Molecular and Cellular Neuroscience 85 (2017) 19–28

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

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

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ARTICLE INFO

Keywords:
DCX
Differentiation
Electromagnetic field
Immunocytochemistry
Nanog
Nestin
Nitric oxide
Oct4
Proliferation
Retinoic acid
Real time qPCR

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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http://dx.doi.org/10.1016/j.mcn.2017.08.005
Received 13 June 2017; Received in revised form 5 August 2017; Accepted 21 August 2017
Available online 24 August 2017
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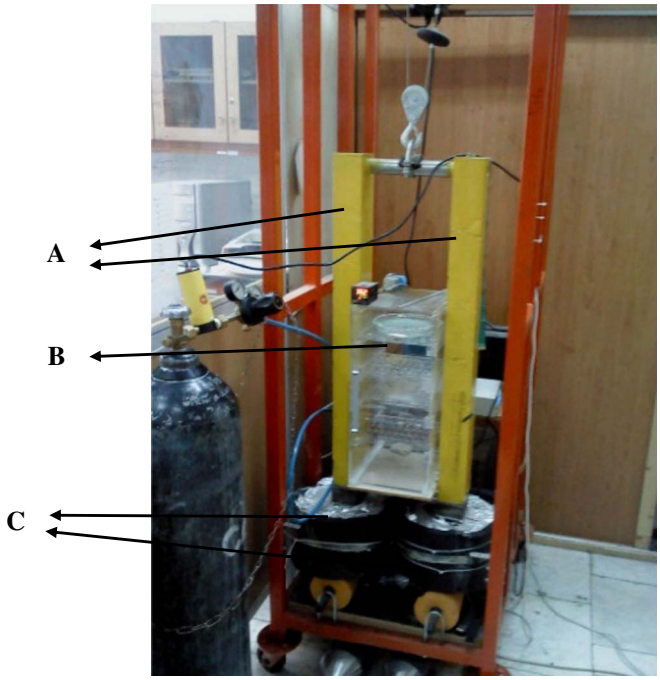

Fig. 1. EMF generator, (A) the iron blades, (B) the incubator, (C) the coils.

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

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