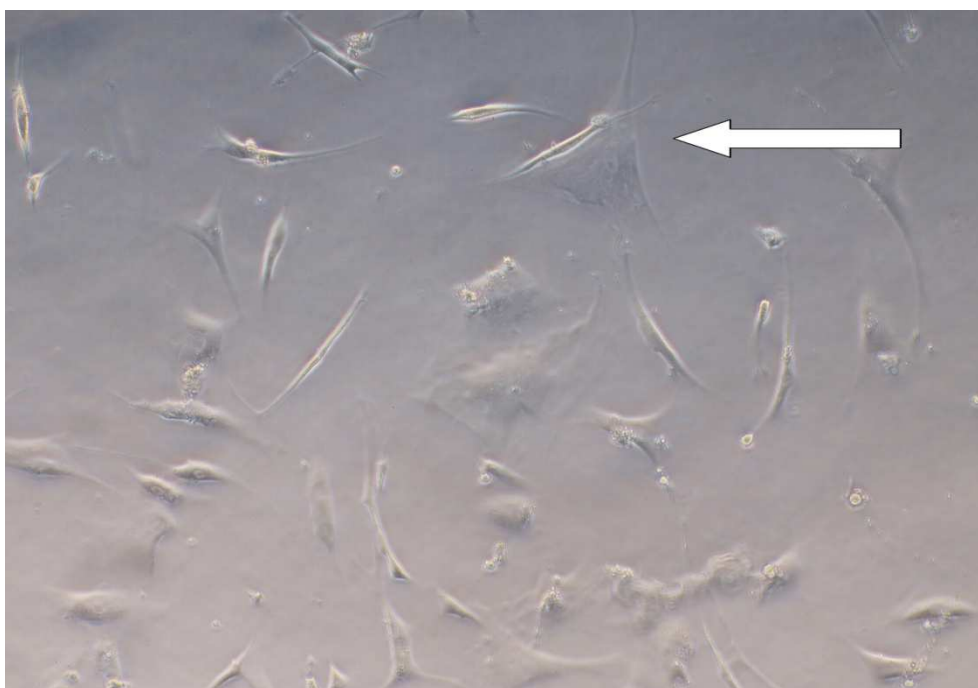
### 4.1. 1-Morphological Changes

MSCs had a typically adherent spindle shape under an inverted microscope. At step 1, changes in cell morphology

could not be observed. During further culturing, with addition of growth factors, the rate of cell proliferation decreased and these spindle-like cells became short and conversed to round epithelial like cells. (figure 2).

