

Bone Marrow Stromal Cell Transdifferentiation into Oligodendrocyte-Like Cells Using Triiodothyronine as a Inducer with Expression of Platelet-Derived Growth Factor α as a Maturity Marker

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ABSTRACT

Background: The present study investigated the functional maturity of oligodendrocyte derived from rat bone marrow stromal cells (BMSC). **Methods:** The BMSC were isolated from female Sprague-Dawley rats and evaluated for different markers, such as fibronectin, CD106, CD90, Oct-4 and CD45. Transdifferentiation of OLC from BMSC was obtained by exposing the BMSC to DMSO and 1 μ M all-trans-retinoic acid during the pre-induction stage and then induced by heregulin (HRG), platelet-derived growth factor AA (PDGFR- α), fibroblast growth factor and T3. The neuroprogenitor cells (NPC) were evaluated for nestin, neurofilament 68, neurofilament 160 and glial fibrillary acidic protein gene expression using immunocytochemistry. The OLC were assessed by immunocytochemistry for O4, oligo2, O1 and MBP marker and gene expression of *PDGFR- α* was examined by RT-PCR. **Results:** Our results showed that the fibronectin, CD106, CD90, CD45 and Oct-4 were expressed after the fourth passage. Also, the yield of OLC differentiation was about 71% when using the O1, O4 and oligo2 markers. Likewise, the expression of *PDGFR- α* in pre-oligodendrocytes was noticed, while MBP expression was detected in oligodendrocyte after 6 days of the induction. **Conclusion:** The conclusion of the study showed that BMSC can be induced to transdifferentiate into mature OLC. *Iran. Biomed. J. 17 (2): 62-70, 2013*

Keywords: Bone marrow stromal cell, Triiodothyronine, Platelet-derived growth factor α

INTRODUCTION

Oligodendrocytes produce the myelin ensheathing axons, which is essential for rapid conduction of nervous impulses [1]. Myelin damage can lead to the loss of nerve function, which is seen in many central nervous system damages such as multiple sclerosis and spinal cord injury. Oligodendrocyte-based cell therapy causes remyelination of a demyelinated axon [2]. In order to obtain oligodendrocytes with a high purity, primary mechanical enrichment was used, while the positive selection was done based on the presence of expressed markers on the cell surface [3]. Several studies have reported the differentiation of various types of stem cells into oligodendrocytes [4], such as olfactory ensheathing cells, neural stem cells, embryonic stem cells and bone

marrow stromal cells (BMSC) [5]. Moreover, *in vivo* administration of BMSC could improve the oligodendrogenesis [6]. Sher and co-workers' finding [5] suggested the possibility of generating oligodendrocytes *in vitro*, which could be a source for cell therapy. One of the most interesting methods to perform a safe transplant was the use of an autologous source such as BMSC, since there was minimal immunological rejection [7]. Therefore, BMSC can be considered as a feasible source for neurological disorder therapy [8]. Although trans-differentiation of BMSC into oligodendrocytes was performed [9], the maturity has not been evaluated. The goal of this study was to improve the induction technique for *in vitro* transdifferentiation of BMSC into oligodendrocyte-like cells (OLC) and to evaluate their maturity.

MATERIALS AND METHODS

Bone marrow stromal cell extraction and culturing.

Sprague-Dawley female rats, weighing 200-250 g (Razi Institute for Serums and Vaccine, Karaj, Iran), were kept under a controlled light/dark cycle (lights on at 6 a.m. and lights off at 6 p.m.) at a temperature of 18-25°C. All animal studies were conducted in accordance with the principles and procedures approved by the Ethical Committee of the Faculty of Medical Sciences, Tarbiat Modares University (Iran). The bone marrow was extracted from rats' long (200-250 g) bones and cultured in DMEM/F12 (Stem Cell Technology Company, Tehran, Iran), supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM/ml L-glutamine and incubated in a humidified incubator with 5% CO₂ at 37°C. The cells were then immunostained for fibronectin, CD45, CD90 and CD106. The neuron D and the stemness gene (Oct-4) were evaluated using RT-PCR.

