Original Article

J Babol Univ Med Sci
Vol 20, Issu 2; Feb 2018. P:42-48

The Role of Iron Oxide Nanoparticles and Magnetic Field on Apoptosis and Bax Gene Expression in Rat Hippocampus after Ischemic Reperfusion

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J Babol Univ Med Sci; 20(2); Feb 2018; PP: 42-8

Received: Jan 7th 2017, Revised: Oct 22th 2017, Accepted: Dec 12th 2017.

ABSTRACT

BACKGROUND AND OBJECTIVE: Stroke is the second leading cause of mortality in the worldwide. After a stroke, many neurons in the ischemic penumbra will undergo apoptosis. The aim of this study was investigation of effects of iron oxide nanoparticles and magnetic field on apoptosis reduction after ischemic reperfusion in rat model.

METHODS: In this experimental study, 50 male Wistar rats weighing 220-250g were randomly divided into five groups of 10 rats each: including control, sham (ischemic reperfusion model), ischemic reperfusion+iron oxide nanoparticles (10mg/kg), ischemic reperfusion +magnetic field (1 Tesla, 20 min in 4 days), and ischemic reperfusion+iron oxide nanoparticles and magnetic field groups. Injections were performed intraperitoneally. After Four days, the hippocampi were removed for studying of Apoptosis Induction (by TUNEL technique) and changes in *Bax* gene expression (by Q-PCR method).

FINDINGS: After induction of ischemic reperfusion, TUNEL+ cells number treated with iron oxide nanoparticles (7±2) and or the magnetic field (12±2) had significant decrease ($p<0.01$) relative to ischemic reperfusion group (27±5) during 4 days. But simultaneous treatment with nanoparticles and magnetic field (23±2.6) did not show significant difference compared to ischemic reperfusion group (27±5) during 4 days. Furthermore *Bax* gene expression decreased in iron oxide nanoparticles treated group (2.46±0.22) or the magnetic field exposed group (3.28±0.33) significantly ($p<0.01$)compared to ischemic reperfusion model (5.21±0.73)..

CONCLUSION: It seems that iron oxide nanoparticles as well as magnetic field to be two effective methods in decrease of apoptosis after ischemic reperfusion.

KEY WORDS: *Bax, Iron Oxide, Ischemia, Magnetic Field, Nanoparticles, Reperfusion*

Please cite this article as follows:

Bagheri Tari Sh, Khazaei Koohpar Z, Falahati M. The Role of Iron Oxide Nanoparticles and Magnetic Field on Apoptosis and Bax Gene Expression in Rat Hippocampus after Ischemic Reperfusion. J Babol Univ Med Sci. 2018;20(2):42-8.

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[DOR: 20.1001.1.15614107.1396.20.2.6.2]

[DOI: 10.18869/acadpub.jbums.20.2.42]

Introduction

Stroke is the second cause of death (1) and is one of the most common causes of adult disability (2) in the world. For treatment of brain damaged parts caused by stoke there are different therapies for neurogenesis, angiogenesis, increase axonal length, and synaptogenesis are available, including the transplantation of neural, mesenchymal, fetal, stem cells or multi-potential stem cells (1). Considering the key role of the hippocampus in learning and memory (3), reducing the damage to this part and restoring it to stroke damage is very important. The use of stem cells in the hippocampus to repair neurological defects today is considered as an important and effective policy in the treatment of neurological diseases (4).

So that induction, proliferation and differentiation of the neural stem cells in the sub-ventricular layer leads to an improvement in the repair of nerve lesions (5). Today, iron oxide nanoparticles are used for various purposes, such as cell tracing, or anticancer drugs tracing along with magnetic resonance imaging (MRI) (6). Conducting medications, enzymes, and antibodies with iron oxide nanoparticles under the influence of a magnetic field to the limb, tissue, or cancerous position can be another therapeutic application (7). The potential advantage of iron oxide nanoparticles in the treatment of neurological lesions is their high power in exchanging materials between the tissue and the blood after ischemia (8). It has also been shown that magnetic fields result in changes in the permeability of the cell membrane and in interaction with ions and organic molecules, such as proteins and nucleic acids, and thus increase cell growth (9, 10).

In this study, the effect of iron oxide nanoparticles and magnetic field on the mouse brain after ischemic reperfusion (IR) was evaluated on apoptosis and Bax gene expression, given that ischemia causes irreversible damage to the brain.

