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Gene Expression Profiles of *BAD* and *Bcl-xL* in the CA1 Region of the Hippocampus Following Global Ischemic/Reperfusion and FK-506 Administration

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Abstract

Background: The hippocampus is a tiny nub in the mammalian brain that is involved in forming, organizing, and storing memories. Global cerebral ischemia (GCI) and reperfusion induced apoptosis lead to cell injury and death. FK-506 is a strong immunosuppressant drug that has neuroprotective effects on the hypoxic-ischemic effects of brain damage. *BAD* and *Bcl-xL* are pro-apoptotic and anti-apoptotic genes, respectively. These genes belong to The B-cell lymphoma-2 (Bcl-2) family.

Objectives: In this study, we assessed the neurotrophic properties of FK-506 on expression of the *BAD* and *Bcl-xL* genes in the hippocampus following global ischemia and reperfusion.

Materials and Methods: In the present experimental study, adult male Wistar rats were obtained and housed under standard conditions in the Tehran University of Medical Science in Iran. Rats were equally distributed in groups of three among the following groups: normal control, treated-1 (ischemia/reperfusion), and treated-2 (ischemia/reperfusion followed by FK-506). Global ischemia was induced for animals in the treated-1 and treated-2 groups. In treated-2, two doses of FK-506 were injected: one dose as an IV injection immediately after reperfusion and another as an intra-peritoneal (IP) injection after 48 hours. Then, the hippocampus tissue was removed after anaesthetizing the rats. RNA was isolated, cDNA was synthesized, and real-time PCR was performed. Finally, the obtained data were analyzed statistically (P value < 0.05).

Results: The quantitative results of real-time PCR show that the mRNA expression ratio of *Bcl-xL* down-regulated was 0.75 ± 0.06 in the ischemia/reperfusion group versus 1.57 ± 0.09 in the control group (P value < 0.001), whereas *Bcl-xL* gene expression was greater in the ischemia/reperfusion +FK506 group (1.93 ± 0.15) than in the ischemia/reperfusion group. Moreover, the mRNA expression ratio of *BAD* up-regulated in the ischemia/reperfusion + FK506 group was 3.65 ± 0.49 compared to Normal control (1.39 ± 0.09) and Ischemia/reperfusion + FK506 was 1.09 ± 0.20 (P value < 0.001).

Conclusions: The analysis of the pro-apoptotic gene to anti-apoptotic gene expression ratio (*BAD* /*Bcl-xL*) confirmed that expression of the pro-apoptotic gene significantly decreased (P value < 0.001) under the ischemia/reperfusion condition. In contrast, the expression of the anti-apoptotic gene increased after administration of FK-506 (P value < 0.001).

Keywords: Ischemic/Reperfusion, Real-Time RT-PCR, Hippocampus, Tacrolimus, *Bcl-xL* Gene, *BAD* Gene

1. Background

Tacrolimus (FK-506) is a strong immunosuppressant drug that has neuroprotective effects on the hypoxic-ischemic effects of brain damage in adult animal models (1). Tacrolimus is chemically known as a macrolide. It reduces peptidyl-prolyl isomerase activity through binding to immunophilin FKBP-12 (FK-506 binding protein) creating an innovative complex. This complex (FKBP12-FK506) interacts with and inhibits calcineurin, thereby inhibiting both T-cell signal transduction and IL-2 transcription (2). FK-506 has different applications. It is commonly used after organ transplantation to suppress the patient's immune system and reduce the risk of organ rejection (3).

The hippocampus is a tiny nub in the mammalian brain that is involved in forming, organizing, and storing memories. It belongs to the limbic system and plays an important role in long-term memory and spatial navigation. The hippocampus is anatomically composed of three main histological subdivisions: the dentate gyrus (DG), CA1, and CA3 (4, 5). The CA1 region is composed of pyramidal neuron cells, receives input from the entorhinal cortex, and operates as a uni-directional (monosynaptic) network (6).

Global cerebral ischemia (GCI) commonly occurs after a variety of clinical conditions, including cardiac arrest

(CA), shock, and asphyxia (7). The result is cell injury and death, which are initially localized; however, it eventually becomes systemic if the inflammatory reaction is passed over (8). Various lines of evidence suggest that GCI leads to hippocampal damage and disruption of spatial learning and memory.

