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The expression of pluripotency and neuronal differentiation markers under the influence of electromagnetic field and nitric oxide

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ABSTRACT

Nitric oxide (NO) is a diatomic free radical compound that as a secondary messenger contributes to cell physiological functions and its variations influence proteins activity and triggering intracellular signaling cascades. Low frequency electromagnetic field (EMF) alters the cell biology such as cell differentiation by targeting the plasma membrane and entering force to the ions and small electrical ligands. The effect of these chemical (NO) and physical (EMF) factors on the expression of the stemness and neuronal differentiation markers in rat bone marrow mesenchymal stem cells (BMSC) was investigated. The cells were treated with low (50 micromolar) and high (1 mM) concentrations of Deta-NO as a NO donor molecule and 50 Hz low frequency EMF. The expression of pluripotency and neuronal differentiation genes and proteins was investigated using real time qPCR and Immunocytochemistry techniques. The simultaneous treatment of EMF with NO (1 mM) led to the down-regulation of stemness markers expression and up-regulation of neuronal differentiation markers expression. Cell proliferation decreased and cell morphology changed which caused the majority of cells obtains neuronal protein markers in their cytoplasm. The decrease in the expression of neuronal differentiation Nestin and DCX markers without any change in the expression of pluripotency Oct4 marker (treated with low concentration of NO) indicates protection of stemness state in these cells. Treatment with NO demonstrated a double behavior. NO low concentration helped the cells protect the stemness state but NO high concentration plus EMF pushed cells into differentiation pathway.

1. Introduction

Nitric oxide is a gaseous, short-lived free radical produced from l-arginine by mediation of nitric oxide synthase (NOS) enzyme (Beltran-povea et al., 2015). This paramagnet diatomic and highly reactive molecule reacts with molecules such as oxygen, iron, nucleic acids and proteins and quickly converts into nitrate and nitrite (Heinrich et al., 2013). There are low concentrations (nano to pico molar) of NO in the cell physiological conditions. NO acts as a secondary messenger molecule and activates the guanylyl cyclase enzyme that leads to produce cGMP and trigger the intracellular signaling cascades in low concentrations (Miller and Megson, 2007). Therefore, NO low concentrations contribute to immune response and blood pressure regulation. In addition, it acts a neurotransmitter in neurons and help cells growth and proliferation (Tuteja et al., 2004). The increase of NO concentration (mM) causes post-translation modifications in proteins like nitration and nitrosylation of tyrosine and cysteine amino acids, which in turn leads to changing proteins' activity (León et al., 2016), producing the nitrite and nitrate, coupling to prosthetic group of proteins and finally influencing gene expression regulation, apoptosis, cell fate and differentiation process (Rath et al., 2014). The recent studies indicated nitric oxide contribution to cell differentiation process. High concentrations of NO increased the apoptosis and cell death, the remaining cells were polarized, and their morphology changed and progressed into differentiation. During embryonic development, NO concentration fluctuated (sometimes increased and decreased at other times), which indicated the role of this free radical in the embryonic differentiation (Beltran-Povea et al., 2015). NO high concentrations cause cell migration (Zhan et al., 2016) and displayed antibacterial effects (McMullin et al., 2005). It was shown that NO high concentration led to down-regulation of pluripotency markers expression like Oct4, Nanog (Mora-Castilla et al., 2014) and up-regulation of differentiation markers expression (Ciani et al., 2004). However, NO low concentration increased the pluripotency markers expression and helped the stemness state of

Abbreviations: BMSC, bone mesenchymal stem cells; DCX, doublecortin; EMF, electromagnetic field; NSE, enolase 2; NO, nitric oxide; NOS, nitric oxide synthase; RA, retinoic acid

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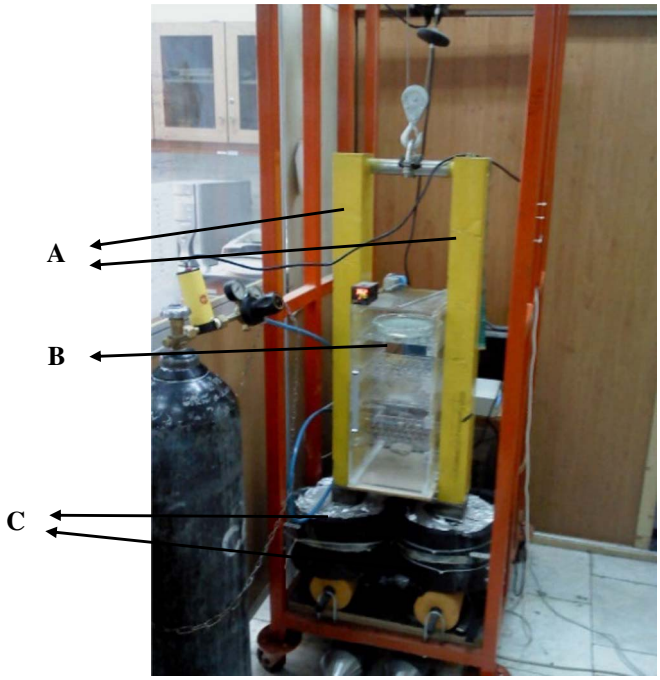


Fig. 1. EMF generator, (A) the iron blades, (B) the incubator, (C) the coils.

Table 1
Selected treatment groups.

Numbers	Groups (Real time qPCR)	Groups (Immunocytochemistry)
1	Ctrl (none treatment)	Ctrl (none treatment)
2	Retinoic acid (RA)	Retinoic acid (RA)
3	High concentration of NO (NO H)	EMF
4	Low concentration of NO (NO L)	High concentration of NO + EMF (NO H + EMF)
5	EMF	
6	High concentration of NO + EMF (NO H + EMF)	

stem cells (Beltran-povea et al., 2015).

Moving electric charge and solenoid including electrical flow produce magnetic field in their surrounding area. Changing electrical flow direction per time unit leads to alternative magnetic field. In addition, the change of magnetic field produces electric field or vice versa. These two fields exist simultaneously and are referred as electromagnetic field (EMF). The energy of magnetic field is less than the energy required to break chemical bonds, but it can alter the angle of bonds (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002). EMF enforces the ions and small ligands such as NO, influences on their velocity and dynamic and affects their binding to the receptors (Luo et al., 2014; Ross et al., 2015). The extent of EMF influence is more than that of electrical field in the cell and affects the organelle scale (Ross et al., 2015). EMF increases the free radicals stability and lifetime via the Zeeman effect (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002). The main target of EMF is plasma membrane. It changes the number of ions and ligands in the cell by influencing the conformation of membrane proteins, ion diffusion and ligands' binding to receptors (Ross et al., 2015). The change of ions and ligands number affects the intracellular signaling cascades and eventually influences the gene expression, histone Methylation and acetylation, transcription factors phosphorylation, cell fate and differentiation (Leone et al., 2015). Recent report indicated that EMF increased ROS via activating the membrane NADH oxidase. The increase of ROS via the signaling MAPK cascades phosphorylated CREB transcription factor and phosphorylated CREB stimulated the neuronal differentiation genes expression (Park et al., 2013). Another study showed that EMF facilitated BMSC differentiation into functional neural cells and increased the expression of neuronal specific genes by frequency and strength of 50 Hz and 5 mT, respectively (Bai et al., 2013). Other study showed that EMF increased ratio of differentiated neurons and promoted neurite outgrowth of embryonic neural stem cells by extremely low frequency (50 Hz, 1 mT) for 1, 2, 3 and 4-day treatment with 4 h per day. In addition EMF increased expression of pro-neural NeuroD and Ngn1 genes which are crucial for neuronal differentiation and neurite outgrowth (Park et al., 2013).