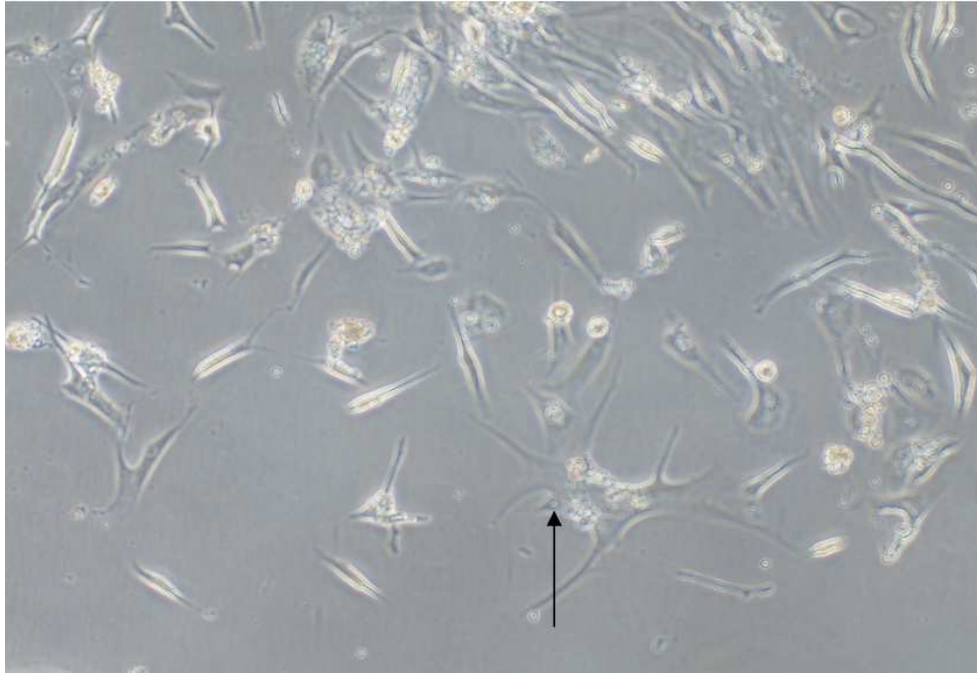


**Fig. (2a).** Inverted microscope image x 200 of differentiated cells day 3D post differentiation.



**Fig. (2b).** Inverted microscope image x 200 of differentiated cells day 3D post differentiation.





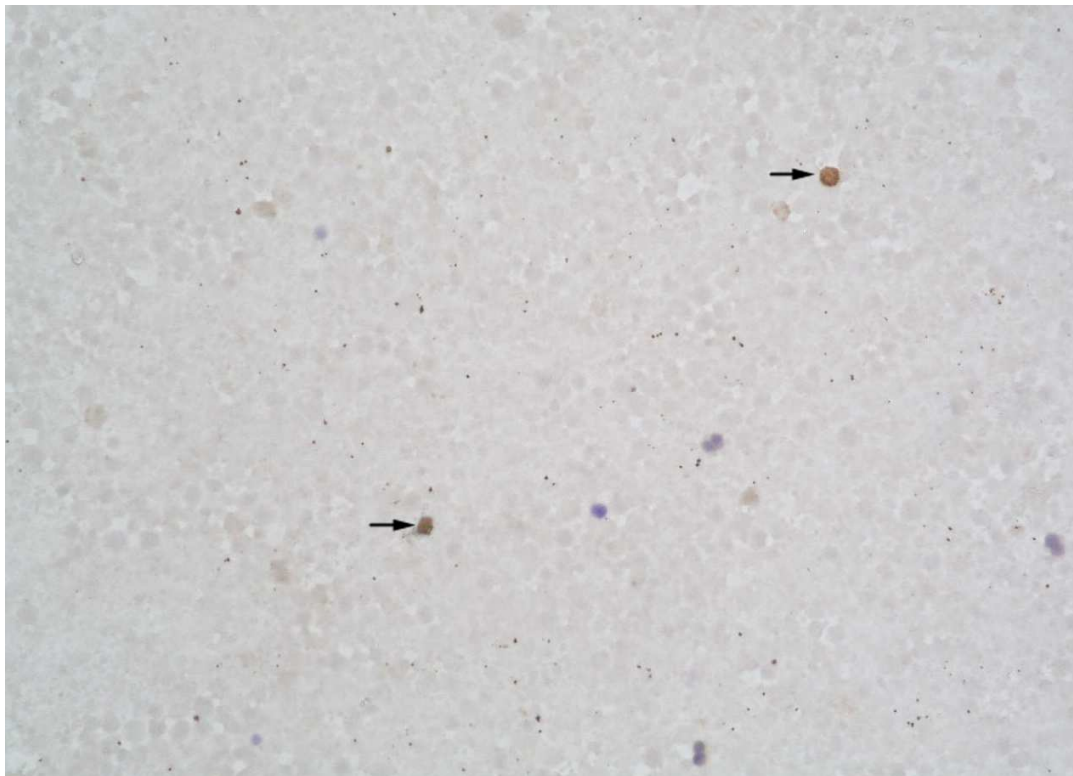
**Fig. (2c).** Inverted microscope image x 200 of differentiated cells day 6 post differentiation.