Methods

Animals: This experimental study was approved by the Ethics Committee of the Islamic Azad University Pharmaceutical Sciences Branch in Tehran, with code of 45.1393auz.REC. It was done on 50 male Wistar rats weighing 220-250 grams. Rats were kept in a room with 12-hour darkness and 12-hour lighting and 25±2 ° C, and water and food were provided. Rats were divided into five groups of 10 (1) Control: healthy animals, 2) Sham: Animals were treated with reperfusion ischemia (IR), which were treated only with normal saline (solvent); 3) Experimental group 1: Animal models of ischemic brain reperfusion treated with iron oxide nanoparticles 10 mg/kg; 4) Experimental group 2: Animals of the cerebral reperfusion ischemia model that was under 1 Tesla's power for 4 days; and 5) Experimental group three: Animal models of brain reperfusion ischemia was treated with 10 mg/kg of nanoparticles of iron oxide and was under 1 Tesla's power daily by the magnetic field. Then, for the induction of anesthesia in animals, the combination of xylazine (Alfasan, Netherlands) and ketamine (Rotexmedica, Germany) were used. Which were prepared with a ratio of 1: 5 (5 ml of ketamine+1 ml of xylazine) and injected 10 mg/kg based on the weight of each animal intraperitoneally.

Induction of cerebral reperfusion ischemia in rat: After anesthesia, a vertical incision was made in the neck area, followed by observation common carotid arteries on both sides. After separating the vagus nerve, carotid arteries were blocked by microsurgery clamp for 20 minutes and the blood flow to the brain was stopped. At the time of ischemia, the temperature of the animal's body was monitored regularly and after 20 minutes the clamps were removed and the blood circulation restored. After induction of ischemia, the muscles were placed in their anatomical position and the section was cut, and stitched.

Iron oxide nanoparticle suspensions: Iron oxide nanoparticles (Fe2O3) (Iron Oxide (II III) Magnetic Nanoparticles powder) were purchased in 10-nanometer powder form from Sigma-Aldrich (Germany). Iron oxide nanoparticles with a final concentration of 10 mg/ml were added to a saline solution at 35-40 ° C for 5 minutes and vortexed to prepare a suspension.

Animal treatment: For animal treatment in different groups, after grouping and surgical treatment of animals, the groups were treated with the following pattern.

Group 1 (control group): healthy animals were treated intraperitoneally 20 minutes after neck fracture with normal saline solution (nanoparticle solvent), then the animal skin was sutured without induction of reperfusion ischemia.

Group 2 (sham group): In this group, animals were treated with normal saline solution for 20 minutes after brain injury induction.

Group 3 (Group treated with iron oxide nanoparticles): Animals were treated intraperitoneally 20 minutes after

[DOR: 20.1001.1.15614107.1396.20.2.6.2]
[DOI: 10.18869/acadpub.jbums.20.2.42]

induction of brain injury at a dose of 10 mg/kg of iron oxide nanoparticles.

Group 4 (group exposed to magnetic field): Animals were placed in a magnetic field of 1 Tesla, in anesthetic state for 20 minutes after induction of brain injury for 4 days (once upon 24 hours and 20 minutes each time) (Fig 1).

Group 5 (Group exposed to magnetic field and iron oxide nanoparticles): Animals were treated intraperitoneally 20 minutes after induction of brain damage with iron oxide nanoparticles (10 mg/kg), and then animals were placed in a magnetic field of 1 Tesla, in anesthetic state for 4 days (every 24 hours and 20 minutes each time). On the fourth day, after different treatments, the animals were anesthetized in all groups and, after separating the head, the rat's brain was placed in a cold saline solution.

Figure1. Magnetic field generator and animal skull placement in 1Tesla magnetic field for 20 minutes per day.

Frozen section of brain and TUNEL staining: To determine apoptosis in neurons after induction of reperfusion ischemia and change in death rate after different treatments, specific apoptosis staining (TUNEL staining) was used. For this purpose, the paraffin block was prepared from brain tissue stored in -80 °C freezer and placed in liquid nitrogen. Each block was then placed in a frozen microtome, and sections of 3-5 µm were prepared and placed on a slide containing OCT solution. To determine the apoptotic cells, the Tunnel staining was performed using the In situ Direct DNA Fragmentation (TUNEL) Assay Kit (Abcam, UK). First, the tissue sections were incubated with Proteinase K solution for 15-30 minutes at 21-27°C, and then washed with PBS. Tissue exposure to hydrogen peroxide3% was performed for 10 minutes at room temperature in darkness and then washed with PBS. In order to increase the permeability of the desired tissue, tissue components were placed on ice for 2 minutes and then the TUNEL Reaction Mixture was added to each tissue section and incubated for 60 minutes at 37°C and then washed with PBS. Subsequently, the converter-POD solution 3 was added and incubated for 30 minutes at 37 °C and then washed with PBS. After washing, DAB was added to the tissues and was incubated for 5-10 minutes at 15-25 °C and then washed with PBS. Finally, three slides were randomly selected from each group and five fields were detected in each slide with an optical microscope with an x400 magnification.

