Mesenchymal stem cells that located in the electromagnetic fields improves rat model of Parkinson's disease

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**Objective(s):** The main characteristic of mesenchymal stem cells (MSCs) is their ability to produce other cell types. Electromagnetic field (EMF) stimulates differentiation of MSCs into other cells. In this study, we investigated whether EMF can effect on the differentiation of MSCs into dopaminergic (DA) neurons.

**Materials and Methods:** An EMF with a frequency of 50 Hz and two intensities of 40 and 400 µT 1hr/day was generated around the cells for a week. Afterwards, these cells were injected into the left ventricle of Parkinsonian rats. The rats survived for 2 weeks, and then sampling was performed.

**Results:** The injected cells differentiated into DA neurons and sporadically settled in the substantia nigra pars compacta (SNpc). Transplanted rats exhibited significant partial correction of the PD model. Transplantation of MSCs significantly reduced motor deficiency compared to Parkinsonian rats (5.0±0.1 vs 7.5±0.08). Results demonstrated that endogenous serum and brain derived neurotrophic factor (BDNF) were altered in all experimental groups. The greatest increase was in group of 400 µT EMF in comparison with Parkinsonian rats (398±15 vs. 312±11.79 pg/ml). Current study has shown that 6-Hydroxydopamine can cause severe loss of dopaminergic neurons (68±6.58), but injected MSCs that exposed to 40 and 400 µT EMF increased dopaminergic neurons in SNpc (108±2.33 & 126±8.89) (P<0.001).

**Conclusion:** Electromagnetic fields with particular frequencies stimulate MSCs. So, these cells had anti-Parkinsonian properties in our studies.

**Introduction**
Parkinson disease (PD) is a prevalent neurodegenerative disorder in old people. It is caused by severe loss of dopaminergic neurons (DA) in different part of central nervous system. The greatest reduction of DA is in the substantia nigra pars compacta (SNpc) of mid brain (1). Although the particular cause of neuronal death or loss in PD is currently unknown, research has shown that both apoptotic (2, 3) and non apoptotic cell death may occur (4). 6-Hydroxydopamine (6-OHDA) is the most frequently used toxins to induce Parkinson’s disease-like models. Injection of this toxin into SNpc results in selective lesion to DA via uncoupling mitochondrial oxidative phosphorylation resulting in energy deprivation (4-6). The advantage of this model is the ease of evaluation of motor deficiency by using tests such as apomorphine-induced rotation test (7) and spontaneous motor test. Injection of 6-OHDA into the nigrostriatal pathway causes the loss of motor stability (8).

Many biological activities can be modulated by electromagnetic fields (EMF) (9, 10). In particular, biological effects of EMF exposure is thought to be through changes in ion channels (especially for H+, K+ and Ca2+) (11), which leads to gene up regulation, cell fate, cell differentiation and cell behavior during normal tissue turnover and regenerative repair (12). It was also reported that the low frequency of EMF causes differentiation of BMSC to pituitary cells (13). Moreover, EMF exposure can modulate the stem cells differentiation,
so it is useful for tissue engineering or regenerative medicine. Previous studies have revealed that electrical stimulation on mouse stem cells (ESCs) promotes differentiation of these cells (14). However, effects of electrical stimulation on these cells were little known. Other studies have shown that the exposure of cells to 1 mT EMF does not have significant effects on structure and cytoskeleton of the cells and proteins. The cytoskeleton is the main structure of the cells that is responsible for cell shape. According to findings, it is thought that 40 and 400 µT provide appropriate EMF type to cause least damage to the cells and also stimulate differentiation and proliferation of mesenchymal stem cells (MSCs) into neuron. Therefore, this study was performed to investigate the effects of low intensity EMF on the proliferation and differentiation of MSCs into dopaminergic neurons, to evaluate the survival and activity of these cells in rat model of Parkinson’s disease.