Pre-induction. BMSC pre-induction and induction was done according to Kaka *et al.* [9]. Briefly, The BMSC were pre-induced (4th passage) using DMSO (2%) in DMEM/F12 medium without fetal bovine serum for 1 day. Then, the medium was replaced with DMEM/F12 containing 15% FBS and 1 μM all-trans-retinoic acid (Sigma-Aldrich, St. Luis, MO, USA) for the following 3 days. The BMSC were plated on gelatin-coated flasks (BD-Biosciences, India) or on 6-well plates containing gelatin-coated glass coverslips. The pre-induced cells were evaluated with nestin, neurofilament 68 (NF68), neurofilament 160 (NF160) and glial fibrillary acidic protein (GFAP).

Induction. At the induction stage, the cells were initially incubated with DMEM/F12 medium containing 5 ng/ml platelet-derived growth factor AA (PDGF-AA) (Sigma-Aldrich, St. Luis, MO, USA), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Luis, MO, USA) and 200 ng/ml heregulin (HRG) (Sigma-Aldrich, St. Luis, MO, USA) for 2 days, followed by induction with different concentrations of triiodothyronine (T3): 0, 5, 12.5, 25, 50, 100, 200 ng/ml (Sigma-Aldrich, St. Luis, MO, USA) for 2 days. The cells were immunostained for O4, oligo2, O1 and myelin basic protein (MBP), while platelet-derived growth factor α (PDGFR-α) was evaluated by RT-PCR.

Viability assay. The dye exclusion test (trypan blue exclusion test) was used to determine the number of viable cells that present in cell suspension. Live cells possess intact cell membranes that exclude certain dyes such as trypan blue, whereas dead cells do not. Cell suspension was simply mixed with dye and then

visually examined to determine whether cells take up or exclude the dye. In the protocol presented here, a viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm.

Immunocytochemical method. The cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. After permeabilization, cells were blocked by 5% bovine serum albumin for 30 min. Immunostaining was done on BMSC, pre-induced and induced cells. We used mouse anti-fibronectin monoclonal antibody (1:100), mouse anti-CD45 monoclonal antibody (1:100), mouse anti-CD106 monoclonal antibody (1:200) and mouse anti-CD90 monoclonal antibody (1:200) specific markers for mesenchymal stem cells. In addition, mouse anti-NF68 monoclonal antibody (1:50) and rat anti-NF160 monoclonal antibody (1:100), markers for neuroprogenitor cells NPC and neurons, respectively; rat anti-GFAP monoclonal antibody (1:100), a specific marker for astrocyte cells; mouse anti-O4 monoclonal antibody (1:100), mouse anti-O1 monoclonal antibody (1:100) and mouse anti-oligo2 monoclonal antibody (1:100), specific markers for immature OLC (all antibodies from EMD Millipore Corporation, Billerica, MA, USA) and mouse anti-MBP monoclonal antibody (1:1000, Covance, Berkeley and CA), a specific marker for mature oligodendrocytes were applied. The cells were incubated with FITC conjugated rabbit anti-mouse secondary antibody (1:100, EMD Millipore Corporation, Billerica, MA and USA) for 2 hours at room temperature. The cells were counterstained with 1:10,000 ethidium bromide (Sigma-Aldrich, St. Luis, MO, USA) for 1 min.

RT-PCR. The BMSC at the end of the fourth passage, rat neonate brain cells (controls), pre-induced cells and induced cells were evaluated for the expression of Oct-4, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), *NeuroD* and *PDGFR-α* genes. Using the RNX-Plus Kit (Fermentas Inc., Maryland, USA), 2 μg of total RNA from each sample was treated with DNase I (Fermentas Inc., Maryland, USA). The purity and integrity of the extracted RNA were evaluated by optical density measurements and electrophoresis on 1% agarose gel. Extracted RNA (1 μg) was converted to cDNA using the First Strand cDNA Synthesis Kit (Fermentas Inc. Maryland, USA). An amount of 50 ng of cDNA was added to the PCR reaction for 35 cycles with denaturation at 95°C for 45 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 30 seconds. After amplification, the products were separated on 2% agarose gel and visualized using ethidium bromide under UV light. Each experiment was repeated at least 3 times in order to ensure reproducibility. Primer sequences (forward

