

Original Article



Induction of neural stem cell proliferation by iron oxide nanoparticles and magnetic field and *Ki67* gene expression in rat hippocampus after ischemia/reperfusion

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Abstract

Background and aims: Ischemic stroke is considered as the second leading cause of death in the world and yet one of the causes of disability in adults. The present study aimed to evaluate the effects of iron oxide nanoparticles and the magnetic field on neural stem cells proliferation after ischemia/reperfusion in the rat model.

Methods: This experimental study was conducted on a total of 50 male Wistar rats aged 6-7 weeks and weight of 220-250 g, which were divided into sham (i.e., ischemia-reperfusion model), control, iron oxide nanoparticles treated-, magnetic field exposed-, and simultaneously iron oxide nanoparticles and magnetic field exposed- groups. The brain ischemia/reperfusion was performed for 20 minutes by blocking the animal carotid arteries. In addition, neural stem cell proliferation was evaluated in the hippocampus of the 5 groups after 4 days by bromodeoxyuridine (BrdU) staining method. Then, the expression of *Ki67* gene involved in the cell proliferation was quantitatively studied among the 5 groups by the quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The results of BrdU staining revealed that iron oxide nanoparticles and the magnetic field separately increased cell proliferation after ischemia/reperfusion after 4 days in the hippocampus. However, simultaneous treatment with nanoparticles and magnetic field failed to show a significant difference compared to the sham group for 4 days. Conversely, the expression of *Ki67* gene increased significantly in the group treated with iron oxide nanoparticles or the group exposed to magnetic field compared to the ischemia-reperfusion model.

Conclusion: In general, iron oxide nanoparticles and magnetic field can separately be regarded as 2 effective methods for increasing the neural stem cell proliferation after ischemia/reperfusion.

Keywords: Iron oxide nanoparticles, Ischemia/reperfusion, *Ki67*, Magnetic field, Q-RT-PCR

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Introduction

Stroke (ischemia) is the result of a reduction in blood flow in an area of the brain (1), which causes damage to the nerve tissue due to the blockage in an artery (2). In addition, it is one of the causes of mortality (3) and disability in the world (4), which causes a disorder in the function of the nervous system and the blood-brain barrier, and as a result, leads to functional disability in the person. So far, no specific treatment has been able to improve the neurological disorders related to the stroke in patients. Using stem cells to relieve the injuries caused by the stroke is regarded as one of the therapeutic methods that has attracted the attention of researchers. Neural stem cells are a group of undifferentiated and multipotent nerve cells with self-renewal power that are differentiated

into 3 cell lines including nerve cells, astrocytes, and oligodendrocytes (3). Neural stem cells are located in the subventricular zone, the subgranular zone of the hippocampus, and posterior periventricular in adulthood (5). Further, stroke stimulates neurogenesis in some areas of the brain including the subventricular zone, and this is effective in repairing the injuries from stroke and increasing the hope for recovery (6).

Researchers are looking for appropriate therapeutic methods in order to reduce the injuries due to the stroke through stimulating the neurogenesis, angiogenesis, axonal elongation, and synaptogenesis (7). Accordingly, one of the therapeutic strategies currently evaluated is to use the stem cells in the treatment (7) or tissue plasminogen activator in order to protect the nerve cells

against the injuries caused by the stroke (8). Furthermore, employing iron oxide nanoparticles (9), cerium oxide (10), and magnetic field are some other therapeutic approaches under investigation (11) so that specified in the studies, iron oxide nanoparticles have a high potential for exchanging the substances between the tissue and blood after ischemia and thus, cellular protection (12). Moreover, magnetic fields may lead to changes in the cell membrane permeability after cerebral ischemia and increase cell proliferation by influencing the ion exchange, as well as nucleic acids and proteins (13,14). The present study was performed to evaluate separate and simultaneous effects of iron oxide nanoparticles and magnetic fields on neural stem cell proliferation after the ischemia/reperfusion in male Wistar rats and on the expression of the *Ki67* gene, which is an effective gene in cell proliferation.