Apoptosis study of tissue specimens: All tissues were prepared to evaluate the apoptosis rate in the CA1 region of the hippocampus by TUNEL staining. In this type of staining, apoptotic cells (TUNEL +) have a chromatic nucleus (black to brown) with a fragmented structure, and healthy cells lack these structures. The total number of TUNEL+(apoptotic bodies) cells in the CA1 region of the animal's brain indicates apoptosis in this part of the animal's brain. The TUNEL+cells were counted using the Image Tools software.

RNA purification: RNX-Plus Solution (Sinaghen, Iran) was used to purify the RNA from the CA1 region of the hippocampus. RNA purified from different specimens was quantitatively evaluated using a spectrophotometer of Nanodrape 1000 (Thermo, USA) and qualitatively investigated in 1% agarose gel.

Expression of Bax gene by Q-PCR method: Q-PCR reaction was used to evaluate the expression of Bax gene. Initially cDNA synthesis was performed using extracted RNA and the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). According to the manufacturer's instructions, materials were prepared in micro tube for the synthesis of cDNA in the Applied Biosystems 2720 Thermo Fisher Scientific (USA) device. In this study, the differences in the expression level of Bax gene in Q-PCR were compared to the reference gene of β-actin using the SYBR Green Master Mix kit (Yekta Tajhiz, Iran).

The reaction in the iCycler iQ ™ Real-TimePCR Detection System (Biorad, USA), according to following program, a single initial denaturation phase at 95°C for 15 seconds, 40 cycles at 95°C for 25 seconds, 59°C for 20 seconds, 72°C for 20 seconds, and final expansion at 72°C for 1 minute. The sequence of used primers mentioned in Table 1. The β-actin gene was used as reference gene. All experiments were repeated at least three times and the results were analyzed using the 2-ΔΔCT equation.Data were analyzed by t-test, one-way ANOVA and Tukey's complement test. $p<0.05$ was considered significant.

[DOR: 20.1001.1.15614107.1396.20.2.6.2]
[DOI: 10.18869/acadpub.jbums.20.2.42]

Table1. Characteristics of primers used in this study

Gene		Sequence	Product length
Bax	Forward	5-AGACAGGGGCCTTTTTGCTAC -3	Bp132
	Reverse	5- AATTCGCCGGAGACACTCG -3	
β-actin	Forward	5-TCCTGTGGCATCCACGAAACT-3	Bp185
	Reverse	5-GGAGCAATGATCTTGATCTTC-3	

Results

The staining results of the studied groups using the TUNEL kit: The images taken from the CA1 region of the hippocampus of rats in the studied groups with TUNEL staining are shown in Fig. 2. This method is widely used for quantitative evaluation of apoptosis. Evaluation of apoptosis in different experimental groups: The results of TUNEL test showed that the formation of ischemic reperfusion (IR) resulted in a significant increase ($p<0.01$) in the number of TUNEL+(27±5) cells compared to the healthy group (33±58/3).

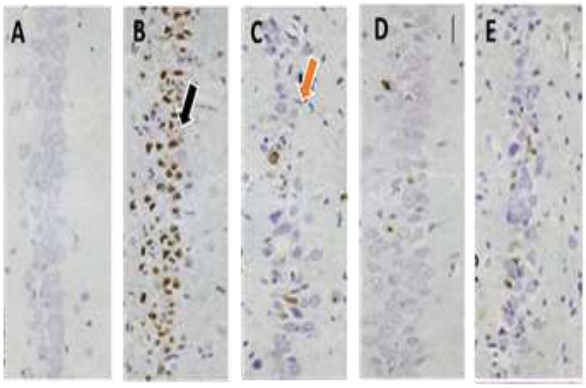

Figure 2. CA1 region of rat hippocampus in the five study groups. TUNEL staining, 400x magnification (black arrow: apoptotic cells and apoptotic bodies) and red arrow: healthy cells. A) Healthy group; B) IR group; C) IR+Fe2O3 group; D). IR+Magnetic field group and E) IR+Fe2O3+Magnetic Field group