Retinoic acid (RA) is one of the most important morphogen chemical molecules and its embryonic distribution correlates with neural differentiation and positional specification in the developing central nervous system. All-trans retinoic acid (RA) and other active retinoids are generated from vitamin A (retinol) (Okada et al., 2004). RA binding initiates changes in interactions of RA receptors (RAR) with co-repressor and co-activator proteins, activates transcription of primary target genes, alters interactions with proteins that induce epigenetic changes. These changes induce the transcription of genes encoding transcription factors and signaling proteins that further modify gene expression (Gudas and Wagner, 2011). RA influences neural development in the early stage of CNS (Rhinn and Dolle, 2012). High-concentration of RA has been shown to promote neural genes expression and repress mesodermal genes expression. RA is one of the most important extrinsic inductive signals that can be used for neural differentiation of mesenchymal stem cells in vitro environment (Okada et al., 2004).

Due to the increase of neurodegenerative diseases, harmless physical factors like low frequency magnetic fields can be used to create polarity and produce neuronal cells for the treatment of such patients so in this study, the differentiation of Rat bone marrow mesenchymal stem cells into neuronal like cells was investigated through exposure to electromagnetic field with the frequency of 50 Hz and strength of 20 mT in the presence and absence of nitric oxide (1 mM and 50 μM) and retinoic acid (200 μM). For this purpose, created stable changes in the cells were considered by estimating the pluripotent and neuronal

Table 2
Gene specific primers.

Gene	Primer sequences	PCR conditions
Gapdh (NM_017008.4)	5′ CCC ATT CTT CCA CCT TTG ATG 3′ F5′ CCT GTT GCT GTA GCC ATA TTC 3′ R	95 °C/5 min; 95 °C/30s 61 °C/60s, 40 cycles
DCX (NM_053379.3)	5′ CTC CTA TCT CTA CAC CCA CAA G 3′ F5′ GGA ATC GCC AAG TGA ATC AG 3′ R	95 °C/5 min;95 °C/30s 61 °C/60s, 40 cycles
Nanog (AB275459.1)	5′ TCA AGG ATA GGT TTC AGA GGC 3′ F 5′ CAA TGG ATG CTG GGA TAC TC 3′ R	95 °C/5 min;95 °C/30s 60 °C/30s, 40 cycles
Oct4 (EU419996.1)	5′ GGG TTG AGT AGT TGT TTA GGG 3′ F 5′ GGG AGG TGG GTA TAG AGA AA 3′ R	95 °C/5 min;95 °C/30s 60 °C/60s, 40 cycles
Nestin (NM_012987.1)	5′ CAG ATG CTT GAG AGA CTG ATA G 3′ F 5′ CTG GTT CCT GCT TTC TAG TG 3′ R	95 °C/5 min;95 °C/30s 61 °C/60s, 40 cycles