#### 4.2. Results of Immunostaining

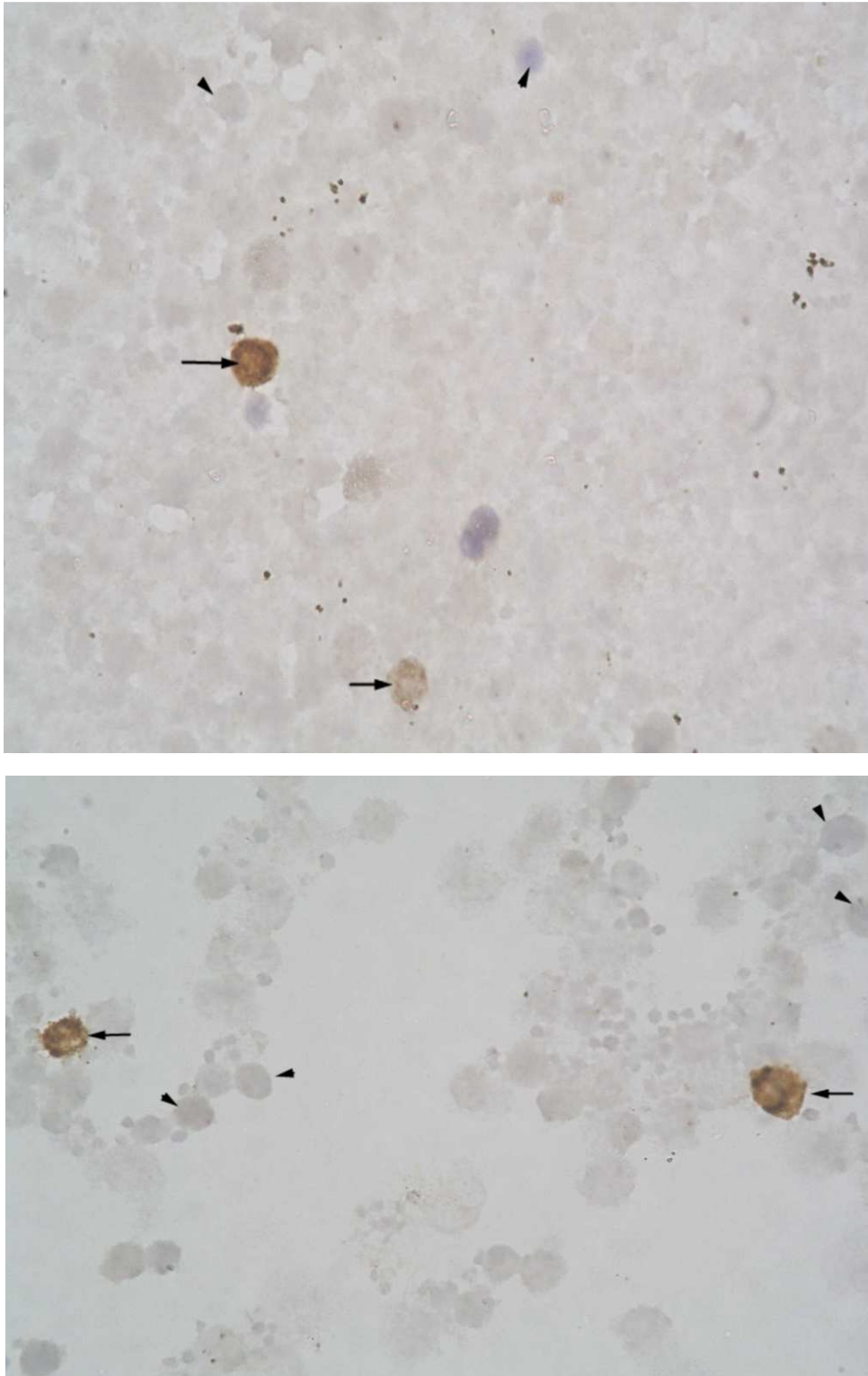
Sections of rat midbrain which were used as positive control showed large number of cells with positive neurofilament (NF) and tyrosine hydroxylase (TH) immunostaining. This immunostaining appeared as brown cytoplasmic deposits. The cultured specimens that were used

as negative control showed no brown cytoplasmic deposits within the cells.

All the cultured specimens immunostained with anti NF antibody showed some scattered immunopositive cells with brown cytoplasmic deposits after staining (Figs. 3- 4 A and B).