The number of TUNEL+(7±2) cells in the group treated with iron oxide nanoparticles with a concentration of 10 mg/kg was significantly lower than the IR group (27±5). In the group exposed to magnetic field of 1 Tesla (for 20 minutes in 4 days), the number of TUNEL+(12±2) cells decreased significantly compared to the IR group (27±5). In group with iron oxide nanoparticles and magnetic field (23±6.2), there was no significant difference in the number of TUNEL+cells in comparison with the IR group (27±5). But the significant increase in the number of TUNEL+cells was shown in comparison with this group (combined treatment) (23±2.6) with the group treated with iron oxide nanoparticles alone (7±2) ($p<0.01$) or magnetic field (12±2) ($p<0.05$). Also, the results of this experiment showed that the group treated with iron oxide nanoparticles (7±2) had a lower TUNEL+number than the group exposed to the magnetic field (12±2) ($p<0.05$) (Fig 3).

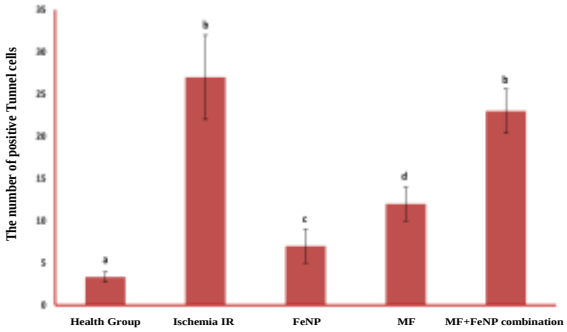

Figure 3. The number of positive TUNEL cells or apoptotic bodies in the CA1 region of the hippocampus were tested in different groups. Non-identical letters: the difference is significant.

Expression of Bax gene expression in the studied groups: After assuring the accuracy of extracted RNA (Fig 4), the expression of Bax gene was quantitatively evaluated in different experimental groups. The results of statistical analysis showed that the formation of reperfusion ischemia (IR) significantly increased the expression of Bax gene expression (5.21±0.73) in comparison with the healthy group (±0.02). However, treatment of rats with iron oxide nanoparticles (10 mg/kg) resulted in significant reduction of Bax gene expression (2.46±0.22) compared to IR (5.21±0.73). Also, in the IR group after exposure to magnetic field (MF) (1 Tesla, for 4 days and 20 minutes each day), the Bax gene expression (3.28±0.33) was significantly lower than the IR group (5.21±0.73). Simultaneous use of magnetic field with intensity of 1 Tesla and iron oxide nanoparticles with concentration of 10 mg/kg (4.17±0.59) did not show significant difference with IR group (5.21±0.73). While the Bax gene expression in the group under treatment with iron oxide nanoparticles alone (2.46±0.22) or in the exposure to magnetic field alone (3.28±0.33) was significantly

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decreased ($p<0.05$) compared to the group under treatment with simultaneous use of them (4.17±0.59). Also, the Bax expression reduction rate in the group treated with iron oxide nanoparticles alone (2.46±0.22) was higher than the expression of Bax expression in the group exposed to the magnetic field (3.85±0.33) ($p<0.05$) (Fig 5).

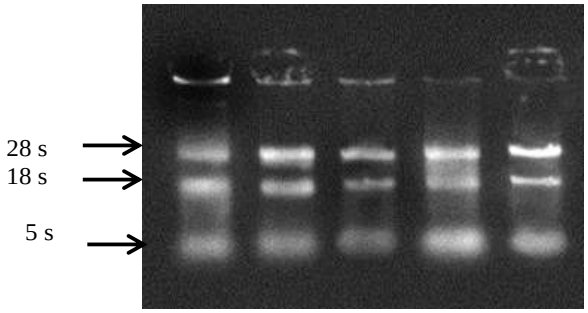

Figure 4. Electrophoresis result of five RNA samples extracted from different experimental groups in agarose gel 1%. The presence of three clear bands (28SrRNA, 18SrRNA and 5 SrRNA) in the agarose gel indicates the correctness of the purification and non-degradation of RNA specimens.