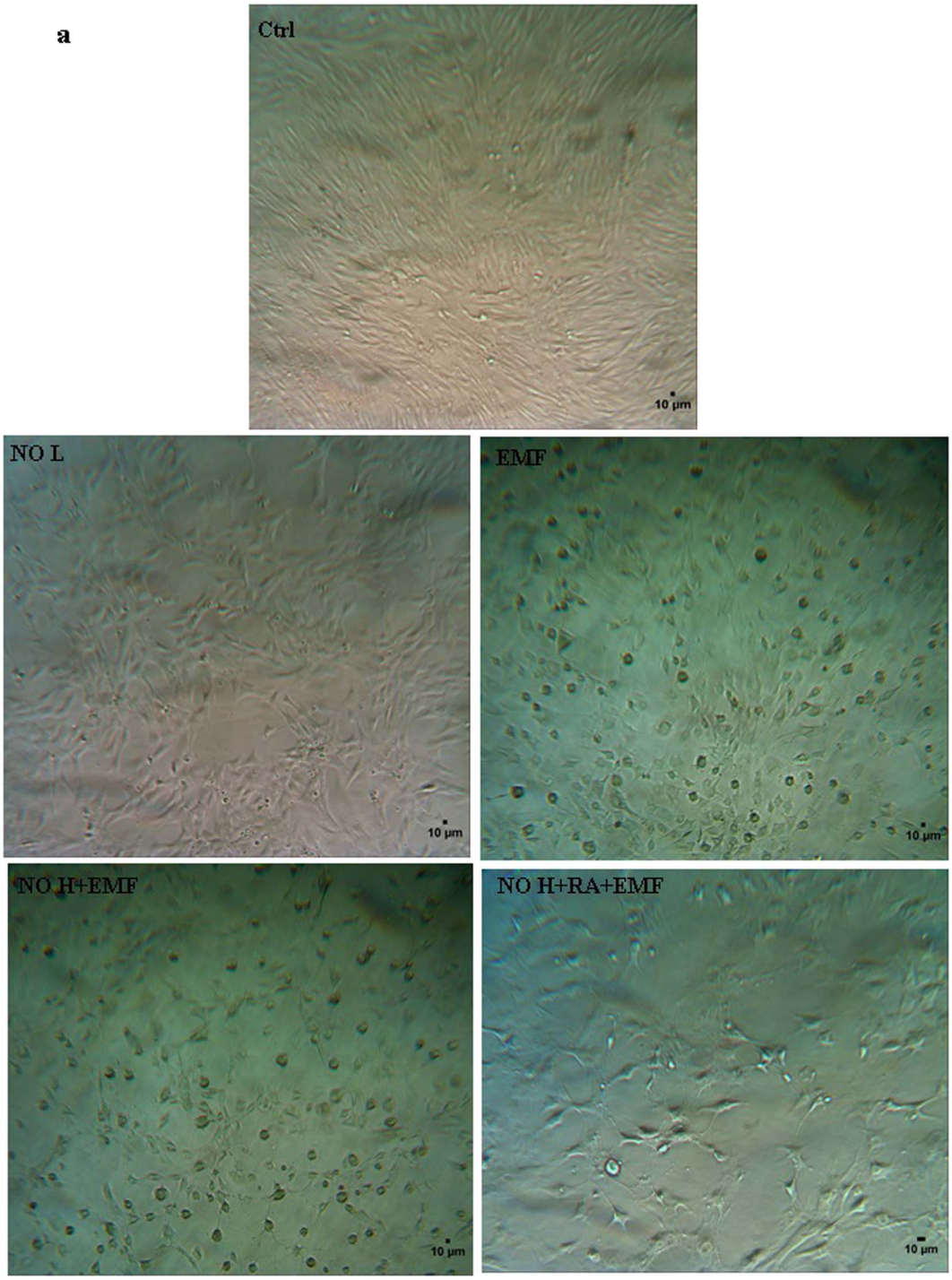


Fig. 2. BMSC passage 4 at different treatment conditions. (a) Microscope images (× 10). (b) Microscope images (× 40).

differentiation genes expression at the transcription and translation levels.

2. Material and methods

Mesenchymal stem cells were isolated from the femur and tibia bone of 6 to 8 weeks Wistar Rat weighting 240–250 g. The isolated cells along with isolated hematopoietic cells were transferred to α-MEM (Gibco, Germany) culture medium supplemented with 15% fetal bovine serum (FBS) (Gibco, Germany) and 1% penicillin/streptomycin (Gibco, Germany) and incubated at 37 °C with 5% humidified for 48 h. Non-adherent hematopoietic cells were discarded while adherent mesenchymal stem cells continued their growth after being washed with phosphate saline buffer (PBS). After cell proliferation and 80% confluence, cells were passaged by trypsin 1%. After 4 times passaging the cells and ensuring the removal of hematopoietic cells, the given treatments were investigated for the differentiation of BMSC into neuronal like cells.

2.1. Preparation of treatments

Deta-NO as a NO donor molecule was purchased from Germany Abcam Company. Different concentrations of this molecule were prepared and added to culture medium with respect to its molecular

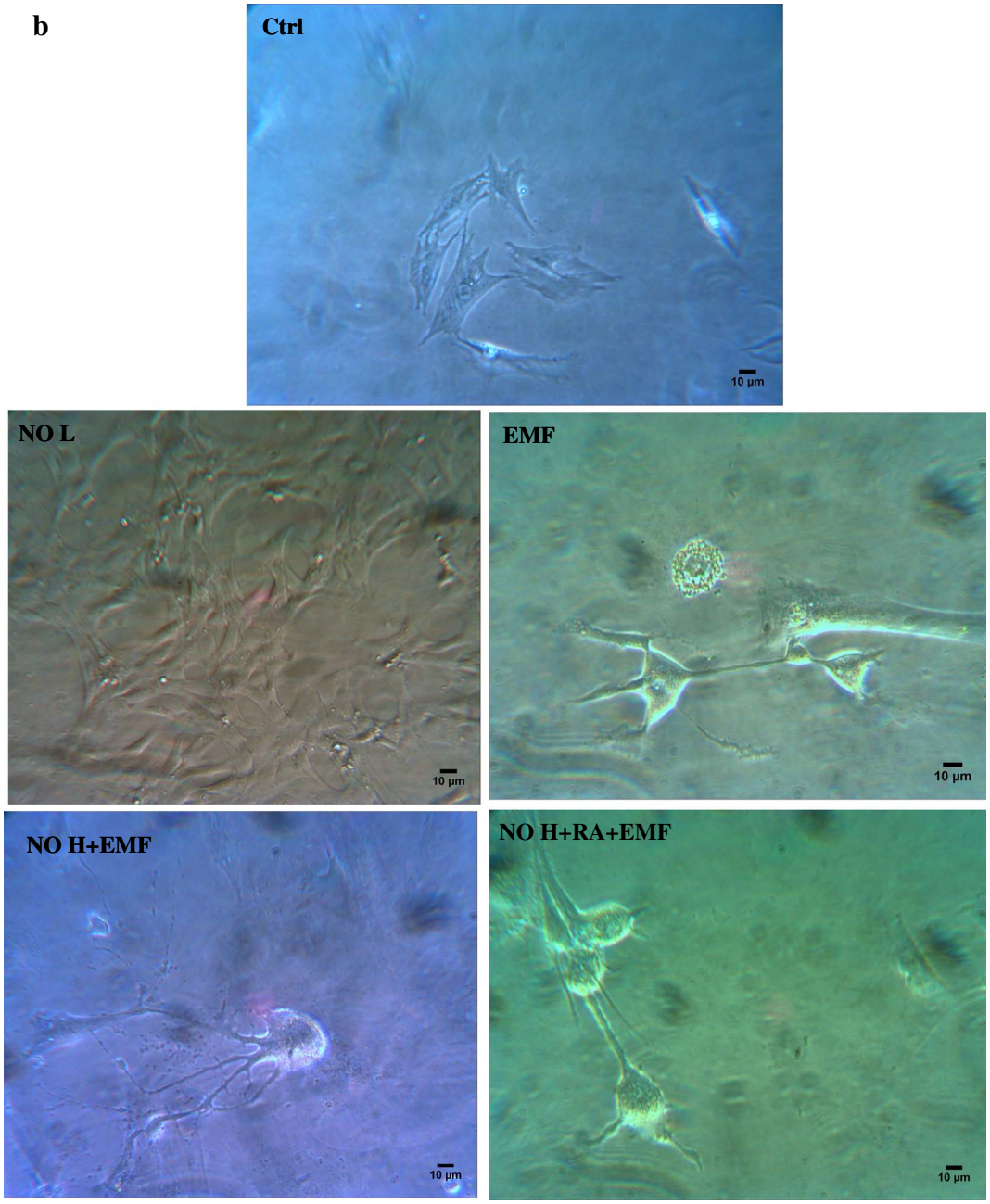


Fig. 2. (*continued*)

weight. In this study, all-trans retinoic acid (Sigma, USA) was used as a molecule facilitating neuronal differentiation. Exposure to EMF was performed by a locally designed EMF generator (Fig. 1). The magnetic field generator consisted of two coils. The coils were made of a wire (30 mm diameter) which was resistant to heat up to 200 °C. Wire length in each coil was about 1 km and each coil weighted approximately 40 kg. Coils had a total resistance and inductance of 3 Ω and 2 H, respectively. EMF affects numerous biological functions such as gene expression, cell fate, and cell differentiation, but will only induce these effects within a certain range of low frequencies (50 Hz) as well as low amplitudes (20 mT) (Ross et al., 2015) so by connecting the AC power to this system, an electromagnetic field was generated (with 50 Hz frequency and 20 mT strength). These two coils guided the generated EMF through two iron blades with 1 m heights and 10 cm^2 surface areas. Using three different sensors, the controller system was able to control the temperature, humidity and CO_2 level. In order to cool off the system, a gas cooler with optimum control on temperature was used. This system was consisted of evaporator, motor, condenser and gas. The evaporator covered the outer surface of coils, which caused to cool off the coil effectively. The cell samples were laid in incubator between two iron blades. Table 1 shows selected different groups.