and reverse), the size of the product and PCR conditions were as follows: expression of rat *Oct-4* gene (a marker for BMSC stemness) was done using the 5' AAGCTGCTGAAACAGAAGAGG 3' *Oct-4* forward primer and the 5' ACACGGTTCTCAATGCTAGTC3' forward and backward primers, (210 bp, accession number: N001009178, annealing at 62°C). GAPDH has served as an internal control gene: 5' CCACAACCTCTCCATTCTC 3' and 5' CCAAGATTCACGGTAGATAC 3', forward and backward primers, respectively (400 bp, accession number: NP_002037.2, annealing at 58°C). The expression of rat *PDGFR-α* gene, (a marker for immature oligodendrocytes) was assessed using the 5'CTAATTCACATTCGGAAGGTTG 3' and 5'GGACGATGGGCGACTAGAC 3' (forward and backward primers, respectively, 190 bp, accession number: M63837.1, annealing at 57°C). The expression of rat *NeuroD* (a neuroprogenitor marker) was assessed using the 5'CAGATGATGGCACAAAGGGTAG3' and 5'GACCGAGAGCATCGCATATTG 3', (forward and backward primers, respectively, 220 bp, accession number NM-001105729.3, annealing at 59°C).

Statistical analysis. All data were compared by one way analysis of variance (ANOVA) with Turkey's test and Student's *t*-test method.

RESULTS

After the fourth passage of the isolated BMSC from the rat bone marrow, the viability of the cells was $98.18 \pm 0.94\%$ (mean \pm SEM). The cellular phenotype was characterized by immunocytochemistry for fibronectin, CD90 and CD106 (Fig. 1). The percentages of immunoreactive cells were $94.32 \pm 0.45\%$, $95.48 \pm 0.24\%$ and $97.16 \pm 0.82\%$, respectively. Also, none or very few of the cells expressed CD45, nestin, NF68, NF160, O4, O1, oligo2 and GFAP (data not shown).

Pre-induction. The viability of BMSC treated with pre-inducers ($79.36 \pm 4.82\%$) (DMSO-retinoic acid) was significantly lower viability than untreated BMSC ($94.26 \pm 1.44\%$) (Fig. 2A). The immunostaining for Nestin, NF68 and NF160 was used to study the pre-induction of BMSC (Fig. 1). Also, the pre-induced cells expressed the NeuroD protein (Fig. 3, upper panel). The expression of fibronectin was decreased to $3.10 \pm 0.49\%$ during the pre-induction stage (Fig. 2B). The pre-induced BMSC were evaluated for nestin and NF68 antibodies (markers for NPC). The mean percentages of immunoreactive cells to nestin and NF68 were $73.2 \pm 2.64\%$ and $71.34 \pm 2.65\%$, respectively (Fig. 2B).

Induction. The expression of Oct-4 was not detected at induction stage, while untreated BMSC expressed this gene (Fig. 3). The percentages of undifferentiated and pre-induced cells were estimated by assessing immunopositivity for fibronectin, nestin, NF68, NF160, O1, oligo2, O4 and GFAP (Fig. 2B). Figure 3C depicts the marker expression in the induced BMSC (bFGF, PDGF and HRG), followed by treatment with T_3 at 0, 5, 12.5, 25, 50, 100 and 200 ng/mL. The viability in the T_3 -treated group at the induction stage was the highest with no significant differences at concentrations of 25, 5 and 12.5 ng/mL. However, the viability was significantly lower at concentrations of 50, 100 and 200 ng/mL (Fig. 4D). Figure 5 demonstrates the immunostaining of BMSC induced by O4, oligo2 O1 and MBP and the transdifferentiated cells were immunoreactive to these markers.

RT-PCR. The results of RT-PCR of NeuroD, *Oct-4* and *PDGFR-α* showed that *NeuroD* was expressed in pre-induced cells, Oct-4 expressed in BMSC and NPC and *PDGFR-α* expressed in pre-oligodendrocytes, while NPC showed no band (Fig. 4).

DISCUSSION

The generation of OLC from BMSC necessitates the generation of NPC at the pre-induction stage and our results correlate with findings of Neri *et al.* [10], who reported the generation of oligodendrocyte from the neural stem cells.

The results of this research showed that when the fourth passage of the BMSC was grown in the existence of DMSO and followed by retinoic acid, it produced mainly NPC, which were expression to *NeuroD*. *NeuroD* expression was not detected in the untreated BMSC, which is consistent with the results of Yeu *et al.* [11], who found no detectable levels of *NeuroD* in undifferentiated BMSC. However, there were reports confirming the *NeuroD* gene expression in BMSC and in marrow stromal cells at very low levels [12]. This issue may be due to the spontaneous differentiation of BMSC into neuron-like cells [13]. The expression of *NeuroD* in rat neonate brain (used as a positive control in this study) is consistent with other investigations that have reported the expression of *NeuroD* in rat neonate brain [14]. Also, we identified its expression at pre-induction stage, which is in agreement with the finding of Naghdi *et al.* [15].