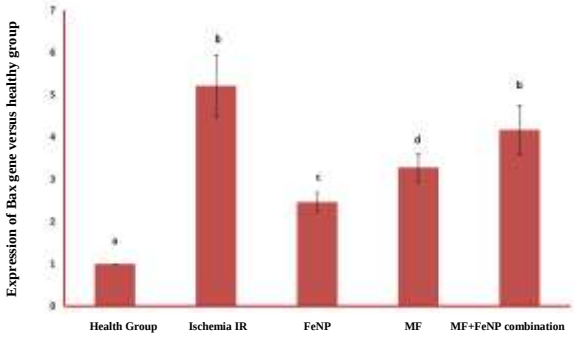

Figure 5. Comparison of Bax gene expression in different groups studied. Non-identical letters: significant difference

Discussion

In this study, it was found that iron oxide nanoparticles and magnetic field alone play an effective role in reducing apoptosis after induction of reperfusion ischemia in the rat hippocampus. Kim et al., in their study, found that the survival rate and attachment of nerve cells to these nanoparticles increased significantly in In vitro conditions (11). In the present study, the decrease in the death of hippocampal cells, in other words, the increase in the survival of cells that were exposed to iron oxide nanoparticles was observed. After ischemia, with the return of blood flow, a high level of free radicals is produced, which causes damage to the organelles and, consequently, to neural cells (12). Iron oxide nanoparticles seem to reduce apoptosis by reducing free radicals and increase cell survival. In the study of Estevez et al., the use of oxidized sodium nanoparticles, due to its strong ability to inhibit free radicals, reduced the complications of ischemia in the rat's brain and reduced the death of neurons after ischemia (12).

Therefore, it seems that iron oxide nanoparticles also have the potential to inhibit free radicals in the survival of nerve cells. In the study of Apopa et al., was found that iron oxide nanoparticles have the potential to increase vascular endothelial permeability. Based on the results of this study, these nanoparticles increase vascular permeability by producing reactive oxygen species (ROS) and stabilizing microtubules. These results suggest that iron oxide nanoparticles can be used to increase the efficacy of central nervous system treatments (8).

In the present study, iron oxide nanoparticles alone were able to reduce the death of neuronal cells, so that the results of the TUNEL test and the expression of the Bax gene confirmed the reduction of planned cell death after induction of ischemia. The study by Palizvzn et al showed that iron oxide nanoparticles have high potential for increased neural protection and repair of nerve lesions (13). Also, the use of magnetic field in this study showed the effect of decreasing the death of neuronal cells after ischemia, although its effect was less than nanoparticles alone. It seems that the magnetic field can lead to cell growth and inhibition of apoptosis by altering the membrane permeability through interaction with ions and organic molecules such as proteins and nucleic acids. Iron oxide nanoparticles and magnetic fields could increase mitochondrial activity in Parkinson's mice (14). Therefore, this method can inhibit the effects of free radicals on mitochondria and reduce the induction of cell death from ischemia.

On the other hand, the simultaneous use of these two treatments had a reverse effect and did not show any significant changes in damaged brain without treatment. It is necessary to increase the simultaneous effect of these two treatments, like the study by Pita-Thomas et al., use different voltages or changes in adjacency with the magnetic field (15) to increase the effectiveness of nanoparticles. The results of this study and other studies show that iron oxide nanoparticles alone and the magnetic field alone reduce the induction of death after ischemia in the rat hippocampus.

[DOR: 20.1001.1.15614107.1396.20.2.6.2]
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However, the reduction in induction of apoptosis in brain-damaged cells that was in contact with iron oxide nanoparticles was much greater than the magnetic field adjacency.

This seems to be the result of increased cell proliferation at the injury site, reducing the harmful effects of free radicals and increasing the survival of important cellular components due to the higher conductivity of vital materials into the cell through iron oxide nanoparticles compared to the magnetic field. On the other hand, in simultaneous treatment with nanoparticles and magnetic fields, reduction of Bax pro-apoptotic gene expression was lower than ischemic rat, which confirms that continuation of treatment with magnetic field nanoparticles for longer or with different voltages may decrease cellular death and increases the protection and repair of neurons after induction of ischemia.

The results of this study showed that the concentration of 10 mg/kg of iron oxide nanoparticles and the magnetic field of 1 Tesla (20 minutes) for 4 days alone could reduce cell death after induction of reperfusion ischemia in rats.

Acknowledgments

Hereby, we would like to thank Dr. Ahmad Reza Dehpour, the head of the Imam Khomeini Hospital laboratory as well as personnel of the laboratory to cooperate in the investigation.

[DOR: 20.1001.1.15614107.1396.20.2.6.2]
[DOI: 10.18869/acadpub.jbums.20.2.42]

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