2.2. Gene expression estimation using real time qPCR

Since, the gene expression need a relatively long time so the $BMSC_s$ cells were treated for a week with Retinoic acid (200 µM), Nitric oxide low concentration (50 µM), Nitric oxide high concentration (1 mM) and exposure to EMF (with frequency and strength of 50 Hz and 20 mT, respectively). Total RNA was then extracted from $BMSC_s$ using the RNAsol reagent (Noyafan, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran) according to manufacturer instruction (Simms et al., 1993). The reading of extracted RNA absorption was at 260, 230 and 280 nm wave lengths in order to measure RNA concentration and ensure that there is no phenolic and protein

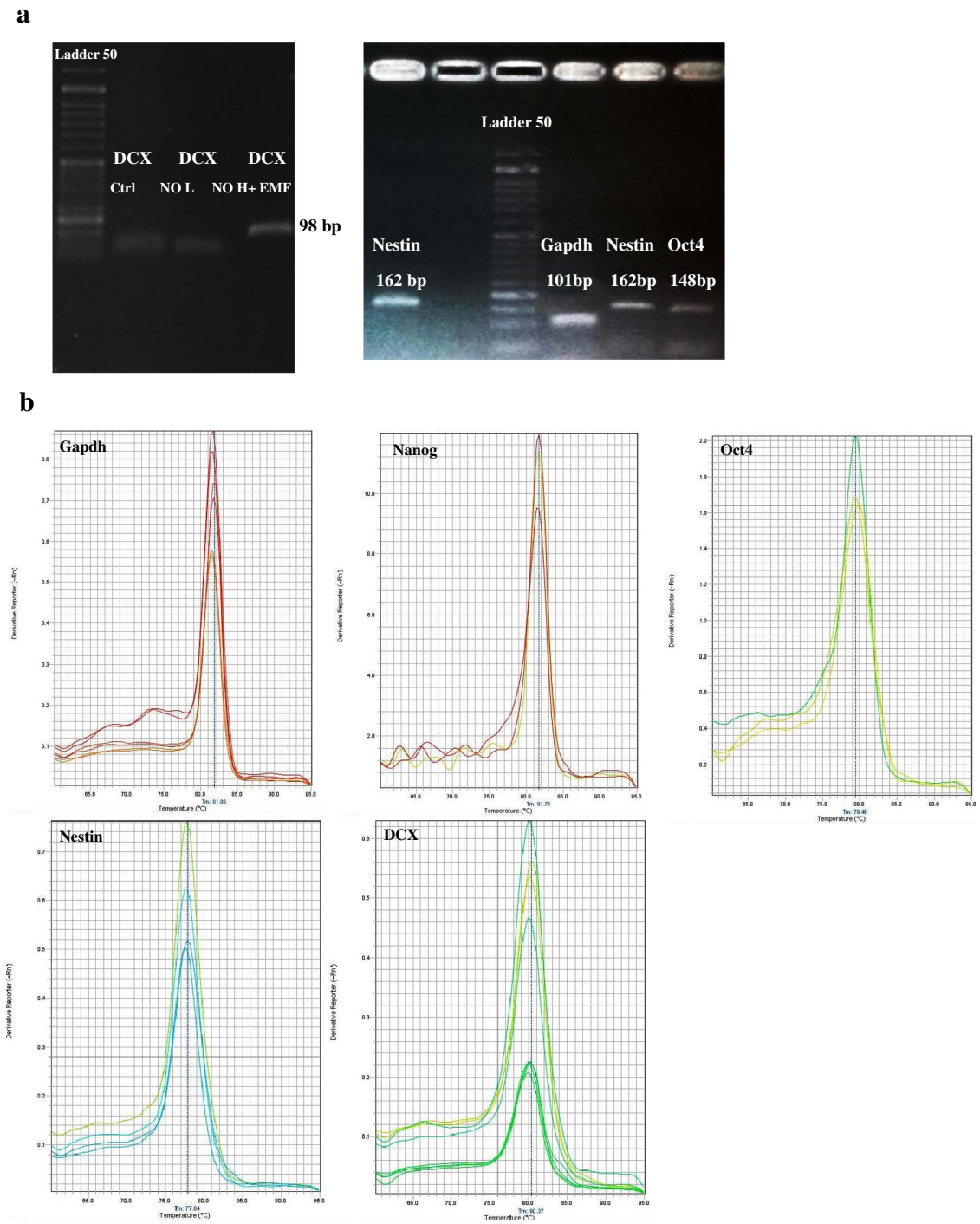


Fig. 3. Effect of RA, low and high concentrations of NO and EMF on expression of pluripotency (Nanog and Oct4) and neuronal differentiation (Nestin and DCX) genes in Rat bone mesenchymal stem cells. (a) RT-PCR gel electrophoresis. (b) Amplicon melting curves. (c) Real time qPCR analysis. mRNA levels were normalized with respect to Gapdh, chosen as a reference gene, Data are means ± SD, n = 3. star refer to significant ($p \leq 0.05$).

contamination. RNA was run on the agarose gel in the horizontal electrophoresis, while 28 s, 18 s and 5 s bands were observed. RNAs with concentration higher than 100 ng/μl were used for cDNA synthesis in presence of random hexamer and reverse transcriptase in the revert-kit (Noyafan, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran). Primers were designed and analyzed by Idt quest primer server for real time qPCR (Table 2). Gapdh was selected as reference gene. Nanog and Oct4 genes were chosen as pluripotency markers that should be up-regulated in the stem cells. Nestin is an intermediate filament and DCX is a microtubule-associated protein which their expression increases in the neural differentiated cells. Real time qPCR reaction was carried out using Noyafan protocol by master mix containing cyber green intercalating dye. Data were present as C_t value and fold variation was calculated after Gapdh normalization.