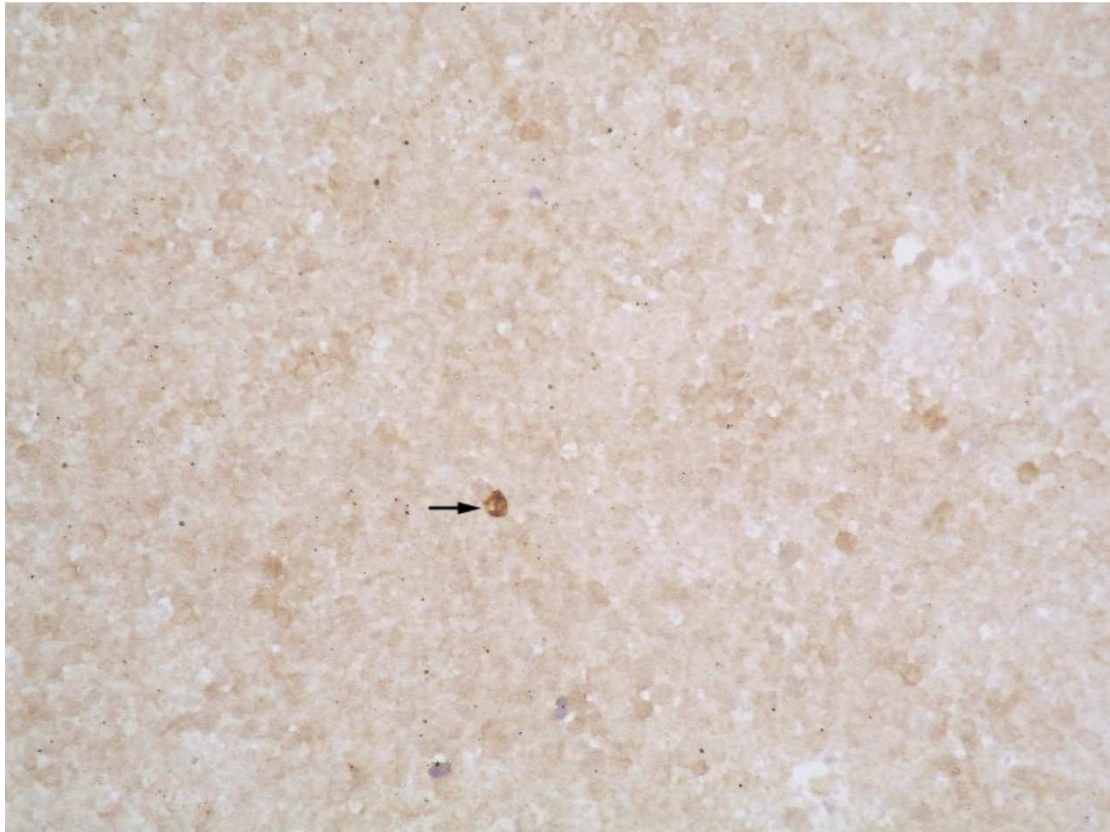
**Fig. 3.** A photomicrograph of a specimen of the cultured MSCs (taken from the donor number four) differentiated into nerve cells, showing scattered cells with brown cytoplasmic deposits (→) (i.e. positive NF immunoreactivity). (Immunohistochemical stain for NF, x400).



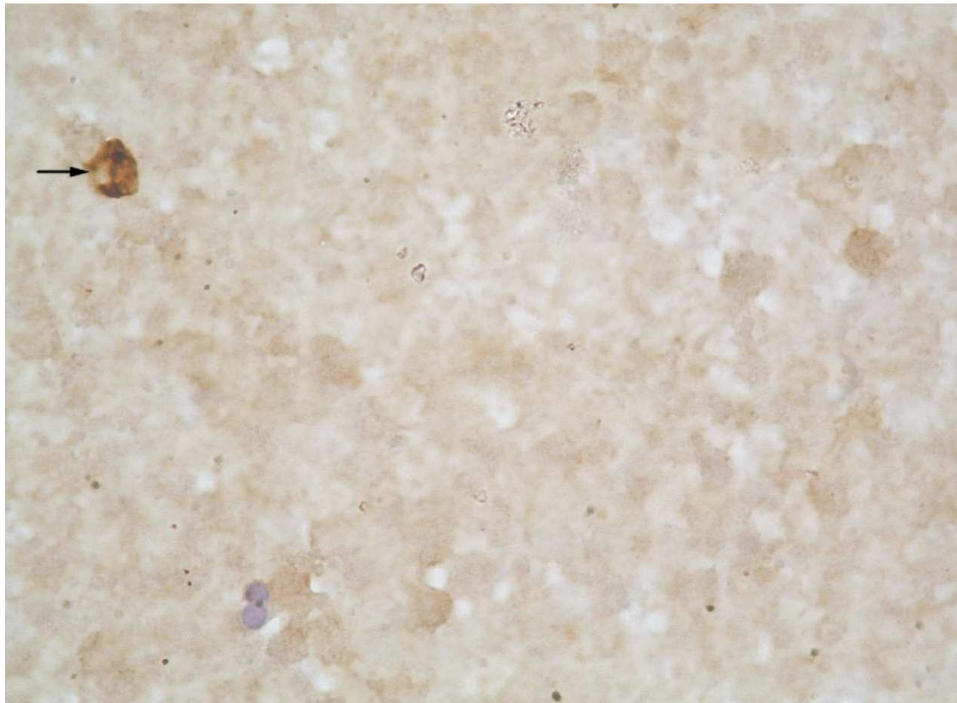
**Figs. 4. a** (above) and **b** (below): Higher magnification of the previous specimen showing positive ( $\rightarrow$ ) and negative ( $\blacktriangleright$ ) NF immunoreactive cells. (Immunohistochemical stain for NF,  $\times 1000$ )

In addition, the immunostained specimens stained with anti TH antibody showed fewer cells with brown cytoplasmic deposits (Figs. 5-6). TH stains dopaminergic (DA) neurons specifically.