In reperfusion, blood flow returns to tissues and reintroduces oxygen. These processes destroy cellular macromolecules and plasma membranes, resulting in indirect redox signaling and apoptosis.

Cell deaths have been classified into various forms, including apoptosis, necrosis, necroptosis, autophagy, and cornification (9). Apoptosis, also referred to as programmed cell death, is a signal-dependent, suicidal form of cell death that is required to control cell generation and maintain self-tolerance within cells. Programmed cell death is a specific and morphological aspect of cell loss characterized by cell membrane destruction, cell contraction, chromatin condensation, and genomic fragmentation (10). *Bad* and *Bcl-xL* are pro-apoptotic and anti-apoptotic genes, respectively (10). These genes belong to The B-cell lymphoma-2 (Bcl-2) family. The protein products of the Bcl-2 family regulate mitochondrial dysfunction and play an important role in maintaining the integrity of the cell (11).

2. Objectives

Because the brain is an important organ during ischemic shock, it is essential to investigate the molecular mechanism of FK-506 in apoptosis using gene expression quantification of pro-apoptotic and anti-apoptotic genes. In this study, we assessed the neurotrophic properties of FK-506 on the expression of *BAD* and *Bcl-xL* genes in the hippocampus following global ischemia and reperfusion.

3. Materials and Methods

3.1. Animal and Drug Administration

In this experimental study, adult male Wistar rats were obtained from the Tehran University of Medical Science in Iran. The animals were housed in cages (three rats/cage) under conditions of 12:12 hour light/dark cycles (light cycle: 08:00-20:00 and dark cycle: 20:00 - 08:00) at constant room temperature (22 - 24°C). Weights and body temperatures of animals were recorded before the surgical procedure. All procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals and approved by the European Communities Council Directive of November 1986 (86/609/EEC).

Animals were equally distributed in groups of three among the following groups: normal control, treated-1 (ischemia/reperfusion), and treated-2 (ischemia/reperfusion followed by FK-506). FK-506 was dissolved in phosphate buffer saline (PBS) and injected intravenously (IV). The animals that were given FK-506 (treated-2 group) received two doses of 6 mg/kg, one dose as an IV injection immediately after reperfusion and another as an intraperitoneal (IP) injection after 48 hours.

3.2. Global Ischemia/Reperfusion

All animals were temporarily anaesthetized with pentobarbital sodium (40 mg/kg). Then, two temporal subcutaneous thermally sensitive resistors were placed, one adjacent to the skull and one in the rectum, to measure temperature during the surgical procedure. Pericardial and core temperatures were strictly controlled at 37 ± 0.5°C by a heating pad and an overhead incandescent lamp. Subsequently, the ventral region of the neck was incised, and global ischemia was induced by obstructing the common carotid arteries with aneurysm clips for 20 minutes. After ischemia was induced, the clips were removed to initiate reperfusion, and plasticity of the arteries was confirmed by visual assessment. Reperfusion was performed, and the incisions were sutured. Before animals returned to the cages, they were kept in a warm chamber for 24 hours to maintain the body temperature at approximately 37°C and eliminate the protective effects of hypothermia.

3.3. Tissue Collecting

The experimental animals were deeply anesthetized by IP injection with pentobarbital sodium (40 mg/kg) 48 hours after the ischemia/reperfusion step. The brains were then removed, and the hippocampi were rapidly separated, dissected, and retained immediately in RNAlater™ (Qiagen, Germany) liquid to inhibit ribonuclease and avoid RNA degradation. The hippocampi were then stored at -20°C.

3.4. RNA Isolation and cDNA Synthesis

RNAlater™ liquid was removed, and hippocampus tissue was washed with PBS to remove inhibitor agents. Then, total RNA was extracted from 5 mg of hippocampus tissue using a High Pure RNA Isolation Kit (Roche, Germany). In accordance with the kit handbook, the samples were treated with the DNase-I enzyme supplied in the kit to digest DNA contamination. Lastly, light absorbance at 260, 280, and 230 nm was measured using a Nano-photometer 2000c (Thermo Science, USA). The RNA samples with optimum A260/A280 and A260/A230 ratios ($\geq$ 1.7) were selected to synthesize complementary DNA.