Materials and Methods

Animals

In this experimental study, the code of ethics was first received from the Ethics Committee of Islamic Azad University of Pharmaceutical Sciences Branch in Tehran. Then, 50 male Wistar rats weighing 220-250 g were purchased from Tehran University of Medical Sciences (Department of Pharmacology). The rats were housed under conditions of 12:12 hours light/dark cycles and at a temperature of $25\pm 2^{\circ}\text{C}$.

Induction of anesthesia in animals

A combination of xylazine (Alfasan, the Netherlands) and ketamine (Rotexmedica, Germany) was used in rats in order to induce the anesthesia. Additionally, a drug combination with a ratio of 1:5 (5 mL ketamine + 1 mL xylazine) and at a concentration of 110 mg/kg was prepared and injected intraperitoneally.

Induction of ischemia/reperfusion

After anesthesia, a vertical incision was made in the neck area of the rats and then ischemia was induced by obstructing the common carotid arteries by the vascular clamp for 20 minutes. During the ischemia, the body temperature of the animal was regularly checked and the clamps were removed and the blood flow was restored after 20 minutes. Next, the isolated muscles were placed in their anatomical position after induction of ischemia and the cut area was stitched.

Preparation of the suspension of iron oxide nanoparticles

Nanoparticles of iron oxide (i.e., iron oxide (II, III) magnetic nanoparticles powder) were purchased from Sigma-Aldrich (Germany) in the form of a powder at a size of 10 nm. Then, these nanoparticles with a final concentration of 10 mg/mL were dissolved in normal saline solution at $35\text{-}40^{\circ}\text{C}$ and the solution was mixed at 250 rpm for 5 minutes in order to prepare the suspension.

Treatment of animals using iron oxide nanoparticles and magnetic field

After grouping and animal surgery, the models were treated with iron oxide nanoparticles and magnetic field based on the following pattern:

Group 1: Healthy animals that were treated with normal saline solution (i.e., nanoparticle solvent) intraperitoneally 20 minutes after the incision in the neck. Then, their skin was stitched without ischemia-reperfusion (IR) induction (control group).

Group 2: The animals were treated with normal saline solution 20 minutes after the IR induction (sham group).

Group 3: The animals were treated intraperitoneally 20 minutes after IR induction at a dose of 10 mg/kg of iron oxide nanoparticle.

Group 4: Twenty minutes after IR induction, the head of the animal was placed in a magnetic field of 1 tesla (once every 24 hours and 20 minutes each time) for 4 days in the anesthetic state.

Group 5: The animals were treated with iron oxide nanoparticles (10 mg/kg) intraperitoneally and 20 minutes after the IR induction, the head of the animal in the anesthetic state was placed in a magnetic field of 1 tesla (once every 24 hours and 20 minutes each time) for 4 days. On the fourth day, the animals in all groups were anesthetized after undergoing different treatments and then their brains were placed in cold normal saline solution after cutting off the head.

Preparation and injection of bromodeoxyuridine to the animals

About 2.5 mL (50 mg/kg) of bromodeoxyuridine (BrdU) solution (Alfa Aesar, USA) was injected to the animals every 2 hours on the fourth day after the surgery in order to inject and mark the stem cells. Then, the animals were killed and their brain tissue was placed in the formalin for immunohistochemical studies 24 hours after the last injection. Next, paraffin sections of the tissue were hydrated. For this purpose, the tissues were placed in fresh Tris solution for 10 minutes and then, the tissues were placed in 100%, 95%, 80%, 70%, 60%, and 45% ethanol (each time for 10 minutes). After the hydration process, the tissue was incubated for 5 minutes by dH_2O .

To remove the calcium, the tissues were placed in hot sodium citrate buffer (pH 6.0) for 10 minutes and were then kept at room temperature for 30 minutes in order to cool down. In addition, the tissues were incubated with 0.5 $\mu\text{g}/\text{mL}$ anti-BrdU (i.e., rat monoclonal antibody, Abcam, England) for 24 hours to mark and specify the BrdU⁺ cells. Then, they were washed with phosphate buffered saline and incubated with secondary antibody (0.5 $\mu\text{g}/\text{mL}$, Proteinsimple, USA) for 24 hours. Finally, the tissues were fixed on the slide and the number of BrdU⁺ cells was counted using an optical microscope.