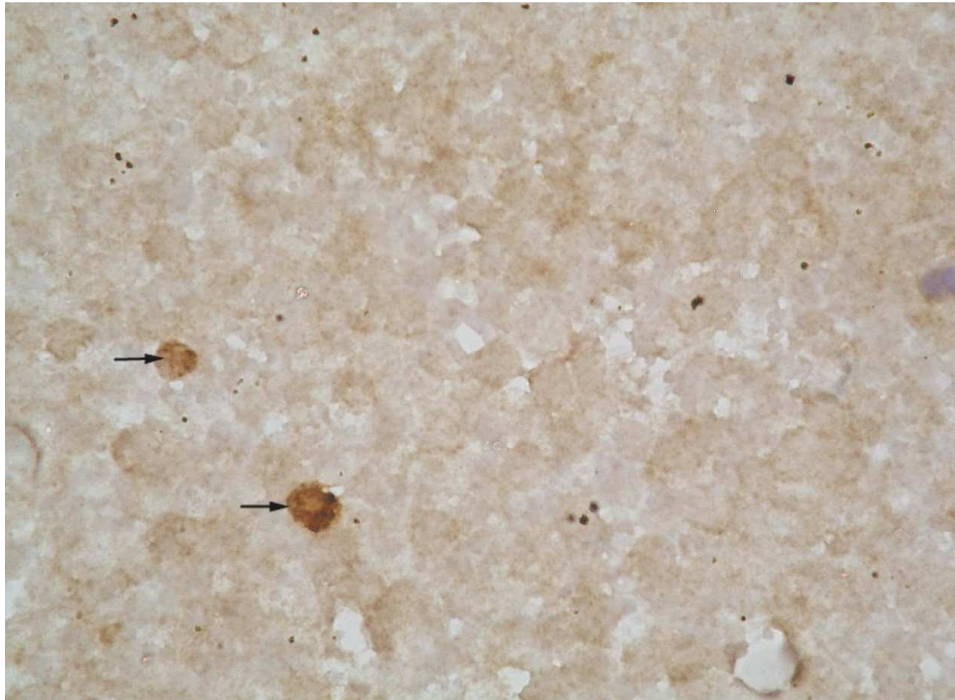
Percentage of cells stained for NF was  $15.38 \pm 3.84$ , while the percentage of cells stained for TH was  $5.94 \pm 0.65$  as shown in figure 7.



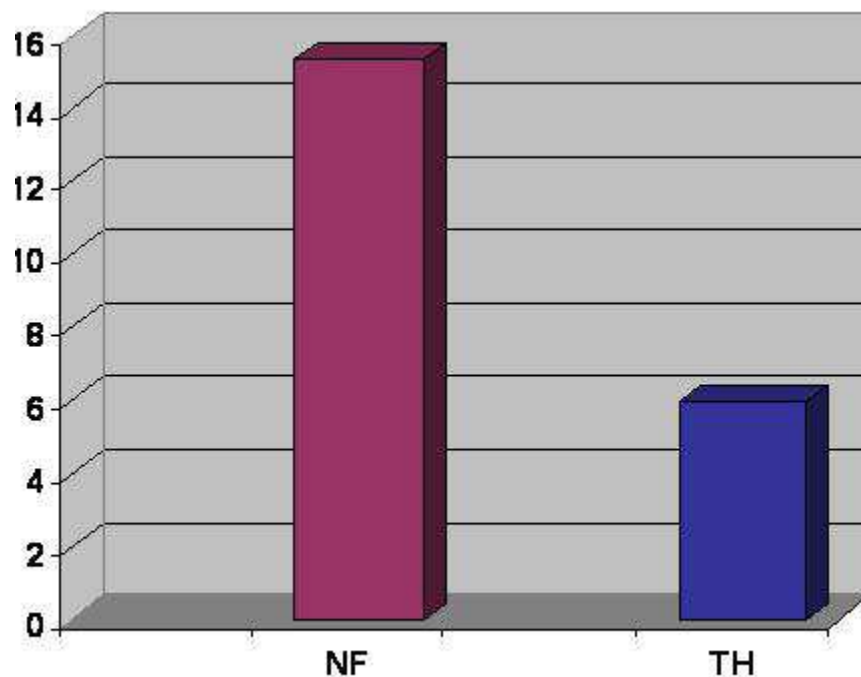
**Fig. 5.** A photomicrograph of a specimen of the cultured MSCs (taken from the donor number seven) differentiated into dopaminergic neurons, showing a single cell with brown cytoplasmic deposits (→) (positive TH immunoreactivity). (Immunohistochemical stain for TH, x400)







