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Dopaminergic-like cells from epigenetically reprogrammed mesenchymal stem cells

Zhiying Zhang, Arshak R. Alexanian *

Department of Neurosurgery, Neuroscience Research Labs, Medical College of Wisconsin, VAMC, Milwaukee, WI, USA

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Abstract

A number of recent studies have examined the ability of stem cells derived from different sources to differentiate into dopamine-producing cells and ameliorate behavioural deficits in Parkinsonian models. Recently, using the approach of cell reprogramming by small cell-permeable biological active compounds that involved in the regulation of chromatin structure and function, and interfere with specific cell signalling pathways that promote neural differentiation we have been able to generate neural-like cells from human bone marrow (BM)-derived MSCs (hMSCs). Neurally induced hMSCs (NI-hMSCs) exhibited several neural properties and exerted beneficial therapeutic effect on tissue preservation and locomotor recovery in spinal cord injured rats. In this study, we aimed to determine whether hMSCs neuralized by this approach can generate dopaminergic (DA) neurons. Immunocytochemisty studies showed that approximately 50–60% of NI-hMSCs expressed early and late dopaminergic marker such as Nurr-1 and TH that was confirmed by Western blot. ELISA studies showed that NI-hMSCs also secreted neurotrophins and dopamine. Hypoxia preconditioning prior to neural induction increased hMSCs proliferation, viability, expression TH and the secretion level of dopamine induced by ATP. Taken together, these studies demonstrated that hMSCs neurally modified by this original approach can be differentiated towards DA-like neurons.

Keywords: mesenchymal stem cells • epigenetic • reprogramming • neural cells • neurotrophins • dopamine

Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by progressive degeneration of nigrostriatal DA neurons.

At present, replacement of the progressively degenerated DA neurons through cells transplantation is considered to have the most potential as a therapy [1,2]. However, any clinically viable treatment option would require a source of replacement cells that are readily available, easily expanded, can differentiate into the dopaminergic neuronal cell type, and most importantly will not be rejected by the host immune system.

Recent advances in generating multi- or pluripotent stem cells *via* genetic reprogramming of adult somatic cells made it possible to generate desirable tissue-specific stem cells from the patient's own somatic cells [3–8]. Although cells generated by this technology can be used with minimal risk of immunorejection, there are several other issues concerning the safety and practicality of the use of these approaches.

Recently, several new reprogramming strategies have been attempted to overcome several safety and efficiency issues associated with genetic approach. The chemical (chemical genetics) approach is one of them which could be a safer, easier and more feasible alternative. Although this new strategy of reprogramming is still in its infancy and mostly has been used to increase the efficiency of some of the iPS reprogramming technologies [9], recently several studies showed the effectiveness of this approach for transdifferentiation (lineage switches) of cells [10–12].

The differentiated state (epigenetic state) of the cells that are going to be used for such manipulations is another important factor to achieve successful reprogramming. It has been demonstrated that less differentiated (immature) cells exhibit higher plasticity and are more pliable for reprogramming [13]. Mesenchymal stem cells (MSCs) are an example of the cells with high plasticity as they can be induced to differentiate into multiple cell lineages. MSCs in their immature state express a variety of genes of three germ layers at relatively low or moderate level that might explain their phenomenal plasticity [14,15]. Numerous recent studies demonstrated that at

*Correspondence to: Arshak R. ALEXANIAN, Ph.D., VMD,
Associate Professor – Department of Neurosurgery, Neuroscience Research Labs, Medical College of Wisconsin, VAMC, 5000 W. National Ave 151, Milwaukee, Wisconsin 53295, USA.
Tel.: +414 384 2000, Ext. 41468
Fax: +414 384 3493
E-mail: aalexan@mcw.edu

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appropriate conditions *in vitro* and *in vivo* the expression of different set of these genes can be up-regulated, turning MSCs into variety of cell lineages of mesodermal, ectodermal and endodermal origins [15]. Although transdifferentiation of MSCs is still controversial, these unique properties make MSCs an ideal autologous source of easily reprogrammable cells.