2.3. Immunocytochemistry

$BMSC_s$ were seeded in the 3.5 cm petri dishes and incubated at 37 °C with 5% humidified for 24 h. Then, $BMSC_s$ were treated with retinoic acid (200 μM), NO (1 mM) and exposure to EMF. Since, the expression and translation of protein markers need a relatively long time so after 10 days, culture medium was discarded. To remove proteins, samples

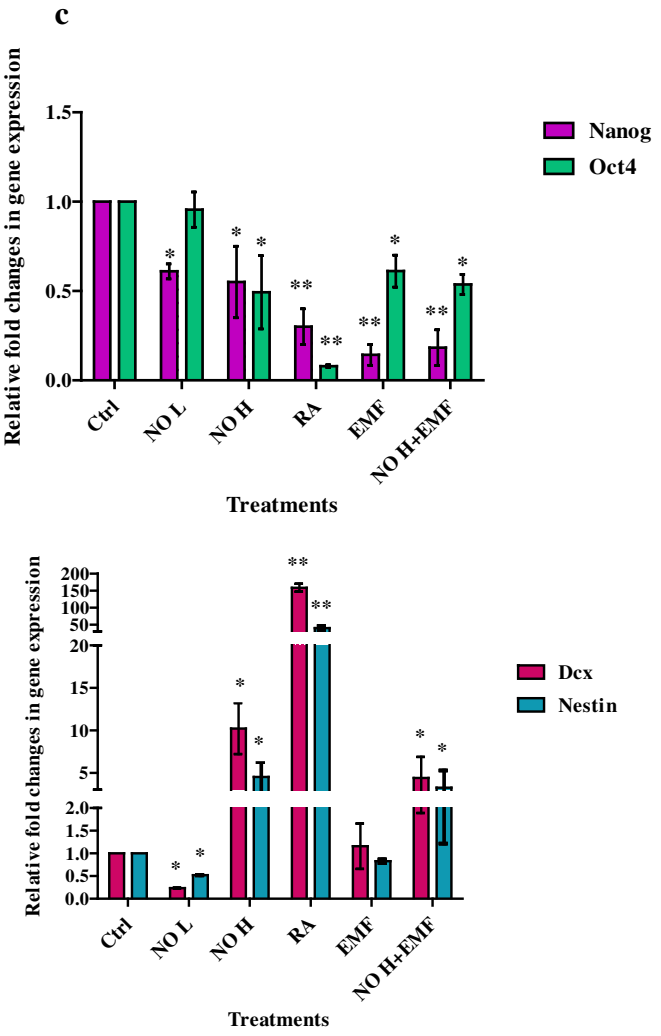


Fig. 3. (*continued*)

were rinsed with 3 ml warm PBS. The petri dishes were transferred immediately to 2 ml ethanol 70% and were incubated 40 min at room temperature for cells fixing. After fixation, cells were rinsed with 3 ml PBS/0.1% Triton X-100. To prevent the nonspecific binding of the antibodies at this stage, 20-min incubation in 2 ml PBS/5% normal goat serum was used. Cells were treated with primary and secondary antibodies. Samples were incubated with 100 µl primary antibody against the given cell markers at the appropriate dilution in PBS/0.1% Triton X-100 for 40 min at 37 °C in a humidified chamber. All samples were washed with PBS/0.1% Triton X-100 for 15 min. 100 µl fluorescently labeled secondary antibody (goat anti-rabbit polyclonal IgG-fluorescein isothiocyanate FITC) was added to the samples which were incubated for 40 min at 37 °C in a humidified chamber. Secondary antibody–exposed petri dishes were washed. Cell nucleus was stained with propidium iodide (PI). Prepared samples were observed and analyzed by florescent microscope. Oct4 was selected as primary antibody binding to pluripotent protein marker (Abcam, Germany). In addition, Nestin and NSE were selected as primary antibodies binding to neuronal protein markers (Abcam, Germany). Oct4 is a transcription factor that involves in self-renewal of undifferentiated stem cells and its accumulation in cell nucleus is more than cytoplasm. Nestin is an intermediate filament protein that its expression increases in the neuronal precursor cells. Neuron specific enolase (NSE) or enolase 2 is an enzyme that is expressed in the mature neurons and cells with neuronal origin. Nestin and NSE markers are stored in cytoplasm.

2.4. Statistical analysis

The results were expressed as mean ± SD (n = 3). Statistical comparisons were performed using one-way ANOVA and Tukey test using SPSS, v.16.0. A p-value < 0.05 was significant statistically. $p \leq 0.05$ indicated that the results were significant. For the real time qPCR data, logarithmic values were converted to real values by raising 2 to the power of $-\Delta\Delta Ct$.

3. Results

Fig. 2 shows the cell morphology under different treatment conditions. EMF, RA and NO H + EMF treatments caused the decrease of cell proliferation, the increase of cell length and multi-polarization of cells. The morphology of cells was similar to neurons' morphology. The cells which were treated with NO low concentration maintained their stemness state and represented the morphology similar to the mesenchymal stem cells.

The results of real time qPCR showed that Nanog gene expression decreased significantly in all groups compared to control group. RA, EMF and NO H (1 mM) + EMF groups indicated the highest decrease of Nanog expression (> 50% decrease compared to control group) and NO L (50 µM) group indicated the lowest decrease of Nanog expression. The Oct4 self-renewal marker expression decreased in groups treated with NO H (1 mM), RA, EMF and NO H (1 mM) + EMF compared to control group. The group treated with Retinoic acid (RA) indicated the highest decrease (near to 80% decrease compared to control group). Oct4 expression in the group treated with NO low concentration didn't change compared to control group. The decrease in Oct4 gene expression was weaker in all groups compared to Nanog gene expression.

The expression of DCX and Nestin neuronal differentiation markers increased significantly in samples treated with NO H (1 mM), RA and NO H (1 mM) + EMF compared to control group. The highest increase was observed in the group treated with RA. Compared to control group, the DCX and Nestin gene expression increased 30 and 100 times, respectively. The DCX and Nestin gene expression in the group treated with NO H (1 mM) + EMF increased 5 times compared to the control group. The DCX and Nestin gene expression decreased significantly in NO L group compared to control group. In addition, DCX and Nestin gene expression didn't change in group treated with EMF alone (Fig. 3).

The immunocytochemistry results showed that in samples treated with RA, NO H (1 mM) + EMF and EMF, fewer cells expressed Oct4 marker compared to the control group. In groups treated with RA, EMF and NO H + EMF about 20, 60 and 20% of the cells expressed Oct4 marker, respectively. The percent of cells expressing Oct4 marker in EMF group was higher than RA and NO H (1 mM) + EMF groups. More cells expressed Nestin protein in all treated groups compared to control group. In group treated with RA, EMF and NO H (1 mM) + EMF, about 20, 80 and 60% of the cells expressed Nestin, respectively. More cells expressed this protein in EMF and NO H (1 mM) + EMF groups compared to RA group. NSE protein was expressed only in group treated with NO H (1 mM) + EMF which in 60% of cells was present in cytoplasm. The expression of this protein was low in the other groups (Fig. 4, Fig. 5).