**Fig. 6. a** (above)&**b**(below): Higher magnification of the previous specimen showing a cell with positive immunoreactivity (→) together with negative TH immunoreactive cells. (Immunohistochemical stain for TH, x1000)



**Fig. 7.** percentage of cells stained for NF was  $15.38 \pm 3.84$ , while the percentage of cells stained for TH was  $5.94 \pm 0.65$ .

## 5. Discussion

The current study explored the in-vitro ability of human MSCs derived from peripheral blood, after mobilization from bone marrow, to differentiate into DA neurons.

In the present study, the donors were given Granulocyte-colony stimulating factor (G-CSF) to mobilize stem cells from the bone marrow. It was previously declared that the

administration of G-CSF daily for 4 to 6 days resulted in a 10 to 30 fold increase in the number of circulating stem cells (18,19). The dose of G-CSF (Filgrastim) used in this study was  $10 \mu\text{g/kg/day}$  injected once daily for 5 days subcutaneously as recommended by other studies (20,21). Collection of peripheral blood MNCs by apheresis was done on the fifth day one hour after injection of the last dose.

Administration of G-CSF and subsequent leukapheresis were usually well tolerated by donors, with short-term side



effects mainly consisting of mild symptoms, such as bone pain, headache and myalgia. Concerning the long-term side effects, Quillen and co-workers reported a 10-year follow-up of unrelated volunteer donors who received multiple cycles of G-CSF and considered that stimulation with G-CSF is safe and not associated with long-term vascular, hematologic or malignant complications (22). MSCs were isolated from MNCs by conventional plastic adherence which is routinely done. MSCs were identified according to the criteria proposed by the International Society of Cellular therapy (23).

In this work, mean MSCs isolated from leukapheresis product was 0.12% with viability index after culture of (84.10%±8.57%). In contrast to some researchers who reported that the number of MSCs isolated from mobilized and non-mobilized PB by conventional plastic adherence was negligible in all the samples tested (24). This may be related to the natural resistance of the cells of mesenchymal origin to migrate out of the organ of residence, other investigators found that the success of plastic adherence method for MSCs isolation from mobilized PB was limited and they could establish cultures in only 50% of six attempts (25)(26).

The culture done in this work was on DMEM media supplemented with 20% FCS as most current protocols for in-vitro culture of MSCs included it. Serum supplementation is important because it provides the cells with vital nutrients, attachment factors and growth factors. There are several published clinical trials using cultured human MSCs for the treatment of various disorders, all of which used FCS for supplementing the culture medium, demonstrating so far that FCS did not cause any significant side effects. Moreover human MSCs grown in FCS were shown to be more efficient in suppressing alloantigen-induced lymphocyte proliferation; suggesting that FCS-supplemented medium is more suitable for preventing or treating alloreactivity-related immune complications (27).

In this study, the differentiation process was performed through two steps. The first step was adding nerve growth factor (NGF, 20 µg/ml) to the culture media for induction of nerve cells from MSCs. Five members of this family have been identified; they are brain-derived neurotrophic factor, nerve growth factor, neurotrophin 3, neurotrophin 7 and neurotrophin 4/5. Neurotrophins could influence neuronal functions such as cell survival and axonal growth. They also exhibit a potent neuroprotective ability as well as promoting regeneration of degenerated neurons (28,29).

The second step in the differentiation process of the present work was using ascorbic acid (AA) for the induction of DA neurons. AA is a water-soluble antioxidant, which acts as a cofactor in various biochemical reactions, and notably in catecholamine synthesis. Yan et al. 2001 mentioned that treatment with AA enhanced the DA differentiation of MSCs leading to more than 10-fold increase in DA neurons induction in untreated cultures. They reported that the dopaminergic effect of AA could not be mimicked by other antioxidants. Therefore, they concluded that pre-treatment of in-vitro expanded cultured stem cells with AA may induce large-scale generation of DA neurons that can be used for

clinical transplantation (16).

Volpicelli et al. in 2004 tested the effect of adding AA for three days after six days of stimulation with NGF, and reported four to seven fold increase in tyrosine hydroxylase (TH) positive cells, as compared with cultures treated for 6 days with NGF alone (30). Some studies showed that the addition of AA enhanced the differentiation of stem cells into both neurons and astrocytes, an action unique to AA and not shared by other antioxidants such as glutathione and vitamin E (31).