Reverse transcription reactions were performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania). In accordance with the kit manual, the reaction volume was 20 μL, and the following components were used: total RNA (1 μg $\approx$ 11 μL), RevertAid RT 200 U/μL (1 μL), RiboLock RNase inhibitor 20 U/μL (1 μL), random hexamer primer (1 μL), dNTP mix 10 mM (2 μL), and reaction buffer 5x (4 μL). Then, samples were incubated for 10 minutes at 25°C, 60 minutes at 42°C, and five minutes at 75°C.

3.5. Target Genes and Primer Design

In this investigation, *BAD* (pro-apoptotic) and *Bcl-xL* (anti-apoptotic) genes were selected as targets. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was also carefully chosen as an internal reference gene. After the sequences of all genes were attained from the NCBI database, primer sets were designed by GeneRunner and PrimerExpress software v.3.0 (Applied Biosystems, Foster City, USA). Finally, in order to avoid secondary structures and homology with other genome regions, the primers were analyzed in a basic local alignment search tool. Oligonucleotide sequences are shown in Table 1.

Table 1. Oligonucleotide Sequences for Interest Genes

Oligo Name Sequence (5′ - 3′)	Amplicon, bp
***Gapdh*-FAAGTTCAACGGCACAGTCAAGG**	22
***Gapdh*-RCATACTCAGCACCAGCATCACC**	22
***BAD* -FGGAGCATCGTTCAGCAGCAG**	20
***BAD* -RCCATCCCTTCATCTTCCTCAGTC**	23
***Bcl-xL*-FGCTGGTGGTTGACTTTCTCTCC**	22
***Bcl-xL*-RGGCTTCAGTCCTGTTCTCTTCG**	22

3.6. Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (real-time PCR) is a molecular technique that monitors the amplification process step by step. In the assay, SYBR Green-I was used as a reporter dye. All the reactions were performed using a Rotor-Gene Q instrument (Qiagen, Germany). Total volume for the PCR reaction was 25 µL, including 12 µL of SYBR Green-I PCR Master Mix (TaKaRa, Japan), and 1 µL of forward and reverse oligonucleotide (400 nM), cDNA template, (300 ng) and ddH_2O. The real-time PCR program was performed for 15 minutes at 95°C, followed by five seconds at 95°C and 20 seconds at 60°C for 40 cycles, with a melting curve analysis ramping from 65°C to 95°C and rinsing 1°C at each step.

Amplification efficiency for target and reference genes was validated using a four-fold dilution series of control cDNA template at 2 000, 200, 20, and 2 ng. Then, a standard curve was drawn by plotting the logarithmic input of cDNA concentration versus mean CT, and the slope was determined. PCR reaction efficiency was calculated using the following formula: $E = (10^{-1/slope}) - 1$.

Expression levels of target genes were calculated using a comparative threshold cycle formula. That is, the expression level of target genes to reference genes in treated samples compared to the normal controls was calculated through formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = [(mCT_{target} - mCT_{reference})_{treated} - (mCT_{target} - mCT_{reference})_{control\ sample}]$) (12).

3.7. Statistical Analysis

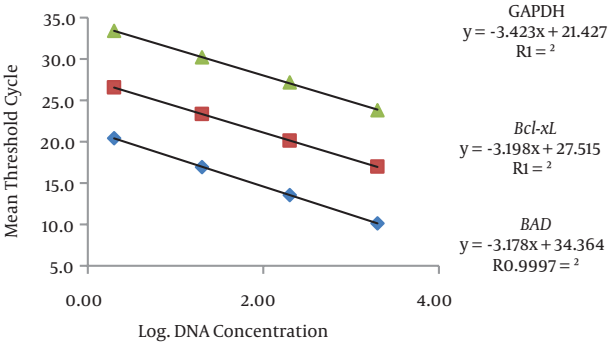
All mathematical calculations were performed using Statistical Package for the Social Sciences software (SPSS Inc. v. 22). The statistical operations included mean ratio (M), standard deviation (SD), confidence intervals (95% CI), and standard error of mean (SEM). Furthermore, significant differences between gene expressions of interest groups were determined, and ANOVA was performed. The significance level was set at P value < 0.05.