**Materials and Methods**

**Animals**

Forty eight adult male albino- Wistar rats (200-250 g) were obtained from the animal center of Semnan University of Medical Sciences, Semnan, Iran. The rats were kept in standard environment, in a temperature (22-24 °C), humidity (40–60%), and light period (12 hr). The rats had free access to food and water. All procedure and maintenance were carried out in accordance with Health Guide for Care and Use of Laboratory Animal that permit by Ethical Committee of Semnan Medical University, Semnan, Iran. (Ethical Committee number 92/299518). Rat were randomly divided into six groups (n=8). Each group was housed in a separate cages, the first group received saline as a control group. The second group was housed in a separate cages, the first group was exposed to 400 µT EMF for a week. The fifth group received MSCs that were isolated from tibias and femurs of rats under deep anesthesia. The bone marrow was flushed onto the Hank’s buffered salt solution (HBSS). For removal of debris, solution was filtered through a cell strainer (100 μm) and then all cells centrifuged and incubated in a 5% CO2 at 37 °C in Dulbecco’s modified Eagle Medium (DMEM, Invitrogen), supplemented with fetal bovine serum (FBS, 10%), amphotericin B (2.5 μg/ml), streptomycin (50 μg/ml). After 3 days, suspended cells were removed with the medium and adherent cells were used as the BMSCs (34). Subsequently, incubation was continued and medium was changed at three days intervals. MSGs were allowed to grow. After third passage, when the density of cultured BMSCs was approximately 5×10^6 cells/cm^2, other cells such as fat and fibroblast were removed and only the MSGs were able to multiply and survive (34). To identify of MSGs used differentiation method to bone and fat cells. After identification, BMSCs with density of 5×10^5 cells in flask, treated with EMF with the frequency of 40 or 400 µT 1hr/day for a week.

**Dil labeling**

Understanding cell morphology is a key part to recognize neuronal cells. Several special staining techniques such as immunofluorescence staining of Dil (1, 1’dioctadecyl-3, 3, 3’, 3’-tetramethylindocarbocyanine perchlorate) have been developed to help the morphological recognition of neurons. The stain agent is a carbocyanine membrane dye that increases the fluorescence upon the insertion of its lipophilic hydrocarbon chains into the lipid membrane of cells. The high photo stability and continual fluorescence of the dye serves as an effective dye for recognition of neuronal structure. Before injection, MSCs were labeled with the fluorescent dye CM-Dil (Molecular Probes, Invitrogen, USA). The MSCs were incubated with 5 μg/10^6 cells Dil for 2 hr at 37 °C in a 95% air per 5% CO2. Then 2×10^5 cells separated and injected in the left ventricle.

**Exposure system and field characteristics**

The exposure system was produced by a horizontal Helmholtz coil (300 turns, distance of 6 cm, and internal diameter of 16 cm) embedded in an open Plexiglas rectangular frame and placed in a CO2 incubator (5% CO2, 37 °C). The 50 Hz electrical current was provided by a signal generator and regulated with a 35 W acoustic amplifier. The EMF at the center of exposure system was measured by a Gauss meter (MG-701, MAGNA Japan), and 40 (the

**Hydroxydopamine lesion**

To obtain unilateral lesion of nigral system, in all rats, 6-OHDA was injected into the left SN. Ketamine hydrochloride/ xylazine hydrochloride (100 mg/kg – 20 mg/kg) (sigma-Aldrich) was used and placed into a stereotoxic device (stoelting, USA) to anesthetize the rats. The skin of skull was exposed by a 2 cm incision, and a single hole was drilled over each side of the skull. For injection in SNpc, the following coordinates were used (47): AP = - 4.8 mm posterior to bregma, M L = - 1.6 mm lateral to the midline, DV = 8.2 mm vertical from the dura, and finally 4 μl of 6-OHDA (2 μg/μl) was dissolved in vehicle 2 mg/ml anti ascorbic acid (Sigma St. Louis, USA) in saline and was then injected to the SNpc. 6-OHDA was injected to the left side at the rate of 1 μl/min.