RNA extraction

The RNX-Plus kit of CinnaGen Co. (Tehran, Iran) was used to extract RNA from the hippocampal tissue of the rat groups treated with iron oxide nanoparticles and magnetic field and the untreated groups. Further, a spectrophotometer (IMPLEN GmbH, Germany) was utilized to measure the quantity of the extracted RNA.

Synthesis of cDNA and the quantitative real-time polymerase chain reaction

For quantitative assessment of *Ki-67* gene expression, cDNA synthesis was first performed using the extracted RNA by the Revert Aid First Strand cDNA Synthesis kit (Fermentas Co., USA). Then, the quantitative real-time polymerase chain reaction (qRT-PCR) was implemented by SYBR Green Master Mix Kit (Yekta Tajhiz Co., Iran) using the Corbett Rotor-Gene 6000 Device (Sydney, Australia). Furthermore, real-time PCR was conducted using the following cycling conditions: 95°C for 5 minutes, as well as 40 cycles at 95°C for 5 seconds and 60°C for 31 seconds. Each complete amplification stage was followed by a dissociation stage at 95°C for 15 seconds and 60°C for 30 seconds. The employed primers are listed in Table 1. Moreover, the β -actin gene was used as the reference gene (internal control) in order to normalize the reaction (15). All experiments were repeated at least 3 times and the results were analyzed using the $2^{-\Delta\Delta CT}$ equation.

Statistical analysis

The results were presented as the mean \pm standard deviation (SD). Additionally, *t* test, one-way analysis of variance (ANOVA), and Tukey tests were applied to compare the significant differences between the groups. Statistical significance was considered as $P \leq 0.05$.

Results

The results of the staining of the hippocampal tissue using bromodeoxyuridine method

BrdU staining was used in different groups after ischemia-reperfusion (IR) induction in order to evaluate the proliferation and growth of the stem cells in hippocampal dentate gyrus of the brains of the rats. The results showed that IR induction in rats resulted in a significant proliferation of the stem cells compared to the healthy group. Moreover, the separate use of the iron oxide nanoparticles with a concentration of 10 mg/kg and a magnetic field of 1 tesla significantly increased the number of stem cells

compared to the IR group. However, the simultaneous use of nanoparticles and magnetic fields failed to show a significant change compared to the IR group. On the other hand, using the iron oxide nanoparticles or magnetic fields alone increased the number of stem cells compared to their simultaneous treatment. The results of this study demonstrated that employing iron oxide nanoparticles duplicates neural stem cells compared to magnetic fields in rats with IR (Figure 1 and Table 2).

The results of *Ki67* gene expression in different groups

Based on the results of the quantitative assessment of *Ki67* gene expression using the quantitative real-time polymerase chain reaction, IR significantly increased the expression of this gene compared to the healthy group ($P=0.009$). In addition, significant increases in *Ki67* expression were observed in the group treated with iron oxide nanoparticles (10 mg/kg) compared to the IR group ($P=0.004$). Further, the effect of the magnetic field of 1 tesla significantly increased the expression of this gene compared to the IR group ($P=0.006$). However, the simultaneous use of a magnetic field of 1 tesla and iron oxide nanoparticles (10 mg/kg) demonstrated no significant difference compared to the IR group. Conversely, applying iron oxide nanoparticles or magnetic field alone caused a significant increase in the expression of the *Ki67* gene compared to simultaneous treatment. Eventually, it was found that iron oxide nanoparticles alone led to higher expression of *Ki67* gene compared to the magnetic field (Table 3).

Discussion

In this study, the effect of iron oxide nanoparticles and magnetic field on the stimulation of stem cells was confirmed in the hippocampal subventricular zone in rats with ischemia/reperfusion and iron oxide nanoparticles were found to increase the stem cells in the hippocampus.