4. Results

4.1. Real-Time PCR (RT-PCR)

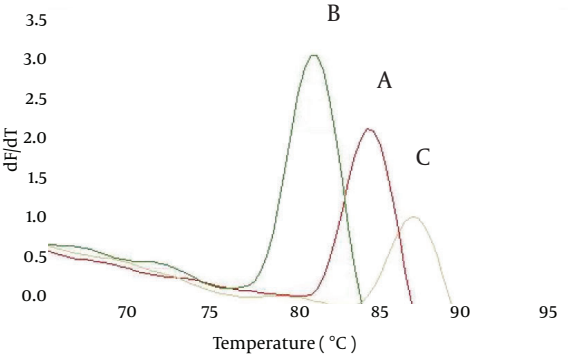
To calculate the PCR efficiency using the gene expression ratio formula ($2^{-\Delta\Delta CT}$), the standard curves for all genes (*Gapdh*, *Bcl-xL*, and *BAD*) were drawn. Then, the slopes were calculated (3.423, -3.198, and -3.178 for *Gapdh*, *Bcl-xL*, and *BAD*, respectively (Figure 1)). Therefore, PCR efficiencies for all interest genes were obtained (95.92% for *Gapdh*, 94.59% for *Bcl-xL*, and 93.64% for *BAD*). The amplification fragments of *Gapdh*, *Bcl-xL*, and *BAD* were melted at 85.3°C, 82.3°C, and 88.0°C, respectively (Figure 2). The analysis of melting curve showed the interest amplification of fragments and any non-specific products (primer dimmers, etc.).

Figure 1. Standard Curve: The Slopes, y-intercept, and Determination Coefficient (R^2)



Gapdh, Blue Diamond; *Bcl-xL*, red Square; *BAD*, Green Triangle.

Figure 2. Melting Curve Analysis of Genes



A, *Gapdh* (85.3°C); B, *Bcl-xL* (82.3°C); C, *BAD* (88.0°C).

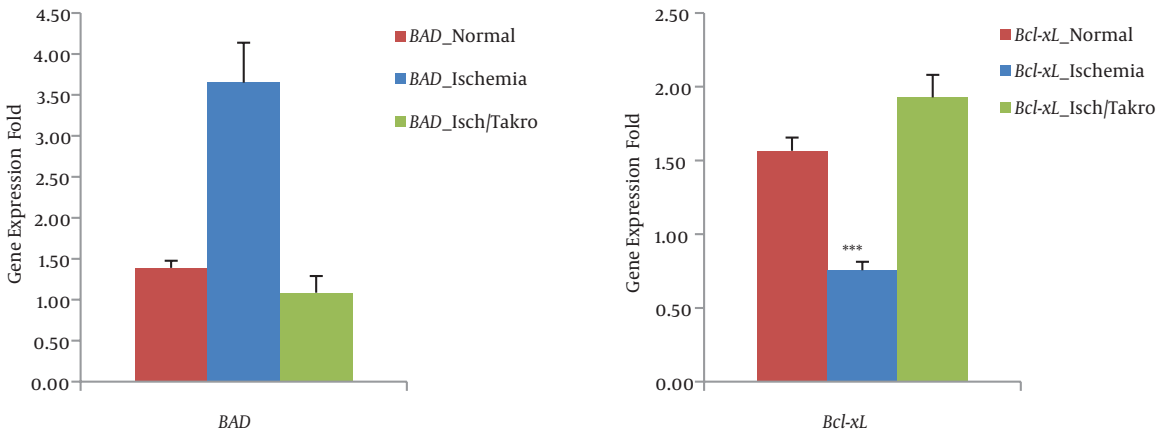


Figure 3. Gene Expression Changes of *BAD* and *Bcl-xL* in Control and Experimental Groups

Table 2. mRNA Expression Ratios of *BAD* and *Bcl-xL* in Experimental Groups

Experimental Groups	*BAD*	*Bcl-xL*
Normal control	1.39 ± 0.09	1.57 ± 0.09
Ischemia/reperfusion	3.65 ± 0.49	0.75 ± 0.06
Ischemia/ reperfusion+FK506	1.09 ± 0.20	1.93 ± 0.15

The quantitative results of real-time PCR show that the mRNA expression ratio of *Bcl-xL* decreased a half-fold in the ischemia/reperfusion group (P value < 0.001) (Figure 3), whereas *Bcl-xL* gene expression increased approximately 2.5 times more in treated-2 (ischemia/reperfusion followed by FK-506) than in treated-1 (Table 2). Moreover, the mRNA expression ratio of *BAD* increased approximately three times more in treated-1 than in the normal control group and treated-2 (P value < 0.001) (Figure 3).