**Cell culture**

BMSCs were isolated from tibias and femurs of rats under deep anesthesia. The bone marrow was flushed onto the Hank’s buffered salt solution (HBSS). For removal of debris, solution was filtered through a cell strainer (100 μm) and then all cells centrifuged and incubated in a 5% CO2 at 37 °C in Dulbecco’s modified Eagle Medium (DMEM, Invitrogen), supplemented with fetal bovine serum (FBS, 10%), amphotericin B (2.5 μg/ml), streptomycin (50 μg/ml). After 3 days, suspended cells were removed with the medium and adherent cells were used as the BMSCs (34). Subsequently, incubation was continued and medium was changed at three days intervals. MSGs were allowed to grow. After third passage, when the density of cultured BMSCs was approximately 5×10^6 cells/cm^2, other cells such as fat and fibroblast were removed and only the MSGs were able to multiply and survive (34). To identify of MSGs used differentiation method to bone and fat cells. After identification, BMSCs with density of 5×10^5 cells in flask, treated with EMF with the frequency of 40 or 400 µT 1hr/day for a week.
same as earth magnetic field) or 400 μT flux density were chosen for exposing the cell cultures, 1 hr/day for a week. The condition of sham group was quite similar to other groups, except that the EMF was turned off (14).

**Immunohistochemical and histological study**

Transcardial perfusion was performed at first with 300 ml normal saline followed by 300 ml 4% paraformaldehyde (PFA) in 0.1 mol/l phosphate buffered saline (PBS). Animals were deeply sedated (pentobarbital, 50 mg/kg IP) and perfused Transcardially with 4% paraformaldehyde. After removing the brain, stems were fixed with 4% paraformaldehyde, the tissue was then cut (5-7 μm thick). Deparafinized, next step was antigen retrieval with tris/EDTA pH 9.0 buffer and 3% hydrogen peroxide, blocked permeabilized was done with 10% normal goat serum in PBS containing 0.5% Triton X-100, stained with an anti-tyrosine hydroxylase (TH) polyclonal antibody (1-200 dilution, with 10% normal goat serum) (ab75875 UK), followed by a biotin – conjugated goat anti-mouse IgG (1-300 dilution) (abcam, UK). After washing 3 times, all sections were incubated with a avidin and biotinylated horseradish peroxidase (HRP) complex then followed by diaminoazobenzidine (DAB) (abcam, UK). Dopaminergic cells were found and quantified using Olympus microscope.

**Behavioral testing**

Motor asymmetry following unilateral lesion of the nigro striatal pathway or DA neurons in SNpc was assessed by apomorphine-induced rotational behavior. All behavioral tests were performed by a technician blinded to the project. All groups were tested for rotational behavior 6 days after the first surgery and after treatment. Rats received subcutaneously 0.5 mg/kg apomorphine hydrochloride (sigma- Germany) dissolved in 0.9% NaCl. Twenty minutes after injection, contralateral turns to the lesion were counted over a period of 60 min. Data were expressed as a contralateral turns/min.

**Morphometric studies**

Ten coronal sections (a section after each 5 sections) from rostral to caudal of the SNpc in each animal were analyzed. The picture of each section was taken by Olympus AX70 microscope and digital camera DP11 with magnification of 40×. An area of 10,000 μm² was measured randomly in the region of SNpc in five separate microscopic fields. To count the number of neuronal cells in Nissl staining (interneuron and neuronal cells) and IHC (TH positive cells), the pictures were transferred to the computer using OLYSIA autoboreport (Olympus optical, Japan) software. A grid was superimposed on the picture and the cells with normal nucleus were counted.