Table 2. The comparison of the number of BrdU⁺ cells in different treatment groups

Group	Mean cell count	SD	P value
Control	48.67	4.73	
IR	126.67	13.05	0.008
Fe NP+IR	167.33	12.90	0.006
MF+IR	147	11.53	0.007
Fe NP+MF+IR	114.33	10.02	0.09

Abbreviations: IR, Ischemia reperfusion; SD, Standard deviation; Fe NP, Iron oxide nanoparticles; MF, Magnetic field; BrdU, Bromodeoxyuridine.

Table 1. The characteristics of the primers used in this study

Target gene		Sequence	Size of PCR amplicon (bp)
<i>Ki67</i>	Forward	5'- CGCAGGAAGACTCGCAGTTT-3'	184
	Reverse	5'- CTGAATCTGCTAATGTCGCCAA-3'	
β -actin	Forward	5'-TCCTGTGGCATCCACGAAACT-3'	185
	Reverse	5'-GGAGCAATGATCTTGATCTTC-3'	

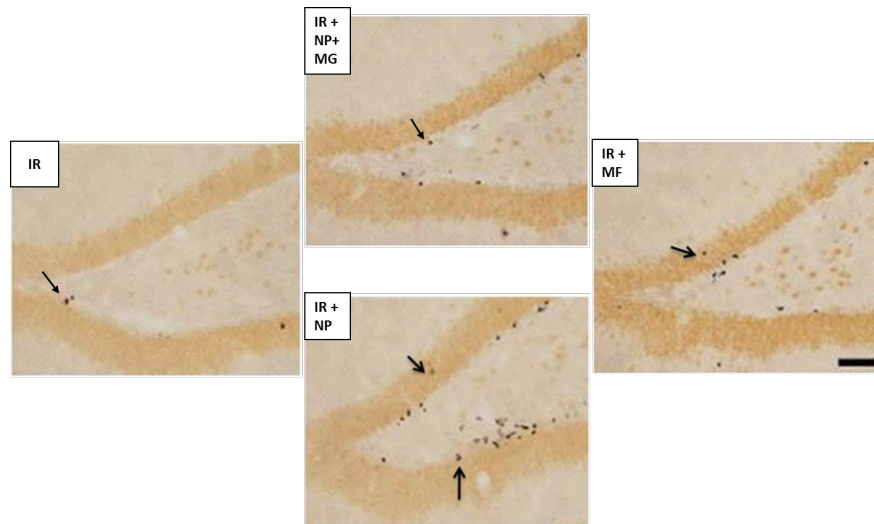


Figure 1. A microscopic view of the hippocampal dentate gyrus of the brain after staining with bromodeoxyuridine (BrdU).

Note. The rats in different groups and the number of BrdU⁺ cells (i.e., cells with dark to dark brown nucleus) stained using immunohistochemistry (100x). (1) IR: A group of animals under cerebral ischemia reperfusion without functional treatment; (2) IR+NP: The animals under cerebral ischemia/reperfusion treated with iron oxide nanoparticles (10 mg/kg); (3) IR+MF: The animals under cerebral ischemic reperfusion treated with a magnetic field of 1 tesla; (4) IR+NP+MF: The animals under cerebral ischemia/reperfusion simultaneously treated with iron oxide nanoparticles (10mg/kg) and a magnetic field of 1 tesla.

In addition, the increased expression of the gene involved in cell proliferation (*Ki67*) confirmed the proliferation of neural stem cells in the hippocampus in this group of treated cells. In a study by Neubert and Brauer, iron oxide nanoparticles were shown to increase the regenerative capacity of neurons after the brain and spinal cord trauma (16). Furthermore, Huang et al indicated that iron oxide nanoparticles increase the proliferation of human mesenchymal stem cells (17). Moreover, Sibov et al demonstrated that iron oxide nanoparticles along with dextran and poly-L-lysine could increase the proliferation of human umbilical cord mesenchymal stem cells while reducing the apoptosis in these cells (18). Additionally, Sadek et al reported that using mesenchymal stem cells reduced the ischemia-reperfusion injury in the kidney of the albino mice (19). Therefore, it seems that utilizing iron oxide nanoparticles plays a role in the proliferation and differentiation of stem cells by increasing the stem cells and may have the ability to reduce the injuries caused by the ischemia reperfusion, which is consistent with the results of other studies.