Immunostaining of cultured cells in the present work was done using anti NF and anti TH antibodies. NF labels neurons, while, TH (involved in the conversion of tyrosine to dopamine) is used as a marker for DA neurons. The percentage of immunopositive cells stained for NF was 15.38±3.84, while the percentage of immunopositive cells stained for TH was 5.94±0.65.

This was in consistent with Farkas et al. 2003 who found that approximately one-third of the cells (20.9%) stained for NF while 8.7% of the cells showed TH positivity (32). In addition, Jiang et al. 2003 found that 65% of cells stained positive for nestin while 25% of cells expressed DA neuron markers (33). Correia et al. 2008 reported 3% TH positive cells in the absence of the growth factor but it increased to 15% in its presence (34). On the other hand, Trzaska et al. 2009 found more than 90% of the induced MSCs expressing NeuN and beta-III-tubulin and 70% expressed TH (35).

## 6. Conclusion

This study suggests that MSCs might have a great therapeutic potential for neurological diseases since they could be easily obtained from peripheral blood after their mobilization using G-CSF. In addition, they could be isolated, expanded and differentiated into DA neurons. However, further studies are needed to ensure the long-term safety and efficacy of these differentiated DA neurons for in-vivo applications.

## References

- [1] Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel (2011): J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *Eur J Epidemiol.*;26 Suppl 1:S1–S58.
- [2] Chiu C, Yeh TH, Lai SC, Wu-Chou YH, Chen CH, Mochly-Rosen D, Huang YC, Chen YJ, Chen CL, Chang YM, Wang HL, Lu CS (2015); Neuroprotective effects of aldehyde dehydrogenase 2 activation in rotenone-induced cellular and animal models of parkinsonism. *Exp Neurol* 263:244-53.
- [3] Muangpaisan W, Mathews A, Hori H, Seidel D. (2011): A systematic review of the worldwide prevalence and incidence of Parkinson's disease. *J Med Assoc Thai.*;94(6):749–755.
- [4] Gaulden J and Reiter JF (2008): Neur-ons and neur-offs regulators of neural induction in vertebrate embryos and embryonic stem cells. *Hum Mol Genet*;17(R1): R60-66.