4. Discussions

Gene expression analysis showed a significant decrease in the expression of pluripotency genes (Nanog and Oct4) and a significant increase in the expression of neuronal differentiation genes (Nestin and DCX) under treatment with RA, NO high concentration (1 mM) and NO high concentration (1 mM) along with EMF. The gradual increase of neuronal genes expression accompanied with neuronal morphological changes as well as the increase in the percent of cells expressing the neuronal marker proteins were observed in the groups treated with NO H (1 mM), RA and NO H (1 mM) + EMF.

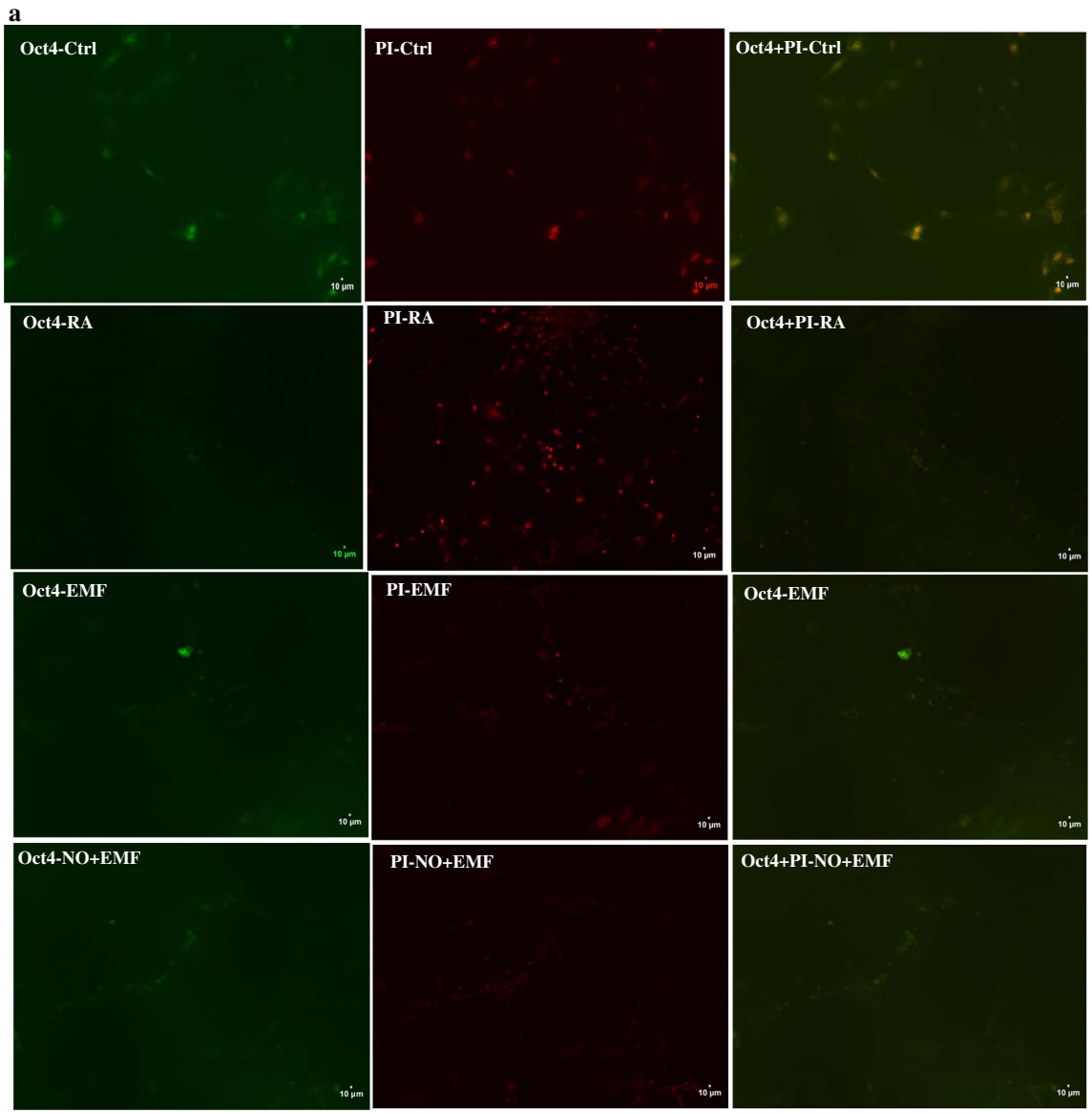


Fig. 4. Immunostaining for Oct 4, Nestin and NSE proteins. (a) Oct4 protein marker. (b) Nestin protein marker. (c) NSE protein marker. Nucleuses were stained with PI.

Delivery of L-arginine, NO donors, NO gas, or overexpression of dependent nitric oxide synthase enzyme (NOS) can increase NO concentration in the cell. We found that treatment with NO high concentration (1 mM) in the presence or absence of EMF promoted neuronal differentiation, but NO low concentration decreased the expression of neuronal differentiation genes which helped the cell proliferation process. These results proved the previous studies in which the low and high concentrations of nitric oxide increased and reduced cell proliferation, respectively (Huang et al., 2010; Napoli et al., 2013). These findings provide new evidence that EMF 50 Hz + NO 1 mM can affect the behavior of $BMSC_s$ and create stable morphology and physiology changes. The self-renewal genes expression at transcription and translation levels decreased in the presence of NO H (1 mM), EMF, NO H (1 mM) + EMF and RA, which in turn led to decreasing cell proliferation and helping cell differentiation (Tapia-Limonchi et al., 2016).

Extracellular Ca^{2+} influx is mediated by EMF which is one of the main mechanisms to explain these effects (Pilla, 2012). Previous studies reported that EMF exposure increases the expression and activity of a Cav_1 (L-type) channel that leads to the increase of intracellular Ca^{2+} ion (Ma et al., 2016). EMF increases the speed of electrical charge carriers such as Ca^{2+} ion, promotes the concentration of intracellular calcium ion and invokes the neuronal differentiation of neuron stem cells (Luo et al., 2014; Ma et al., 2016). When stem cells are exposed to low frequency EMF, the increase of the intracellular Ca^{2+} levels may play an important role as an initial factor in subsequent cell behaviors (Ma et al., 2016). Changes in the intracellular Ca^{2+} concentration mediated by ion channels and receptors launches MAP kinase cascades, regulates the expression of several transcription factors, influences on the acetylation, de-acetylation, methylation and de-methylation of histones and subsequently modulates the expression of proliferation and differentiation genes (Leone et al., 2015). The increased Ca^{2+} ion by binding to calmodulin protein activates calmodulin dependent nitric oxide synthase and leads to produce NO. Unlike neuronal NOS (nNOS) and endothelial NOS (eNOS), inducible NOS (iNOS) displays a high affinity for calmodulin, which is tightly bound to this protein in presence of calcium ion (Xu et al., 2002).