After performing pre-induction with DMSO-retinoic acid, the cells were induced by PDGF, bFGF and HRG, followed by different concentrations of T_3 (dose response). The highest percentage of OLC was observed at T_3 concentration of 25 ng/mL. However, at higher concentrations of T_3 , a viability decline

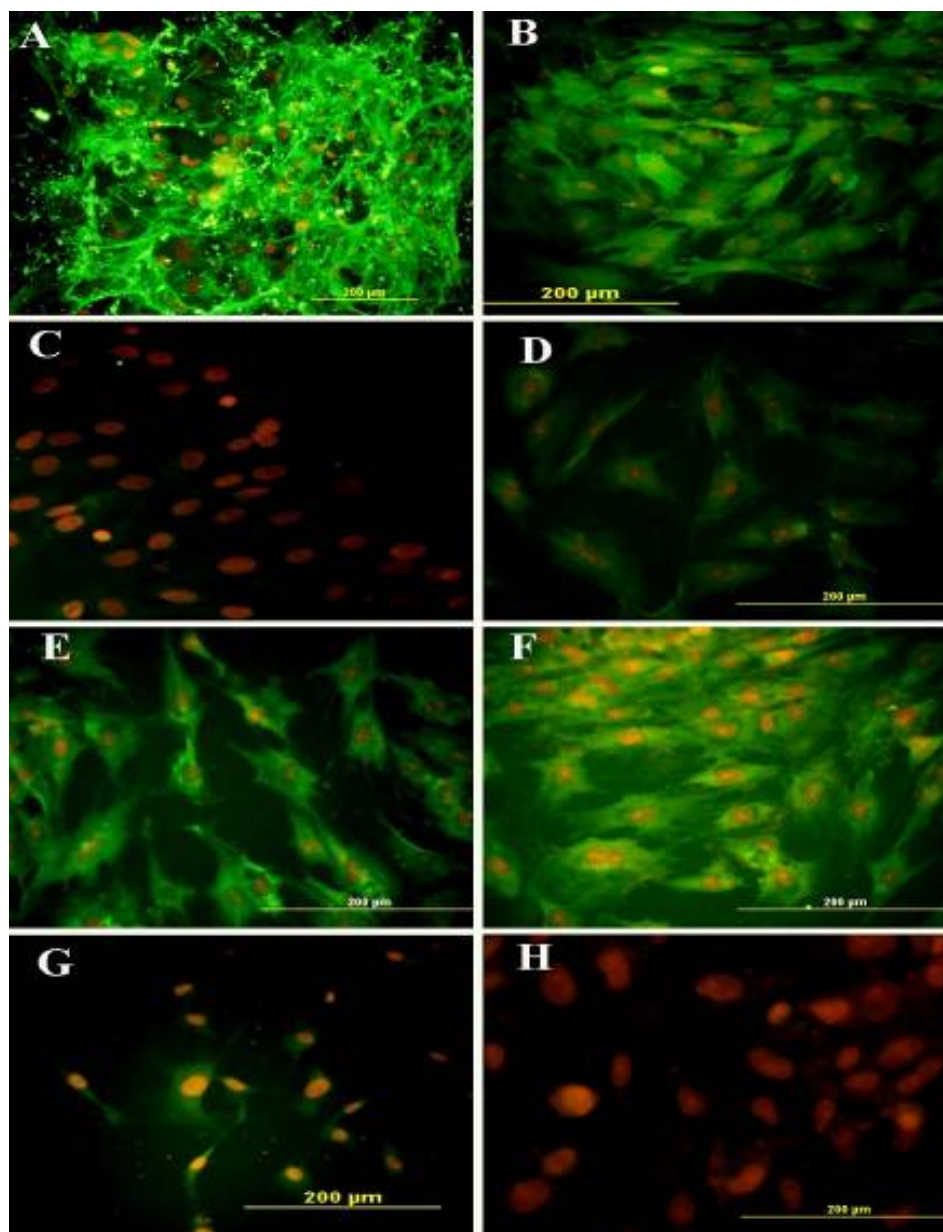


Fig. 1. Immunocytochemistry representation of different cell markers. Following the treatment of bone marrow stromal cells with DMSO-retinoic acid at pre-induction stage, the cells were labeled with primary antibodies, followed by incubation of FITC-conjugated secondary antibody and counterstaining with ethidium bromide. Immunostained cells with (A) anti-fibronectin antibody, (B) anti-CD90 antibody, (C) anti-CD45 antibody, (D) anti-CD106 antibody, (E) anti-nestin, (F) anti-neurofilament 160 (NF-160) antibody, (G) anti-NF68 antibody and (H) anti-glial fibrillary acidic protein antibody.