In a study by Pita-Thomas et al on retinal neurons, the effect of iron oxide nanoparticles in the magnetic

field increased the length of exons by 20 times (20). Similarly, Yuan et al indicated that the magnetic field induced angiogenesis after a heart attack in a mouse model (21). Based on the results of a study by Hao et al, it was found that employing a magnetic field may cause umbilical venous endothelial cells tubulization under in vitro conditions (22). In addition, Guo et al revealed that the magnetic field was able to increase the proliferation of neural stem cells in the mouse brain after the ischemia (23). Further, Podda et al found that low-frequency electromagnetic fields increased the survival of the cell and the levels of the anti-apoptotic protein Bcl-2 in the mouse hippocampus whereas decreasing the expression of the pro-apoptotic protein Bax (24). Furthermore, Abbasnia et al demonstrated that a magnetic field (1 Hz or 30 Hz) could increase the proliferation and differentiation of neural stem cells and progenitor cells in the mouse brain within one week (25). Similar to the results of the above-mentioned studies, employing a magnetic field in the present study increased the proliferation in neural stem cells. Moreover, the magnetic field in the present study had an intensity of 1 tesla and was used for 4 days, which seems to be more effective in increasing the cell proliferation compared to the study by Abbasnia et al. Accordingly, the magnetic field can be considered as one of the stimulants of the neural stem cells in rat hippocampus, which can reduce the damage to the brain caused by the induction of ischemia.

Likewise, Pita-Thomas et al reported that the iron oxide nanoparticles along with the magnetic field increased the length of the exons (20). Additionally, Li et al found that silica-coated superparamagnetic iron oxide nanoparticles alone were unable to increase the proliferation, migration, and tube formation of the endothelial progenitor cells.

Table 3. The comparison of *Ki67* gene expression in different studied groups

Group	Gene expression level	SD	P value
Control	1	0	
IR	2.31	0.27	0.009
Fe NP+IR	3.18	0.28	0.004
MF+IR	2.52	0.23	0.006
Fe NP+MF+IR	2.16	0.15	0.07

Abbreviations: IR, Ischemia reperfusion; SD, Standard deviation; Fe NP, Iron oxide nanoparticles; MF, Magnetic field; BrdU, Bromodeoxyuridine.

However, its combination with the magnetic field improved neurobehavioral outcomes while it reduced the brain atrophic volume in the ischemic perifocal region (26). In other studies, similar to the present study, iron oxide nanoparticles and magnetic fields were separately used to reduce the brain damage and the results promised the effectiveness of these 2 treatment methods in the near future. In addition, 2 potential treatment methods were simultaneously employed in the present study, which revealed no significant difference regarding increasing the proliferation compared to the sham samples. As a result, it seems that it is necessary to use a magnetic field with higher or lower voltages in order to increase the effectiveness of this combined method.

Conclusion

In general, the results of this study indicated that the use of iron oxide nanoparticles at a concentration of 10 mg/kg or magnetic field of 1 tesla (20 minutes) for 4 days caused an increase in the proliferation of neural stem cells and the expression of the gene involved in the cell proliferation (*Ki67*) after inducing the ischemia/reperfusion in rat hippocampus. However, the simultaneous use of iron oxide nanoparticles and magnetic field showed no significant difference in increasing the neural stem cells after the induction of ischemia/reperfusion compared to the sham samples. Therefore, increasing the effectiveness of this combined method necessitates using a magnetic field with lower or higher voltages, which requires further investigations.

Conflict of interests

None.

Ethical considerations

The Ethics Committee of Islamic Azad University of Pharmaceutical Sciences Branch in Tehran approved the study (ethics No. iauz.REC.1393.45).

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