Recently, using chemical genetics approach for reprogramming (by using a specific combination of small cell-permeable biological active compounds that involved in the regulation of chromatin structure and function, and interfere with specific cell signalling pathways that promote neural differentiation), we have been able to generate neural-like cells from hMSCs [16,17]. NI-hMSCs exhibit several neural characteristics such as neural morphology, expression of specific neural markers, secretion of neurotrophic factors, formation of synapses and electrophysiological properties of maturing neurons. Most importantly, NI-hMSCs survive and differentiate after transplantation into the injured spinal cords of rats, promote tissue preservation and significantly improve locomotor recovery in injured animals [18]. Thus, hMSCs neurally modified by this method may provide an alternative source of autologous adult stem cells that could be useful for the treatment of several neurological disorders.

With this study we aimed to determine whether hMSCs neuralized by this approach can generate DA neurons and whether hypoxic preconditioning will modulate proliferation and viability of cells and improve DA differentiation.

Materials and methods

Expansion of hMSCs

Human bone marrow MSCs (frozen at passage 1) was provided by Tulane University Center of Gene Therapy. According to the product specification sheet, human bone marrow aspirate was drawn and mononuclear cells were separated using density centrifugation. The cells were plated to obtain adherent human marrow stromal cells, which were harvested when cells reached 60–80% confluence. These specimens were considered passage zero (P0) cells. These P0 cells were expended, harvested and frozen at passage 1 (P1) for distribution. Prior to release, two trials of the frozen P1 cells were analysed over three passages for Colony-Forming Units, cell growth, and differentiation into fat, bone and chondrocytes (at P2 only). These characterized hMSCs from P1 were expanded in MEM-Alpha Medium containing 10% foetal bovine serum (FBS; StemCell Technologies, Vancouver, Canada), 100 μ/ml penicillin, 100 mg/ml streptomycin and 2 mM Glutamine (Sigma-Aldrich Corp., St. Louis, MO, USA), and used for additional studies.

Hypoxic preconditioning

Before neural induction expanded hMSCs were grown in hypoxic condition. The hypoxia chamber (Modular Incubator Chamber; Billups-Rothenberg Inc., Del Mar, CA, USA) filled with a mixture of 4% O_2, 5% CO_2 and 91% N_2 was used. Before neuronal induction treatment, hMSCs were incubated in hypoxia chamber for 48 hrs.

Cell viability assay for hMSCs grown under normoxic and pre-hypoxic conditions

HMSCs were plated onto 96-well tissue culture treated plates at a cell density of 1000/well and incubated in DMEM containing 15% FBS at hypoxic (4%O_2, 5% CO_2, 91%N_2, 37°C) or at normoxic (21%O_2, 5% CO_2, 37°C) conditions for 24 hrs. Later different dosages of H_2O_2 at the concentrations of 0, 20, 60, 120, 200 and 300 μM were added to the medium and incubated for another 24 hrs. To investigate the protective effect of pre-hypoxia on cell viability, a MTT assay (colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple colour) was performed. Ten microlitres of MTT (5 mg/ml) reagent was added to each well, including controls and incubated for 4 hrs until a purple precipitate was clearly visible within the cells under the microscope. The culture medium was then discarded and replaced with 100 μl of a detergent reagent to each well, including controls. The plate was incubated in the dark for 4 hrs and quantified spectrophotometrically at a wavelength of 570 nm with a reference wavelength of 630 nm. The following formula was used to calculate cell viability: percentage cell viability = (absorbance of the experiment samples/absorbance of the control) 100.

Cell proliferation assay

HMSCs were plated on to tissue culture treated plates (22.1 cm^2) at a cell density of 220 cell per cm^2 in MEM-Alpha medium containing 15% FBS and L-glutamine and grown in hypoxic or at normoxic conditions for 5 days. Later cells were trypsinized (0.25% Trypsin/EDTA), counted, and replated with the same density. This process was repeated for cell passages P5–P8. All assays were carried out in triplicate. The statistical significance was assessed using StatView, SAS.

Neural induction

Neural induction was performed by the method described recently [14,17]. Briefly, hMSCs from early passages (P2–P4) were exposed to 200 nM trichostatin A (TSA) (inhibitor of histone deacetylases), 50 μM RG-108 (DNA methyltransferase inhibitor), 300 μM 8-BrcAMP (highly stable, biologically active form of cAMP) and 1 μM Rolipram (inhibitor of phosphodiesterases), in the medium of NeuroCult/N_2 supplemented with 20 ng bFGF.