occurred, probably due to toxicity. This finding was also reported by Kaka *et al.* [9]. On the other hand, the optimum expression of O1, O4 and Oligo2 was achieved at dose of 25 ng/mL. The differentiation of BMSC into OLC at this dose was 71%, while Kaka *et al.* [9] reported a 58% differentiation at T₃ concentration of 10 ng/mL.

T₃ increases morphological and functional maturation of postmitotic oligodendrocytes, as indicated by a well-developed network of branched processes and by the expression of myelin oligodendrocyte glycoprotein and

glutamine synthetase [16]. The T₃ deficiency changes the distribution of oligodendrocyte/myelin markers during oligodendroglial differentiation *in vitro* [9]. However, our results show that T₃ at concentration of 25 ng/mL is more effective than 10-ng/mL concentration. Kang *et al.* [17] have generated NPC by exposing human embryonic stem cells to several inducing agents in DMEM/F12 supplement and then by subjecting them to growth factors EGF, PDGF, bFGF and T₃ (30 ng/mL). By this method, they achieved 81% differentiation into OLC [17]. However,

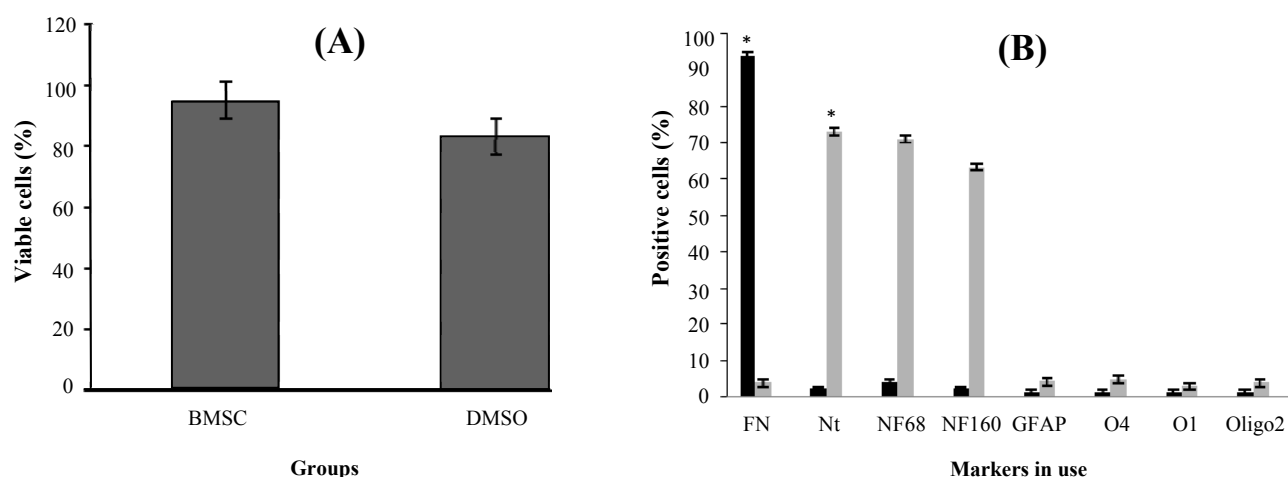


Fig. 2. Histograms of quantitative analysis of viability and different markers by immunocytochemistry at pre-induction and induction stages. **(A)** The percentage of the viable cells in untreated and treated bone marrow stromal cells (BMSC) at pre-induction stage. The histogram shows the viable cells in untreated BMSC (control) and the DMSO-retinoic acid treated cells. The viability was higher in the control group compared to the other group. **(B)** Quantitative analysis of different markers by immunocytochemistry at pre-induction stage. Black columns shows untreated BMSC and light gray columns shows BMSC treated with dimethyl sulfoxide-retinoic acid. The fibronectin (FN) shows a significant difference in each group. *indicates statistical significance between BMSC and the other experimental groups ($P < 0.001$). Nt, nestin; NF, neurofilament; GFAP, glial fibrillary acidic protein

this result may be due to the source of cells used for differentiation, which resulted in a higher percentage of oligodendrocytes. The current study supports the results of previous research on the effect of T_3 on the differentiation stem cells into OLC [18].