- [5] Deierborg T, Soulet D, Roybon L, Hall V, and Brundin P (2008): "Emerging restorative treatments for Parkinson's disease," *Progress in Neurobiology*, vol. 85, no. 4, pp. 407–432.
- [6] Kitada M and Dezawa M (2012): Parkinson's Disease and Mesenchymal Stem Cells: Potential for Cell-Based Therapy. *Parkinson's Disease*. Volume 2012, , Article ID 873706, 9 pages, 2012. doi:10.1155/2012/873706.
- [7] Wyse R.D, Dunbar G.L. Rossignol. (2014): Use of Genetically Modified Mesenchymal Stem Cells to Treat Neurodegenerative Diseases. *J.Int. J. Mol. Sci.*, 15(2), 1719–1745.
- [8] Glavaski-Joksimovic A, Bohn MC (2013): Mesenchymal stem cell and neuroregeneration in Parkinson's disease. *Exp Neurol* 247:25-38.
- [9] Taran R, Mamidi MK, Singh G, Dutta S, Parhar IS, John JP, Bhonde R Pal R, Das AK (2014): In vitro and in vivo neurogenic potential of mesenchymal stem cells isolated from different sources. *J Biosci* 39(1):157-69.
- [10] Gabr H, Abd El-Fattah R, Ahmed D, Farhan M, Mousa S, (2011): Mesenchymal stem cells derived from bone marrow and leukapheresis show different putative subpopulations. *Stem Cell Studie* 1(1):e19.
- [11] E. Mezey, K. J. Chandross, G. Harta, R. A. Maki, and S. R. McKercher (2000). "Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow," *Science*, vol. 290, no. 5497, pp. 1779–1782.
- [12] Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D and Lagneaux L (2005): Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells*; 23: 1105-1112.
- [13] Waller CF, Bronchud M, Mair S and Challand R (2010): Comparison of the pharmacodynamic profiles of a biosimilar filgrastim and Amgen filgrastim: results from a randomized, phase I trial. *Ann Hematol*; 89(10): 971–978.
- [14] Sotiropoulou PA, Perez SA, Salagianni M, Baxevas CN and Papamichail M (2006): Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells*; 24(2): 462-71.
- [15] Yan J, Studer L and McKay RD (2001): Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors. *J Neurochem*; 76(1): 307-11.
- [16] Bagga V, Dunnett SB, Fricker-Gates RA (2008): Ascorbic Acid Increases the Number of Dopamine Neurons in Vitro and in Transplants to the 6-OHDA-Lesioned Rat Brain. *Cell Transp*. 17:763-773.
- [17] Bancroft JD and Cook HC (eds) (1994): Immunocytochemistry. In: *Manual of Histological Techniques and their Diagnostic Applications*. Churchill Livingstone, Edinburgh, London, Madrid, Melbourne, New York and Tokyo, p. 263-325.
- [18] Jin P, Wang E, Ren J, Childs R, Shin JW, Khuu H, Marincola FM and Stroncek DF (2008): Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis. *J Transl Med*; 6: 39
- [19] Helmy KY, Patel SA, Silverio K, Pliner L and Rameshwar P (2010): Stem cells and regenerative medicine: accomplishments to date and future promise. *Ther Deliv*; 1(5): 693-705.
- [20] Hölig K, Kramer M, Kroschinsky F, Bornhäuser M, Mengling T, Schmidt AH, Rutt C and Ehninger G (2009): Safety and efficacy of hematopoietic stem cell collection from mobilized peripheral blood in unrelated volunteers: 12 years of single-center experience in 3928 donors. *Blood*; 114(18): 3757-63.
- [21] Woods I, Tawab-Amiri A, Byrne K, Sabatino M and Stroncek DF (2010): Pilot analysis of cytokines levels in stored granulocyte-colony-stimulating factor-mobilized peripheral blood stem cell concentrates. *Transfusion*; 50(9): 2011-5.
- [22] Quillen K, Byrne P, Yau YY and Leitman SF (2009): Ten-year follow-up of unrelated volunteer granulocyte donors who have received multiple cycles of granulocyte-colony-stimulating factor and dexamethasone. *Transfusion*; 49(3): 513-8.
- [23] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenback I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E (2006): Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4):315-317.
- [24] Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B and Hows JM (2003): Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol*; 121: 368–374.
- [25] Fox JM, Chamberlain G, Ashton BA and Middleton J (2007): Recent advances into the understanding of mesenchymal stem cell trafficking. *Br J Haematol*; 137(6): 491-502.
- [26] Lund TC, Tolar J and Orchard PJ (2008): Granulocyte colony-stimulating factor mobilized CFU-F can be found in the peripheral blood but has limited expansion potential. *Haematologica*; 93(6): 908-12.
- [27] Mannello F and Tonti GA (2007): Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! *Stem Cells*; 25(7): 1603-9.
- [28] Scuri M, Samsell L and Piedimonte G (2010): The role of neurotrophins in inflammation and allergy. *Inflamm Allergy Drug Targets*; 9(3): 173-80.
- [29] Rangasamy SB, Soderstrom K, Bakay RA and Kordower JH (2010): Neurotrophic factor therapy for Parkinson's disease. *Prog Brain Res*; 184: 237-64.
- [30] Volpicelli F, Consales C, Caiazzo M, Colucci-D'Amato L, Perrone-Capano C and di Porzio U (2004): Enhancement of dopaminergic differentiation in proliferating midbrain neuroblasts by sonic hedgehog and ascorbic acid. *Neural plasticity*; 11(1-2): 45-57.
- [31] Harrison FE and May JM (2009): Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic Biol Med*; 46(6): 719-30.
- [32] Farkas LM, Dunker N, Roussa E, Unsicker K and Kriegstein K (2003): Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in-vitro and in-vivo. *The Journal of Neuroscience*; 23 (12): 5178-5186.

- [33] Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, and Verfaillie CM (2003): Neuroectodermal differentiation from mouse multipotent adult progenitor cells. PNAS; 100 (1): 11854–11860.
- [34] Correia AS, Anisimov SV, Li J-Y and Brundin P (2008): Growth factors and feeder cells promote differentiation of human embryonic stem cells into dopaminergic neurons: a novel role for fibroblast growth factor-20. Front Neurosci; 2(1): 26–34.
- [35] Trzaska KA, King CC, Li KY, Kuzhikandathil EV, Nowycky MC, Ye JH and Rameshwar P (2009): Brain Derived Neurotrophic Factor Facilitates Maturation of Mesenchymal Stem Cell-Derived Dopamine Progenitors to Functional Neurons. J Neurochem; 10(3):568-576.