**Measurement of the brain and serum BDNF concentration**

At the end of experiments, the brain and serum level of brain-derived neurotrophic factors (BDNF) were measured with the reagents provided in the BDNF Immunoassay System. The procedure was conducted based on the manufacturer's protocol (R and D Systems, Minneapolis, MN, USA). The heart blood was collected in free tubes with anticoagulant and incubated at room temperature for 30 min. Next, samples were centrifuged for 15 min at 1500 ×g. The supernatants were collected and stored at -70°C. Then rats decapitated, and brains were quickly out. The extraction of BDNF from the brain sample was performed on ice (48). Briefly, the sample was suspended in 5 volume of lysis buffer containing 20 mM Tris-HCl, 137 mM NaCl, 1% NP40, 0.5 mM PMSF, 10% glycerol, 0.5 mM sodium and protein inhibitor (Calbiochem, USA). The suspension was homogenized on ice for 30 min using a sonicator at power level 1. The brain tissue homogenate were then centrifuged at 16000 ×g for 20 min at 4°C. The supernatant was stored at -70°C for subsequent analysis.

**Statistical analysis**

All data were reported as mean± standard error of the mean (SEM) Enzyme activities was expressed as optical density (OD) value. Statistical analysis was performed by computer using Statistical Package for the Social Sciences version 16 (SPSS16.0). One-way ANOVA and Tukey post hoc multiple comparison tests were used to analyze each tissue. Statistical significance was present at P<0.05.

**Results**

**Drug-induced rotational behavior assessment**

As shown in Figure 1, contralateral turns induced by apomorphine hydrochloride were increased in the 6-OHDA group or PD group in the first test (2 weeks after surgery) compared to treatment and control groups (P<0.01). The frequency and duration of the rotation in 6-OHDA group were 8.67± 0.1 circles/min. In the second test, 4 weeks after surgery or 2 weeks after the end of the experiments, the number of contralateral turns observed in the 6-OHDA group was more than all other groups (7.57±0.08, P<0.01). When each group was analyzed considering, in the treatment groups (40 & 400 μT) showed a significant reduction in total contralateral turns (6.50±0.29, 5.0±0.1) but in BMSC treatment group was (7.33±0.52). On the other hand, the number of contralateral turns decreased in all treatment groups, but the decreased value in exposed cell was very noticeable. There were no
significant differences between the second and first tests of the control, sham and 6-OHDA group.

Immunohistochemistry
Tyrosine hydroxylase staining was performed for assessment of the dopaminergic neurons. Injection of 6-OHDA into the SNpc significantly reduced the number of TH immunostaining cells (68±6.58, P<0.001) as illustrated in Figure 2 B,C,D, 3. These cell reduction was observed in all tested groups, but the cell number in the exposed cells treatment groups was higher than other groups (108±2.33 and 126±3.89). Figure 2A shows the boundaries of the SNpc, which were used to measure the cell number in the all tested groups in Nissl and IHC staining. Statistical and comparative light microscopic analyses demonstrated that the number of TH and Nissl stained cell in the 6-OHDA group had a prominent decrease compared to the control and treatment groups (68±6.58, P<0.001). The cell number in the control and sham groups were similar (216±5.59 and 212±4.96, respectively), but in the unexposed cell treated groups the number of Nissl-stained cell was 107 ± 5.77 (Figure 3). So, it can be deduced that BMSCs could protect or immigrate into substantia nigra and differentiated into dopaminergic neurons in degenerative disease in rat.

BDNF assay
Neurotrophic factors, such as the BDNF, have been considered to play a principal role in protection of neurons. In this study, we have demonstrated that endogenous BDNF are altered in PD, and the stem cells can change the BDNF level in the serum and brain. We found that the level of brain BDNF was higher and significant in exposed cells (400 µT) group in comparison with the 6-OHDA group (398±15 vs 312±11.79 pg/mg protein, respectively) (P= 0.001). In this group, the level of BDNF was higher than control group (398±15 vs 342±8.79 pg/mg protein). There were

Figure 3. The mean number of TH-positive neurons in the substantia nigra pars compacta (SNpc) of all groups. The number of TH-positive neurons in the SNpc was significantly higher in group that received MSCs exposed to 400 µT, compared with the Parkinson’s rats. (*P ≤ 0.01)