Our RT-PCR results showed that OLC expressed *PDGFR- α* , while untreated BMSC and NPC did not. It was first reported that PDGF could be the cause of proliferation and differentiation of oligodendrocyte

progenitor cells. The PDGF and its receptors are widely expressed in both embryonic and adult central nervous system [19]. In previous studies, the expression of PDGF in OLC derived from different regions of the central nervous system such as brain has been studied, but the expression on bone marrow-derived OLC has not been studied [20]. Kaka *et al.* [9] could differentiate OLC from BMSC, but *PDGFR* gene expression was not evaluated. Our data showed

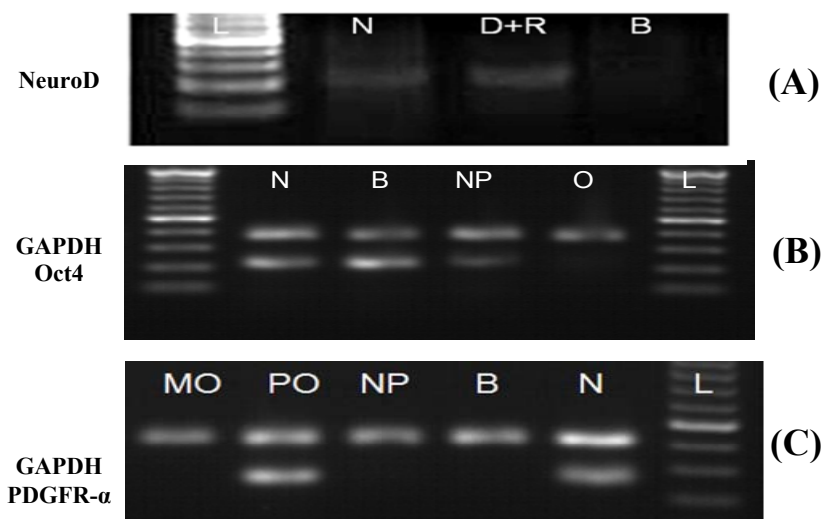


Fig. 3. Electrophorograms of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, 400 bp), *NeuroD* (220 bp), *Oct-4* (210 bp) and platelet-derived growth factor α (*PDGFR- α* , 190 bp) using RT-PCR. **(A)** The electrophorogram of *NeuroD* gene expression profile at positive control (rat neonate brain, N), pre-induction stage (dimethyl sulfoxide-retinoic acid, D+R) and a negative control (untreated bone marrow stromal cells [BMSC], B). **(B)** The electrophorogram of *Oct-4* gene expression profile in rat neonate brain (N), BMSC (B), neuroprogenitor (NP) cells and oligodendrocyte-like cells (O). **(C)** The electrophorogram of *PDGFR- α* gene expression profile in mature oligodendrocyte (MO), pre-oligodendrocyte (PO), neuroprogenitor cells (NP), BMSC (B) and rat neonate brain (N). L shows DNA ladder.