NO is involved in the proliferation and differentiation of neural precursor cells subventricular zone (SVZ) explants. Since neural precursors in the SVZ are naturally exposed to NO-producing neurons in vivo, NO has a direct action on these cells during postnatal and adult neurogenesis (Matarredona et al., 2004).

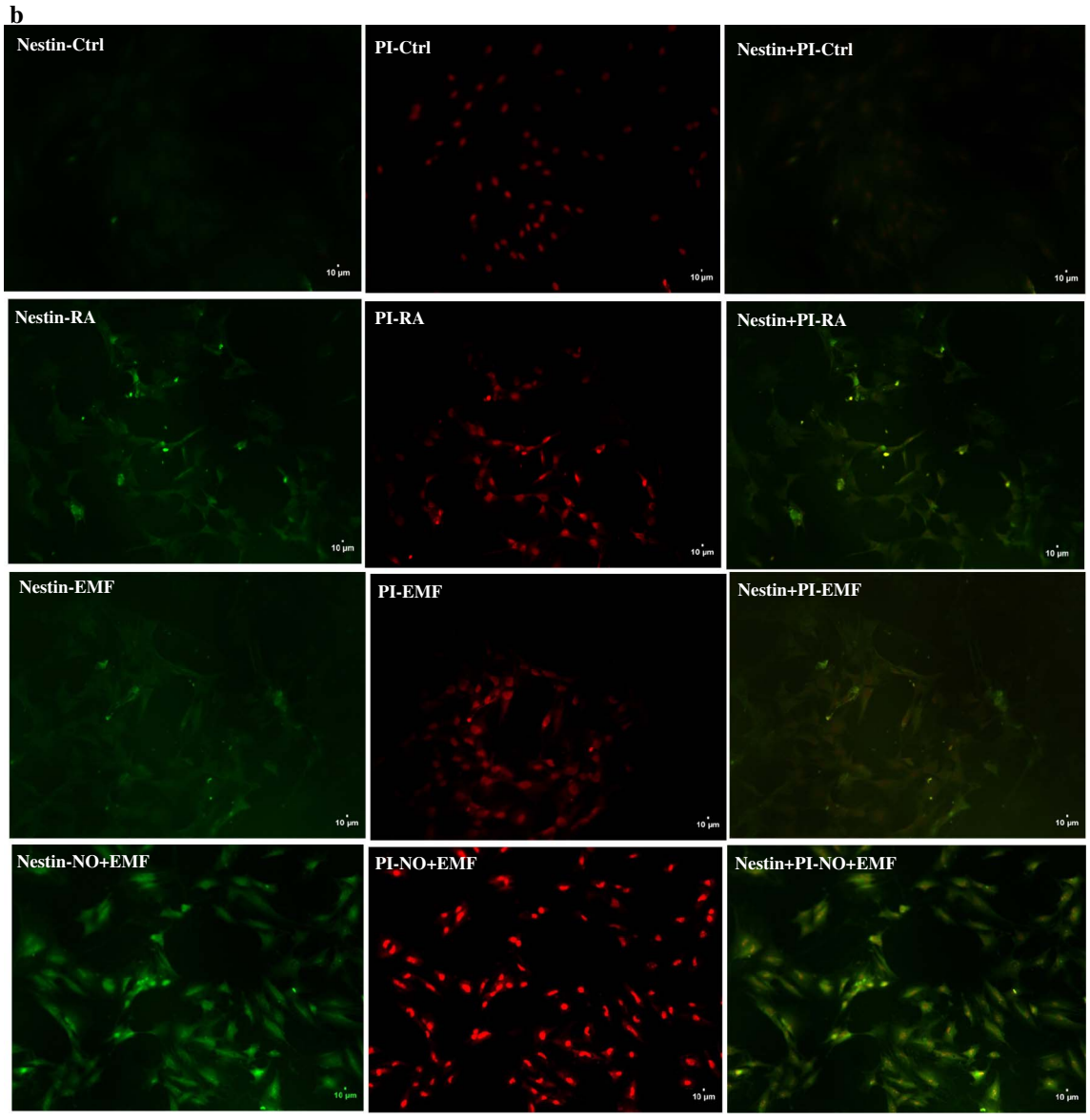


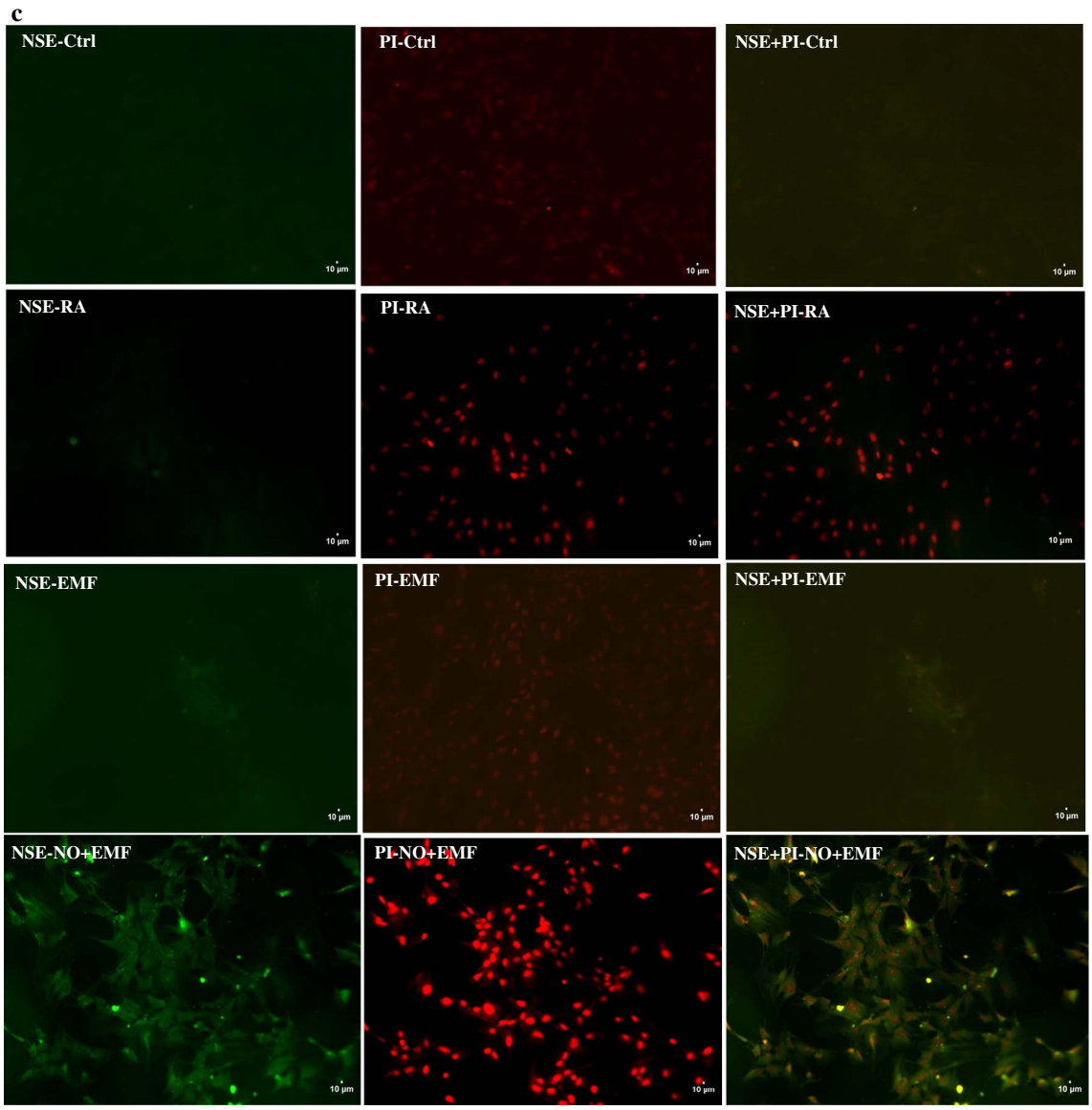
Fig. 4. (*continued*)