Immunocytochemistry

For immunocytochemistry, 2 weeks-treated NI-hMSCs were fixed with 4% paraformaldehyde and stained for early and mature dopaminergic neurons marker Nurr1 and tyrosine hydroxylase (TH). First, cells were permeabilized 10 min. with 0.2% Triton X-100 in PBS, followed by blocking with 5% Goat serum in PBS for 30 min., then incubated for 1 hr with one of the following primary antibodies in PBS, Mouse monoclonal anti-β-III-tubulin (B3T) (1:750, Covance, Madison, WI, USA), Rabbit polyclonal anti-Nurr1/NOT1 (1:300, Millipore, Billerica, MA, USA), Rabbit polyclonal anti-tyrosine hydroxylase (1:150, Millipore), immunoreactive cells were visualized with Texas Red (TxR)-conjugated goat anti-rabbit IgG or fluorescent-conjugated (FITC) goat

anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). To reduce fluorescence quenching, glass cover slips were mounted in ProLong Antifade reagent (Molecular Probes Inc., Eugene, OR, USA) and dried on microscope slides. Representative images were captured by a Nikon inverted microscope equipped with colour digital camera (Spot II). Metamorph software (Universal Imaging) was for analysing the images and counting the cells. Nurr1 and TH immunopositive cells were counted after subtraction of slight fluorescence which observed in 90% of non-modified hMSCs. In the results section, values represent the average from two different experiments with standard deviations.

Western blot analysis

Cells grown in 6-well tissue culture plates for 24 hr, 1 and 2 weeks were used for preparation of whole cell extract. After removing the media, cells were rinsed twice with PBS and incubated in 1 ml/well trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) for 5 min. at 37°C. Cold, sterile 1 × PBS (1 ml/well) was added to each well after incubation. The suspension was centrifuged for 5 min. at 500 g. After cells were resuspended in 60–120 μl lysis buffer (50 mM Tris HCl, 1 mM EGTA, 1% SDS, 1 mM EDTA, 5 μl/ml PMSF, 1% IGPAL, 10 μl/ml protease inhibitor cocktail) and kept on ice for 30 min. The mixture was centrifuged at 16,000 g for 15 min. at 4°C and the supernatants were collected. The protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific Inc., Miami, OK, USA). The samples were frozen at −80°C. For SDS-polyacrylamide gel electrophoresis equal amounts of protein extracted from cells (10 μg) were resolved on 4–15% polyacrylamide gradient (Bio-Red, Richmond, CA, USA). For Western blotting, proteins were transferred to nitrocellulose membrane by Trans-Blot SD Electrophoretic Transfer Cell. After blocking with 5% dry milk reconstituted in Tris-buffered saline, blots were incubated with the following primary antibodies: Rabbit polyclonal anti-Nurr1/NOT (1:1000, Millipore), rabbit polyclonal anti-TH (1:800, Millipore). After incubation with goat anti-rabbit HRP-conjugated secondary antibody (1:6000, Pierce Chemical Company, Rockford, IL, USA), the protein bands were detected using the chemiluminescent substrate SuperSignal West Dura or SuperSignal West Maximum Sensitivity Substrate (Pierce Chemical Company, Rockford, IL, USA) and by capturing and digitizing the images with Kodak Image Station 2000 MMT. To normalize the expression of these genes with actin, the membranes were incubated 25 min. by Restore