Figure 4. Light photomicrograph of rat substantia nigra. Mesenchymal stem cells (MSCs) were detected in mid brain by fluorescence microscope. (A, B) Arrows show that MSCs were labeled with CM-Dil and appeared clear under fluorescent microscopy. The labeled cells were mainly detected in substantia nigra after injection

Figure 2. Preservation of dopaminergic systems of 6-OHDA-lesioned rats by mesenchymal stem cells (MSC) transplantation. A-D: TH-positive fibers in the striatum and neurons in the substantia nigra pars compacta (SNpc) of rats. DAPI staining (blue) was used to identify nuclei. (A) The quadrate shows the boundaries of the SNpc, which were used to measure cell number. (B) Received MSCs transplantation and (C-D) received MSCs that exposed to EMF. Scale bar: 200 μm in (A), 100 μm in (B-C-D)
cells that isolated from the rat bone marrow may be proliferated in vitro, and after injection can be transferred to mid brain. Dopaminergic neurons can be found in different areas of brain and brain stem such as the substantia nigra of midbrain, hypothalamus, some part of retina, and sheet of olfactory bulbs. The most dominant groups of DA neurons stationed in the ventral tegmental area and substantia nigra of the midbrain; both of these areas participate in the formation of extra pyramidal motor system that controls postural reflexes and are responsible for initiation of movement (2). It is estimated that striatal environment and cells might be responsible for producing neurotrophic factors that lead to major differentiation of progenitor cells into TH-positive neurons. Therefore, we injected MSCs into left ventricle, and then cells suspend in the cerebro spinal fluid (CSP) and migrate to damaged area. We observed that the labeled cells that were injected in the left ventricle, reside in midbrain. Some of these cells were in substantia nigra and the others were spread sporadically in the mid brain. Results have shown that MSCs are able to pass through blood brain barrier and be stationed in the affected areas. But, how these cells are capable of interacting with other cells or differentiate into dopaminergic neurons and produce dopamine are not correctly known.

It is widely accepted that EMF can influence several biological functions, modulate intracellular reactive oxygen species (ROS) levels and the cell cycle progression (17-19). Exposing cells to 50 Hz EMF lead to increase in cell proliferation rate (20). Stimulating the cells with 0.1 μT EMF activates the protein kinase C. This activation caused an increase in cell proliferation. An increase in [Ca2+] in cells upon EMF exposure was reported by numerous researchers (21, 22), and it is known that this function is able to modulate proteasome activity (23).

MSCs are multipotential cells and have high capacity for replication. Cells have a potential to differentiate into other lineages of mesenchymal tissues (24). The exposure of MSCs to 600 μT causes the MSCs differentiate into other cells such as adipocytes. Therefore, EMF exposure may also lead to overexpression of lipoprotein lipase and peroxisome (25). However, it is reported that exposure of MSCs to EMF increase cells differentiation (26).

Sinusoidal EMF of 800 μT with frequency of 50 Hz is able to differentiate stem cells. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis shows a significantly increase of GATA-4 and Nkx-2.5 mRNA expression (27). However, exposing of embryonic stem cells with the above described EMF caused differentiation into cardiac cells (27). GATA-4 and Nkx-2.5 mRNA

![Figure 5. Brain derived neurotrophic factor (BDNF) levels in serum and brain of rats. In serum, the ratio was significantly elevated by 6-OHDA treatment in comparison with the control group. At the same time, it was induced in the stem cell treatment. All values are mean±SEM. *compared with the Parkinson’s disease (PD) group (P≤ 0.01)](image)
are essential for encoding zinc finger containing transcription factor and homeodomain, and both of these are essential for cardiogenesis in different species (28), especially in human (29, 30). The EMF has been previously tested on P19 embryonic carcinoma cells (P19 cells) (31). EMF with intensity of 1 mT and frequency of 50 Hz leads to differentiation of P19 cells; however, the result was not very significant. By exposing P19 cells into sever EMF with the intensity of 10 mT, it differentiates into neuronal cells (31). Exposure of bone morrow stem cells to EMF with intensity of 1.1 mT leads to differentiation to osteogenic cells (32). Differentiation of BMSC into osteogenic cells is due to increase of intracellular Ca2+ after EMF stimulation. According to these results, it has been deduced that the elevation of Ca2+ in intracellular is one of the important factors for activation of biochemical mechanism that is responsible for the induction of terminal differentiation (32). The above findings revealed that EMF can cause proliferation and differentiation of stem cells into other cells. And this may open a new prospective in the use of EMF for differentiation of stem cells into a specific cells without the aid of gene transfer technologies.