NO high concentration induces apoptosis in cells. This process is independent of NO/cGMP pathway and leads to arrest cells in G2/M phase of cell cycle (Choi et al., 2002; Liu et al., 2015). Apoptosis process is prerequisite of differentiation. The cells resistant to apoptosis are differentiated into specialized cells (Fimia et al., 1998). NO high concentrations (500 μM to 1 mM) binds tyrosine amino acid in proteins which leads to post translation modifications in proteins, impacts on the activity of proteins and enzymes such as nucleus proteins and transcription factors and thus modulates cell differentiation pathway (Nott et al., 2008).

NO low concentrations (pM-nM in vivo)/(μM in vitro) stimulate the sGC/PKG-Iα pathway and downstream cytoplasmic, mitochondrial and nuclear signaling pathways that exert beneficial effects on BMSCs, such as the increase of cell survival and proliferation. NO high concentrations can have contradictory effects by promoting cell cycle arrest. Mechanisms involving cell cycle proteins, such as cyclins, CDK2, p21 and pRB, seem to be at least responsible for the NO-dependent inhibition of MSC growth and promote the differentiation (Bonafè et al., 2015).

EMF enhances the life-time of free radicals such as NO (Georgiou, 2010) by Zeeman-splitting. The increase of free radicals' stability such as NO reinforce NO contribution in apoptosis process, cell fate, gene expression and cell differentiation in EMF presence. In this study, it was shown that in presence of EMF with high concentration of NO, up-regulation of Nestin and DCX genes were more severe than EMF treatment alone. Neuronal differentiation protein markers (Nestin and NSE) expressed in more cells by treatment with high concentration of NO and EMF in comparison with EMF treatment alone. So combined effect of EMF associated with high concentration of NO through down and up-regulating the stemness and the neuronal differentiation markers, respectively yielded to decrease the cell proliferation rate, increase the cytoplasmic neuronal proteins and the neurites number and ultimately guided $BMSC_s$ into neuronal differentiation that more cells achieved neuronal cell morphology. Under treatment with NO H (1 mM), majority of cells were experiencing necrosis and early apoptosis, However expression of neuronal differentiation markers increased, number of neuronal differentiation cells was low and cell morphology was in-regular and difference with neuronal cells. Presence of EMF helped to decrease necrosis and early apoptosis and increased the percent of neuronal differentiated cells.

According to the results, the expression of Oct4 in the transcription level showed the maximum decrease in the group treated with RA



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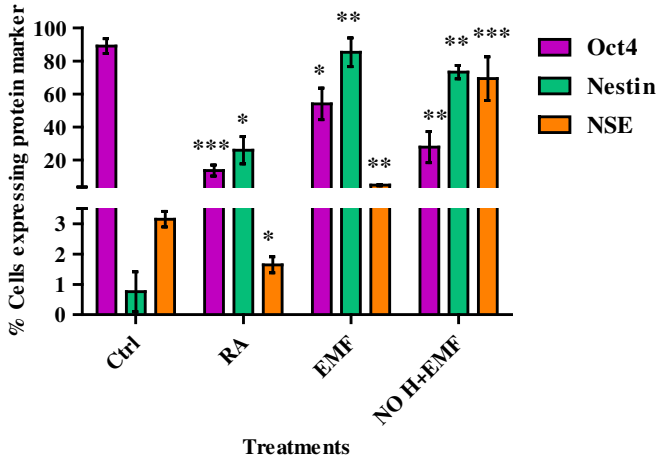

Fig. 5. Immunocytochemistry analysis. Profile of Oct4, Nestin and NSE during of treatment with RA, High concentration of NO (1 mM) + EMF and EMF. Histograms show mean expression values. Data are means ± SD, n = 3. Star refers to significant ($p \leq 0.05$).

compared to other groups. Similarly, the expression of Nanog gene in the transcription level decreased in the groups treated with RA, EMF and NO H + EMF compared to other groups. The groups treated with RA showed the minimum gene expression of pluripotency markers and maximum gene expression of differentiation markers compared to other groups. NO low concentration decreased the expression of Nestin and DCX genes in the transcription level compared to other groups. NO high concentration increased the expression of Nestin and DCX genes more intensely compared to EMF and NO H + EMF groups. This indicates that the effect of a chemical inducer is more than a physical inducer in cell differentiation process.

The different treatments changed the expression of protein markers in translation level differently. These results indicate that RA, NO H, EMF and EMF + NO H each through different routes lead the differentiation of $BMSC_s$ into neuronal cells and the differentiated cells number is different under the different treatments.

5. Conclusions

This study showed that EMF, NO (high concentration) and RA lead to differentiation of Rat bone mesenchymal stem cells into neuron like cells through different mechanisms and impacts. The magnitude of this

impact was not similar in different groups. The greatest impact on the cell morphology, neuronal genes expression, neuronal proteins expression and cell polarity was evident in the group treated with high concentration of NO in the presence of EMF. Low concentration of NO helped the stem cells maintain their stemness state through up-regulation of pluripotency genes and down-regulation of neuronal differentiation proteins. During the differentiation, NO concentration had a critical role in the behavior of BMSCs. It is suggested that the function of differentiated neuronal cells to be investigated in further studies by the use of Patch-Clamp technique. In addition, it is better to produce differentiated neuronal cells by the induction of nitric oxide and electromagnetic field to stem cells in vivo that are used to treat diseases like multiple sclerosis (MS), Parkinson and Alzheimer.

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