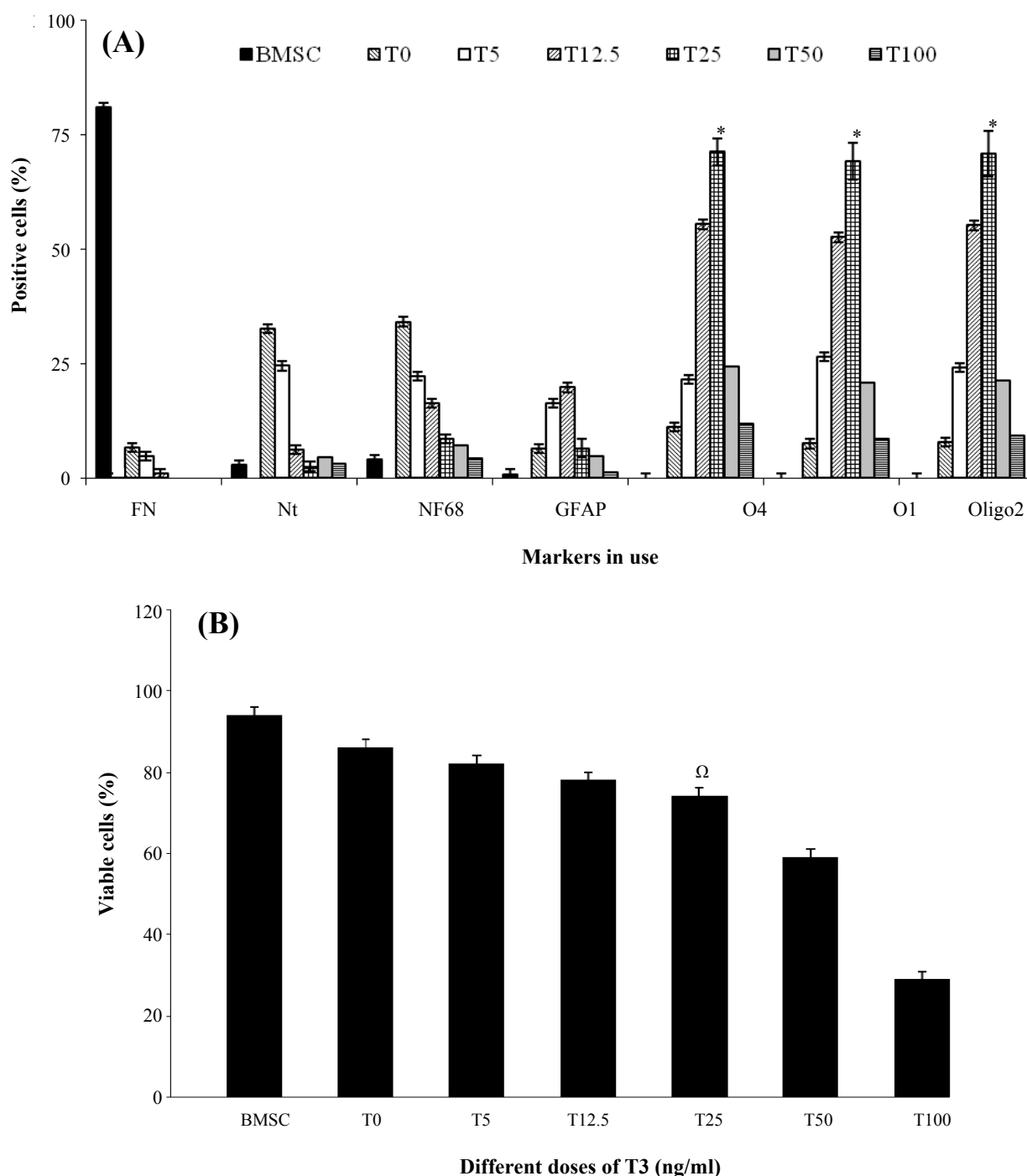


Fig. 4. Histograms of quantitative analysis of viability and different markers by immunocytochemistry at induction stages. **(A)** The percentage of the immunoreactive cells. Triiodothyronine (T3) was added to the induction stage in concentrations of 0, 5, 12.5, 25, 50, 100 and 200 ng/ml. Untreated bone marrow stromal cells (BMSC) were used as a control group. In addition to T3, the cells were pre-induced with dimethyl sulfoxide-retinoic acid and induced with platelet-derived growth factor (PDGF), fibroblast growth factor and heregulin. The assessed markers were: fibronectin (FN), nestin (Nt), neurofilament 68 (NF68), glial fibrillary acidic protein (GFAP), O4, O1 and Oligo2. There were statistically significant differences among the groups in the same stage. A significant increase was noted for percentages of O4, O1 and oligo2 ($P < 0.05$), while NF68 was significantly increased in the pre-induction stage. *indicates a significant difference among cells treated with 0, 5 and 12.5 ng/ml of T3. **(B)** The percentage of viable cells at the induction stage in a dose response of T3 (0, 5, 12.5, 25, 50, 100 and 200 ng/mL) as an inducer following pre-induction with PDGF, basic fibroblast growth factor and heregulin. Increased T₃ concentrations (50, 100 and 200 ng/ml) in the medium caused lower viability. ^Ω indicates a significant difference among cells treated with 0, 5 and 12.5 ng/ml of T3.