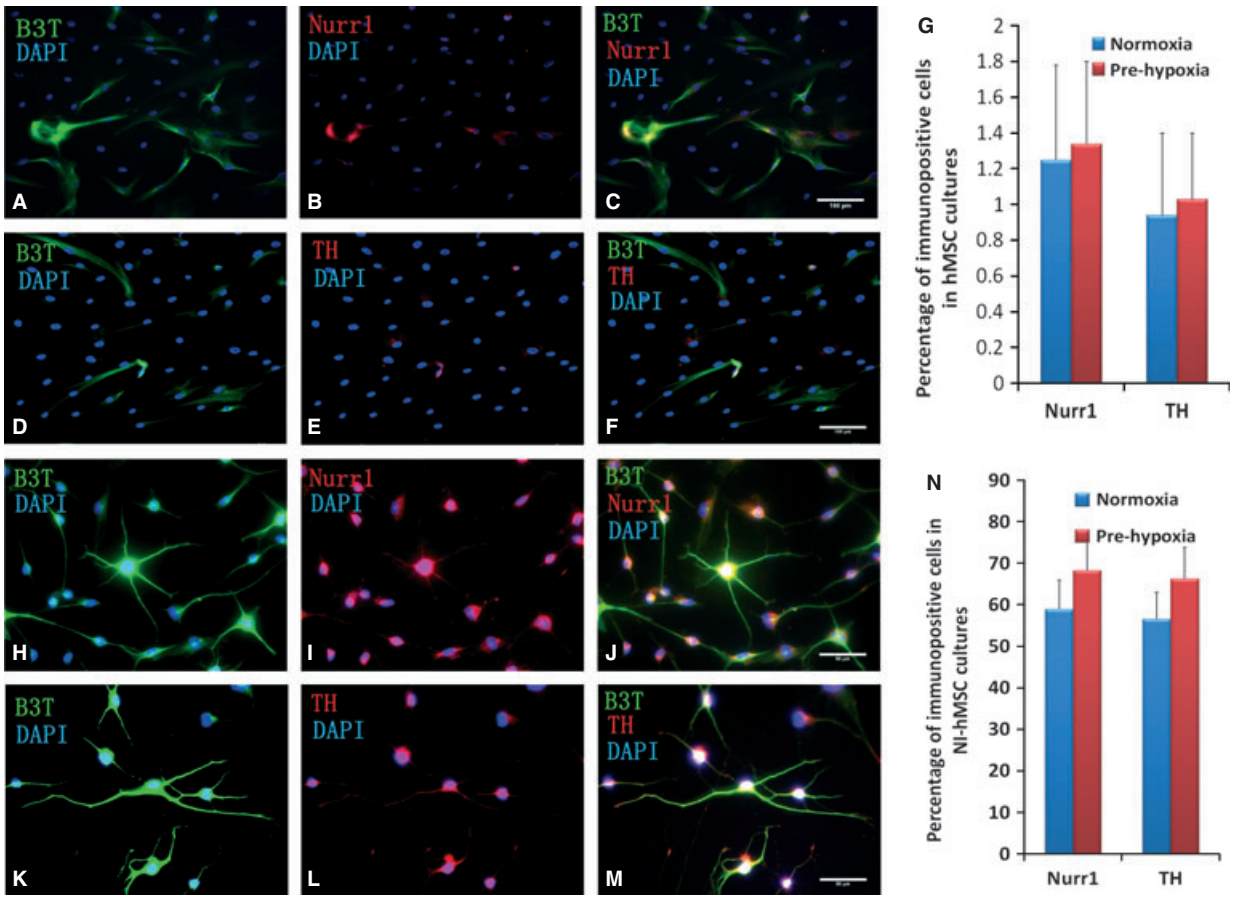

Fig. 1 Representative immunofluorescence images of hMSCs (**A**–**F**) and NI-HMSCs (**H**–**M**) for neuronal marker B3T and DA markers Nurr1 and TH. Cells nuclei were counterstained with DAPI. Graphs represent the percentage of cells positive to each DA marker in hMSCs (g) and NI-hMSCs (n) grown in pre-hypoxic and normoxic conditions.

Western Blot Stripping Buffer (Thermo Scientific), re-blocked with 5% BSA, and incubated with mouse monoclonal anti-β-actin (1:7000, Millipore). After incubation with goat anti-mouse HRP-conjugated secondary antibody the protein bands were detected as described above. The experiments were carried out in duplicates and the data are shown as standard error of the mean. The statistical significance was assessed using one-way ANOVA followed by Tukey's pair comparisons. *P* values < 0.05 were considered significant.

NGF, NT-3, GDNF, BDNF and Dopamine ELISA

To study whether or not differentiated NI-hMSCs release neurotrophic factors, Emax immunoassay system kit for NGF, NT-3, GDNF, BDNF (Promega Corporation, Madison, WI, USA) was used. For dopamine release studies dopamine kit (Labor Diagnostika Nord GmbH & Co. KG, Am Eichenhain, Nordhorn, Germany) was used.