The pro-apoptotic to anti-apoptotic gene expression ratios for the experimental groups were calculated. The *BAD*/*Bcl-xL* ratios were 0.89, 4.83, and 0.56 for the normal control group, treated-1, and treated-2, respectively.

5. Discussion

Ischemia is defined as the reduction of cerebral blood flow (CBF) to a critical threshold that causes brain damage involving the entire brain or one region of the brain. Although reperfusion restores CBF, it can lead to secondary brain injury from the influx of neutrophils and the increase in reactive oxygen species (ROS), cerebral edema, and hemorrhage (7).

Over the past decade, many studies have revealed that apoptosis and necrosis are the temporally distinct processes of neuronal cell death that can occur during cerebral ischemia (13-16). Apoptosis is defined as programmed cell death, and it is an important process associated with DNA fragmentation characterized by cell shrinkage, chromatin aggregation, and preservation of the integrity of cell membranes and mitochondria without inflammation and injury to surrounding tissues (17).

Tacrolimus (FK-506) and cyclosporine are immunophilin and calcineurin inhibitors that attenuate apoptotic cell death (7). FK-506 prevents cerebral ischemia-induced hippocampal neuro-degeneration (18). Some investigators have found that continual administration of FK-506 before the ischemic incident demonstrates neuroprotection in the CA1 region at one week of reperfusion in a rat model of global cerebral ischemia (19). Harukuni et al. reported that pretreatment with cyclosporine and FK-506 inhibits dephosphorylation of the pro-apoptotic protein *BAD* (7).

In the present study, the quantitative results show that global cerebral ischemia and reperfusion induced programmed cell death in the brain, especially in the hippocampus, through up-regulation of *BAD* (a pro-apoptotic gene) and down-regulation of *Bcl-xL* (an anti-apoptotic gene) in the treated-1 group (P value < 0.001). On the other hand, *BAD* gene mRNA levels were lower in the treated-2 group (ischemia/reperfusion followed by FK-506) than in the treated-1 group, while *Bcl-xL* gene expression is up-regulated. These results show that FK-506 is a potent immunosuppressive agent that strongly inhibits apoptosis. In addition, an analysis of the pro-apoptotic gene to anti-apoptotic gene expression ratio (*BAD* /*Bcl-xL*) was performed. The obtained data confirmed that expression of the pro-apoptotic gene significantly decreased (P value < 0.001) under ischemia/reperfusion, but the expression of the anti-apoptotic gene increased after administration of FK-506 (P value < 0.001).

In a parallel study, a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay was performed to detect apoptotic bodies. Sharifi et al. (20) reported that the number of TUNEL-positive cells was significantly in-

creased in the CA1 region of the hippocampus after ischemia. There was a significant difference between the control group and groups 2 (P value = 0.001) and 3 (P value = 0.023), which means that apoptotic cells significantly decreased as a result of repeated injection of FK-506 in this region

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Footnotes

Authors' Contribution: Ramak Badr wrote the proposal, performed the laboratory operations, and prepared the manuscript. Mehrdad Hashemi designed the study, provided technical support, performed data analysis and edited the manuscript. Reza Mahdian analyzed the data and provided technical support. Gholamreza Javadi edited the manuscript and provided technical support. Abolfazl Movafagh performed the statistical analysis and edited the manuscript.