In conclusion, in accordance with the results of other studies, we suggest that the range of EMF, which is effective on differentiation of MSCs, is between 10 µT and 400 µT. Also the differentiation will not occur if the intensity is less than 10 µT. The results of this research have shown that the intensity of 400 µT have more effective on the settle of the cells on injured area and recovery of the disease. According to the data, the number of DA neurons in SNpc in group of 400 µT was more than other groups. Also, these cells that were exposed to EMF are much more active and can differentiate into DA neurons in vivo.

Function and survival of striatal neurons dependent on BDNF, which is chiefly provided by anterograde transport from corticostriatal afferents (33). The present study reports that the elevation of brain and serum BDNF following 6-OHDA treatment support dopaminergic neurons of SNpc. One of important mechanisms that MSCs might protect DA neurons in striate is through the production or up-regulation of neurotrophines such as BDNF. High concentrations of BDNF have not only been found in the central nervous system, but also in other non-neuronal cells, particularly in platelets. A positive correlation between serum and cortical BDNF concentrations has been observed in rats (34, 35) and humans (36). In this study, the level of BDNF was increased in brain in all cell therapy groups, but the level of brain BDNF was only significant in group of 400 µT. It was concluded that there was a constitutive up-regulation of brain BDNF concentrations that might compensate for defective intracellular protein signaling in the SNpc of brain stem. Study of brain tissue from patients with Parkinson’s disease after death has shown that degeneration in striatal neurons are closely related with reduction in BDNF expression. Moreover, in the brain tissue of Parkinson’s, BDNF of striatum is more reduced as compared to age or sex-matched controls (37).

Now, there is no effective treatment for PD patients (38). Neuroprotective growth factors have also been used for PD (36). Among the growth factors, BDNF is the best candidate to modulate the onset and severity of movement and cognitive functions in PD mouse models (35). In animal models, a striatal stab wound in 6- to 8-week old mice increases BDNF level around the injury site and activates microglia and macrophages (39). Furthermore, the production of BDNF occurs in dopaminergic fibers and dopamine-transporter positive neuritis (40). Thus, these studies suggest that increased BDNF expression following administration of activated MSCs protects dopaminergic neurons from death, and supports the residence of new cells in damage area and production of BDNF.

Stem cells have the ability to regulate immune responses (41, 42) and differentiate into special cells with the aim of replacing injured cells (43). These cells produce trophic factors for protection and repair of cells by inhibition of apoptotic pathways (44). Therefore, MSCs might express and produce growth factors such as, epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF), BDNF, and stromal-derived factor (SDF-1α) (45). The neuroprotective effect of BDNF on cultured neurons is through the PI3kinase/Akt pathway by inhibition of neuronal death (46). The most important discovery of this research is that in treatment groups, the number of injected cells was equal, but in cells exposed to 400 µT, the level of brain and serum BDNF was higher than other cell treatment groups. The cells exposed to 400 µT are more active and are able to convert to dopaminergic neurons. Therefore, it can be concluded that EMF with intensity 400 µT and frequency 50 HZ will stimulate and differentiate MSCs to neuronal cells.

Conclusion

MSCs that were exposed to EMF with 400 µT increase brain BDNF and subsequently lead to increased tyrosine hydroxylase neurons in SNpc after 6-OHDA. We propose that these activated MSCs have good effects on Parkinson's diseases. However, future studies are required to reveal further detailed mechanisms of action.
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Conflict of interest
The authors declare no conflict of interest regarding the publication of this paper.

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