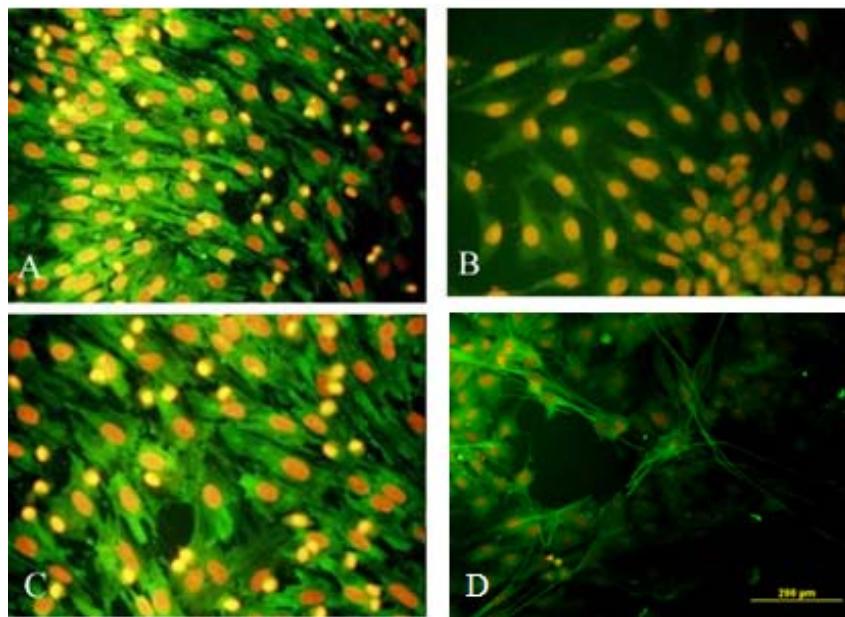


Fig. 5. Immunocytochemistry analysis of the transdifferentiated bone marrow stromal cells into oligodendrocyte-like cells. Immunostained cells with (A) anti-O1, (B) anti-oligo2, (C) anti-O4 and (D) anti-myelin basic protein antibodies.

that OLC-derived BMSC can express. Several studies show that PDGF has a neurotrophic effect, neuroprotection, and neuronal differentiation effect [21] on glial and neural cells. *In vitro*, PDGF is a survival factor and also an effective mitogen for oligodendrocyte progenitor cells, but it prompts only a limited number of cell division [22]. In our study, PDGF along with other inducers was added to culture medium that caused the differentiation and proliferation of NPC into OLC. We observed that apoptosis of OLC was increased in lack of PDGF.

Recently, it has been demonstrated that PDGF is a survival factor for oligodendrocyte progenitors in impaired oligodendrocyte development in the PDGF-A deficient mice [23]. These mice are characterized by a reduction in the numbers of *PDGFR-α* progenitors and oligodendrocytes in the spinal cord and cerebellum and the medulla. Injection of PDGF into mice greatly reduced apoptosis [24]. It seems that PDGF influences on development of oligodendrocyte progenitor cells by blocking the intracellular signaling pathways from the PDGF receptor to the gene expression [25]. We recognized *olig2* expression at induction stage, which is in agreement with the findings of other investigations [26]. OPC and mature oligodendrocyte express *Olig2* transcription factors, the importance of *Olig2* for the differentiation of neural progenitor cells into the oligodendroglial lineage have discovered in other studies [27], over expression of *Olig2* stimulates differentiation of neural stem cells into mature oligodendrocytes *in vitro* [28]. Disruption of *Olig2* causes an interrupted development of oligodendrocytes in the spinal cord and lack of oligodendrocyte

progenitor cells called NG2 cells [29]. Mature oligodendrocytes expressed low levels of *Olig2* transcription factors [30]. The differentiated oligodendrocytes also expressed MBP, which is an important marker for maturity [31].

Moreover, MBP was considered as a functional marker for oligodendrocytes [32] and confirmed in time-lapse imaging [33]. The prior expression of *PDGFR-α* (at pre-oligodendrocytes) with immunopositivity for MBP (in differentiated oligodendrocytes) may indicate the necessity for the presence of PDGF for maturation of these cells [34]. Amur-Umarjee *et al.* [35] reported that MBP mRNA is transported into the oligodendrocytes. Double immunostaining showed the simultaneous expression of MBP and *PDGFR-α*, suggesting the importance of these receptors for the maturation of oligodendrocytes.

Our results show that transdifferentiation of BMSC into the OLC can be achieved by pre-induction with DMSO + retinoic acid and using bFGF, PDGF, HRG and T3 (25 ng/ml) as inducers. The OLC obtained through the described procedure can be used as a potential cell source for transplantation and treatment of central nervous system disorders.

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