Emax immunoassay system is designed for the sensitive and specific detection of neurotrophic factors such as NGF, NT-3, GDNF and BDNF in an antibody sandwich format. For these studies flat-bottom 96-well plates were coated with Polyclonal Antibody (pAb) of one of the neurotrophic factors which binds soluble NGF, NT-3, GDNF or BDNF. Samples (100 μl) from differentiated NI-hMSCs that were grown for 24 hr prior to immunoassay in defined medium such as Neurobasal-A/B27, was added to antibody-coated wells. Next, the captured neurotrophic factors were bound by second specific monoclonal antibodies (mAbs). After washing, the amounts of specifically bounded mAbs were detected using a species-specific antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. The unbound conjugates were removed by washing, and samples incubated with a chromogenic substrate. The colour change was measured at 450 nm on the plate reader within 30 min. of stopping the reaction. The NGF, NT-3, GDNF and BDNF standards provided with this system were used for generation a linear standard curve from 7.8 to 500 pg/ml. Data represent the means of two independent experiments performed in duplicate. Control test did not indicate any neurotrophins in Neurobasal-A/B27.

To study whether or not NI-hMSCs secrete dopamine, a dopamine kit (Labor Diagnostika Nord GmbH & Co.KG) was used. For these studies at the end of 2 weeks of neural induction of hMSCs, the neural induction medium was replaced with a defined Neurobasal-A/B27 medium 700 μl, followed by incubating at 37°C for 5 min. In another two experiments, in addition to medium replacement, the cells were treated with elevated K^+ solution (KCl 56 mM) for 5 min. to induce depolarization, or ATP (100 μM) for 1 min. respectively. To the collected medium, 1 mM EDTA and 4 mM sodium metabisulphite were added to prevent catecholamine degradation. DA was quantitated by Dopamine Research ELISA kit according to the manufacturer's instruction (Labor Diagnostika Nord GmbH & Co.KG). The DA standards provided with this system were used for generation of an exponential standard curve from 0.9 to 30 ng/ml. Data represent the means of three independent experiments performed in duplicate.

Results and discussion

The immunocytochemistry, Western blot and ELISA methods were used to study whether or not NI-hMSCs, reprogrammed by recently developed epigenetic approach, will produce dopaminergic-

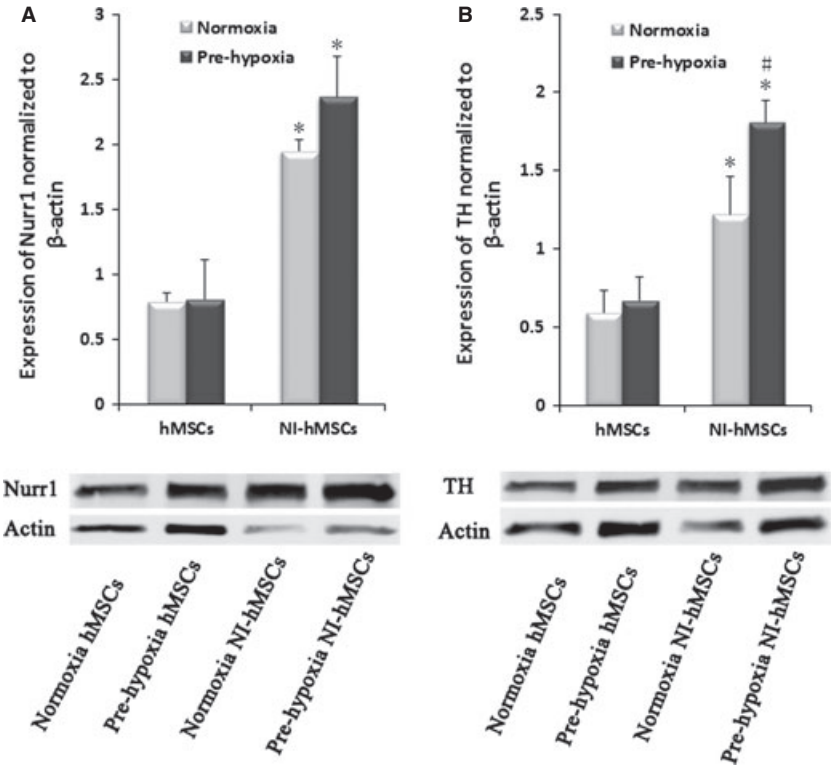

Fig. 2 Western blot analysis of Nurr1 (**A**) and TH (**B**) expression in hMSCs and NI-hMSCs grown in normoxic and hypoxic conditions. Expression of protein normalized to b-actin. $*P < 0.05$, comparison of the expression of Nurr1 and TH between hMSCs and NI-hMSCs; $\#P < 0.05$, comparison of the expression of TH between normoxic and hypoxic pre-conditioned NI-hMSCs.