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References

1. Zhou Y, Xiong Y, Yuan SY. [Effect of tacrolimus on growth-associated protein-43 expression in the hippocampus of neonatal rats with hypoxic-ischemic brain damage]. *Zhongguo Dang Dai Er Ke Za Zhi.* 2009;**11**(1):65–8. [PubMed: 19149927]
2. Nam SH, Ji XY, Park JS. Investigation of tacrolimus loaded nanostructured lipid carriers for topical drug delivery. *Bull Korean Chem Soc.* 2011;**32**(3):5.
3. Rath T. Tacrolimus in transplant rejection. *Expert Opin Pharmacother.* 2013;**14**(1):115–22. doi: 10.1517/14656566.2013.751374. [PubMed: 23228138]
4. Sedat J, Duvernoy H. Anatomical study of the temporal lobe. Correlations with nuclear magnetic resonance. *J Neuroradiol.* 1990;**17**(1):26–49. [PubMed: 2388093]
5. Lee I, Hunsaker MR, Kesner RP. The role of hippocampal subregions in detecting spatial novelty. *Behav Neurosci.* 2005;**119**(1):145–53. doi: 10.1037/0735-7044.119.1.145. [PubMed: 15727520]
6. Eichenbaum H, Schoenbaum G, Young B, Bunsey M. Functional organization of the hippocampal memory system. *Proc Natl Acad Sci U S A.* 1996;**93**(24):13500–7. [PubMed: 8942963]
7. Harukuni I, Bhardwaj A. Mechanisms of brain injury after global cerebral ischemia. *Neurol Clin.* 2006;**24**(1):1–21. doi: 10.1016/j.ncl.2005.10.004. [PubMed: 16443127]
8. Ondiveeran HK, Fox-Robichaud A. New developments in the treatment of ischemia/reperfusion injury. *Curr Opin Investig Drugs.* 2001;**2**(6):783–91. [PubMed: 11572657]
9. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ.* 2009;**16**(1):3–11. doi: 10.1038/cdd.2008.150. [PubMed: 18846107]
10. Malhi H, Guicciardi ME, Gores GJ. Hepatocyte death: A clear and present danger. *Physiol Rev.* 2010;**90**(3):1165–94. doi: 10.1152/physrev.00061.2009. [PubMed: 20664081]
11. Youle RJ, Strasser A. The BCL-2 protein family: Opposing activities that mediate cell death. *Nat Rev Mol Cell Biol.* 2008;**9**(1):47–59. doi: 10.1038/nrm2308. [PubMed: 18097445]
12. Kamyab AR, Shahrokhi F, Shamsian E, Hayat Nosaied M, Dibajnia P, Hashemi M, et al. Determination of sensitivity and specificity of a novel gene dosage assay for prenatal screening of trisomy 21 syndrome. *Clin Biochem.* 2012;**45**(3):267–71. doi: 10.1016/j.clinbiochem.2011.11.013. [PubMed: 22178108]
13. Bhardwaj A, Alkayed NJ, Kirsch JR, Hurn PD. Mechanisms of ischemic brain damage. *Curr Cardiol Rep.* 2003;**5**(2):160–7. [PubMed: 12583862]
14. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science.* 1995;**267**(5203):1456–62. [PubMed: 7878464]
15. Khazaei Koohpar Z, Hashemi M, Mahdian R, Parivar K. The effect of pentoxifylline on BCL-2 gene expression changes in hippocampus after long-term use of ecstasy in wistar rats. *Iran J Pharm Res.* 2011;**12**(3):521–7. [PubMed: 24250658]
16. Sari S, Hashemi M, Mahdian R, Parivar K, Rezayat M. The effect of pentoxifylline on bcl-2 gene expression changes in hippocampus after ischemia-reperfusion in wistar rats by a quatitative RT-PCR method. *Iran J Pharm Res.* 2013;**12**(3):495–501. [PubMed: 24250655]
17. Hashemi M. The study of pentoxifylline drug effects on renal apoptosis and BCL-2 gene expression changes following ischemic reperfusion injury in rat. *Iran J Pharm Res.* 2014;**13**(1):181–9. [PubMed: 24734070]
18. Benetoli A, Dutra AM, Paganelli RA, Senda DM, Franzin S, Milani H. Tacrolimus (FK506) reduces hippocampal damage but fails to prevent learning and memory deficits after transient, global cerebral ischemia in rats. *Pharmacol Biochem Behav.* 2007;**88**(1):28–38. doi: 10.1016/j.pbb.2007.07.001. [PubMed: 17675223]
19. Uchino H, Minamikawa-Tachino R, Kristian T, Perkins G, Narazaki M, Siesjo BK, et al. Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. *Neurobiol Dis.* 2002;**10**(3):219–33. [PubMed: 12270685]
20. Sharifi ZN, Movassaghi S, Foroumadi A, Hashemi M, Jafari Semnani S, Atashi M. The study of inhibitory effect of pentoxifylline on apoptosis of maleWistar rat hippocampus after long-term use of ecstasy. *J DevelopBiol.* 2011;**3**(10):8.