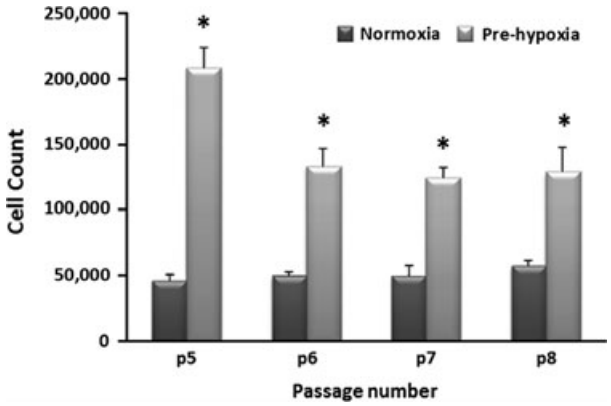

Fig. 3 Cell proliferation assay for normoxic or pre-hypoxic conditioned hMSCs grown for 4 passages. $*P < 0.05$.

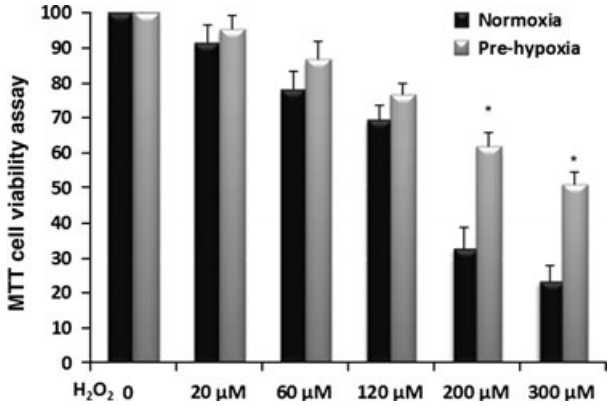

Fig. 4 MTT cell viability assay for hMSCs grown under normoxic and pre-hypoxic conditions. $*P < 0.05$.

like cells *in vitro* and whether hypoxic preconditioning prior to neural induction could affect hMSCs proliferation and viability and DA differentiation. Several recent studies demonstrated that hMSCs grown in hypoxic condition maintained significantly higher colony-forming unit capabilities and expressed higher levels of stem cell genes than hMSC cultured at normoxic conditions [19–22]. Hypoxic preconditioning can also change differentiation potential of MSCs [22].

To investigate DA differentiation ability of cells first we performed immunocytochemistry to study the expression of DA specific markers Nurr1 and TH in hMSCs and NI-hMSCs grown in normoxic (21%O_2, 5% CO_2) or hypoxic (4%O_2, 5% CO_2, 91%N_2) conditions. Results showed that the small percentage of hMSCs were immunopositive to Nurr1 ($1.25 \pm 0.53\%$) and TH (0.97 ± 0.42)% (Fig. 1A–G). Neural induction increased the percentage of Nurr1 and TH to $58.96 \pm 7.02\%$ and $56.64 \pm 6.35\%$ respectively (Fig. 1H–N). The hypoxic preconditioning did not significantly affect the expression of these genes neither in HMSCs nor in NI-hMSCs. Double labelling studies showed that the expression of these genes and neuronal marker B3T were co-localized (Fig. 1H–M). The majority of Nurr1 and TH positive cells exhibited typical neuronal morphology with small bipolar or multipolar cell bodies and extended long neuritic processes. Such a drastic change in morphology and expression profile observed after 2–3 weeks of neural induction. The expression of Nurr1 and TH in hMSCs and NI-hMSCs was confirmed with Western blot. Expression levels of these genes were significantly higher in NI-hMSC (Fig. 2A and B). Expression of Nurr1 in pre-hypoxic conditioned hMSCs and NI-hMSCs showed tendency of increase without any statistical significance. However, TH expression was significantly higher in pre-hypoxic conditioned NI-hMSCs (Fig. 2B). To investigate whether or not hypoxic preconditioning could affect hMSCs proliferation and viability, cell count and MTT assays were used respectively. Cell proliferation assay results showed that hypoxic preconditioning enhanced hMSCs proliferation at least during P5–P8, compared to cells grown in normoxic conditions (Fig. 3). Viability assay results demonstrated protective effect of hypoxic preconditioning on H_2O_2 induced cytotoxicity which was statistically significant for higher doses of H_2O_2 (Fig. 4). To study whether or not NI-hMSCs secrete neurotrophins and dopamine, ELISA approach was used. ELISA studies for neurotrophins demonstrated that hMSCs expressed neurotrophic factors BDNF and GDNF that increased with neural induction (Fig. 5A and B). Hypoxic preconditioning slightly increased (statistically not significant) the release of neurotrophins. For dopamine release studies a kit for dopamine ELISA was used. The studies showed that non-modified hMSCs

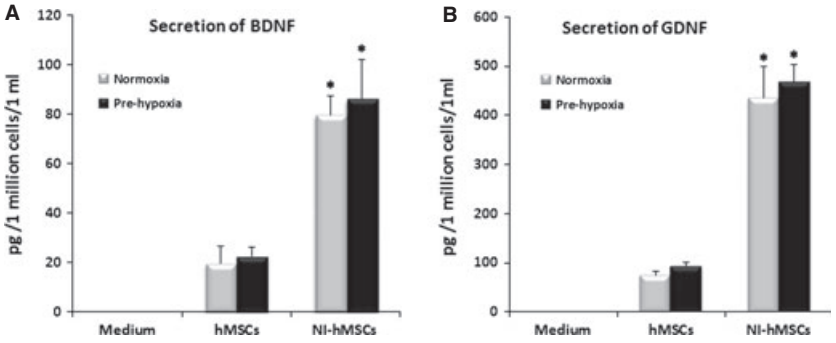

Fig. 5 Secretion of BDNF (**A**) and GDNF (**B**) by normoxic or pre-hypoxic preconditioned hMSCs and NI-hMSCs. $*P < 0.05$.

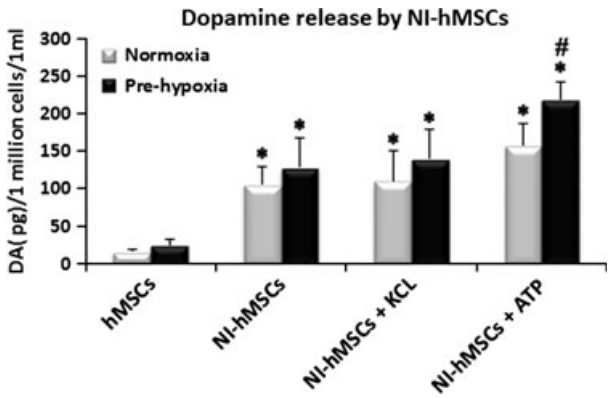

Fig. 6 Secretion of dopamine by hMSCs and NI-hMSCs and by NI-hMSCs treated with KCL and ATP in normoxic and hypoxic conditions. $*P < 0.05$, comparison of the expression of dopamine by hMSCs and NI-hMSCs; $\#P < 0.05$, comparison of the expression of dopamine between normoxic and hypoxic pre-conditioned NI-hMSCs.

secreted dopamine that significantly increased with neural induction (Fig. 6). Although statistically not significant, increased tendency in the secretion of dopamine by NI-hMSCs was observed in the samples that were treated with KCL. If the samples were cell treated with ATP a significant increase in dopamine release was observed in hypoxic preconditioned NI-hMSCs (Fig. 6). Non-responsiveness of hypoxic preconditioned NI-hMSCs to KCl suggests that they were not yet fully differentiated.

In conclusion, the result of this study suggests that bone marrow-derived hMSCs neurally modified by this original approach can be differentiated towards DA-like neurons with higher efficiency (50–60%) in contrast to previous studies (10–40%) [16–20]. Hypoxic preconditioning increased proliferation rate, cell viability to cytotoxic effect of H_2O_2, and DA differentiation efficiency. Higher DA differentiation ability of hMSCs expanded in hypoxic condition could be explained by their high plasticity, which cells usually lose with each passage when grown in normoxic condition. Further *in vivo* studies will reveal whether or not NI-hMSCs can produce mature functional DA neuronal cells *in vivo*.

Acknowledgements

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Conflict of interest

The authors confirm that there are no conflicts of interest

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