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EXPERIMENTAL STUDY

Assessment of global ischemic/reperfusion and Tacrolimus administration on CA1 region of hippocampus: gene expression profiles of *BAX* and *BCL2* genes

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ABSTRACT

BACKGROUND: It is well known that hippocampus has a pivotal role in learning, formation and consolidation of memory. Global cerebral ischemia causes severe damage to pyramidal neurons of the CA1 region and usually results in residual neurological deficits following a recovery from ischemia. Scientists investigate to find the molecular mechanism of apoptosis and to use this cell death for clinical treatment.

OBJECTIVE: In this investigation, we evaluated the molecular mechanism of FK-506 in apoptosis using gene expression quantification of *BAX* and *BCL-2* genes in hippocampus following global ischemic/reperfusion.

MATERIALS AND METHODS: In the present experimental study, adult male Wistar rats were obtained and housed under standard conditions. Each experimental group consisted of five rats and was equally distributed in the normal control, ischemia/reperfusion, ischemia/reperfusion followed by FK-506. Global ischemia was induced for animals in ischemia and drug groups. In the drug group, moreover, two doses of FK-506 were injected as IV injection and intra-peritoneal (IP) injection after 48 h. Then, hippocampus tissue was removed. Consequently, RNA isolated, cDNA was synthesized and Real-Time PCR was performed. Finally, the obtained data was analyzed statistically ($p<0.05$).

RESULTS: The quantitative results showed the *BAX* expression ratio increased approximately 3-times in ischemia/reperfusion (3.11 ± 0.28) group compared to the untreated (NR) and the drug group ($p<0.001$). The statistical analysis showed a significant difference for *BCL-2* expression between the experimental groups ($p<0.001$). The mRNA level of *BCL-2* decreased in the ischemia/reperfusion group, while it was without alteration in the other groups.

CONCLUSION: The results showed that global cerebral ischemia/reperfusion induced *BAX* as pro-apoptotic gene and tacrolimus a neuroprotective drug inhibited *BAX* gene expression and induced *BCL*-2 gene expression as anti-apoptotic gene *(Tab. 2, Fig. 3, Ref. 21)*. Text in PDF *www.elis.sk.*

KEY WORDS: *BAX*, *BCL2*, ischemic/reperfusion, real-time RT-PCR, Tacrolimus.

Introduction

Hippocampus is a tiny part of mammalian brain that is involved in memory forming, organizing, and storing. It belongs to the limbic system structure and is anatomically composed of three main histological subdivisions named dentate gyrus (DG), Cornus Ammonis 1 (CA1), and Cornus Ammonis 3 (CA3) (1). The neurons in the CA1 region (the principal pyramidal cell field in the hippocampus) provide a significant output pathway from the hippocampus, which plays an important role in long-term memory and spatially related tasks.

Global cerebral ischemia (GCI) occurs commonly after a variety of clinical conditions including cardiac arrest (CA), shock and asphyxia. Various lines of evidence indicate that a brain injury following a global cerebral ischemia is exacerbated by hyperoxia during early stages of reperfusion (2). Hyperoxic resuscitation also promotes damage to white matter in gerbils, and hippocampal neuronal death in dogs (3, 4). Global cerebral ischemia is a clinical outcome of cardiac arrest, severe hypotension, or certain operations such as cardiopulmonary bypass and cerebral intervention, which deprive the brain of oxygen and glucose. It causes severe damage to pyramidal neurons of the CA1 region and usually results in residual neurological deficits following a recovery from ischemia (5).

Tacrolimus, commonly known as FK-506, is a drug that suppresses the immune system. It was approved by the FDA in 1994 for liver transplantation and also has been used in patients for the transplantation of the heart, kidney, small bowel, and bone marrow. Tacrolimus has neuroprotective effects on hypoxic-ischemic of brain damage in the adult animal model (6). This drug blocks the activation of calcineurin through the formation of complexes

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with immunophilins. FK506 binds to a different immunophilin than CsA, called FK506-binding protein (FKBP) 12 (7). This complex (FKBP12-FK506) interacts with and inhibits calcineurin, so inhibiting both T-cell signal transduction and IL-2 transcription (7).

Programmed cell death, particularly termed apoptosis, is a signal-dependent suicide form of cell death to control cell generation and maintenance of self-tolerance. Apoptosis is an essential process that occurs during normal development to maintain homeostasis and occurs under pathological conditions (8).This mechanism is probably one of the most widely-studied subjects among cell biologists. There are two basic apoptotic signalling pathways; the extrinsic and the intrinsic pathways (9, 10). The intrinsic apoptotic pathway is triggered by different intracellular stimuli. It depends on a complex termed apoptosome, consisting of procaspase-9, apoptotic protease activating factor (Apaf-1) and cytochrome C (11). The*BCL-2* family members (e.g. *BAX*, *BAK*, *BCL-2*, and *BCL-xL* genes) control the release of cytochrome C by regulating mitochondrial membrane permeabilization. The *BAX* and *BCL-xL* are pro-apoptotic and anti-apoptotic genes, respectively.

In this investigation, we evaluated the molecular mechanism of FK-506 in apoptosis using gene expression quantification of pro-apoptotic (*BAX*) and anti-apoptotic (*BCL-2*) genes in hippocampus following a global ischemic/reperfusion.

Methods and materials

Animal and drug administration

In the experimental study, male Wistar rats (Cellular and molecular research centre, Iran university of Medical Sciences, Tehran, Iran) were obtained and housed in cage (3 rats/cage) under condition light and dark cycles (12:12 h) at the constant room temperature (22–24 °C). Animal's weight and body temperatures were recorded before the surgical procedure. All procedures were performed in accordance with the recommendations for the proper use and care of laboratory animal and confirmed to the European Communities Council Directive of November 1986 (86/609/EEC).

In each experimental group, male rats were equally distributed (5rats in a group), including normal control, ischemia/reperfusion, ischemia/reperfusion followed by FK-506. Tacrolimus was dissolved in phosphate buffer saline (PBS) and injected intravenously (IV). In the Ischemia/Reperfusion+FK-506 group, animals received two doses of FK-506 (6 mg/kg), one dose as IV injection immediately after reperfusion and one dose as intra-peritoneal (IP) injection after 48 h.

Global ischemia/reperfusion

All animals were temporarily anaesthetized with pentobarbital sodium (40 mg/kg). Two temporal subcutaneous thermally sensitive resistors were placed adjacent to the skull and in the rectum to measure the 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. Ventral region of neck was incised and global ischemia was induced through obstructing the common carotid arteries with aneurysm clips for 20 min. The clips, then, were removed to initiate reperfusion. The plasticity of arteries was confirmed by visual assessment during this stage. Finally, the incisions were sutured. Before animal returning to cages, they were located in a warm chamber for 24 h to maintain the body temperature at approximately 37 °C and eliminate the protective effect of hypothermia.

Tissue collecting

The trial animals were deeply anesthetized by IP injection with pentobarbital sodium (40 mg/kg) after 48 h passing from ischemia/reperfusion step. The brain was removed and hippocampus was rapidly separated, dissected and CA1 region segmented and retained immediately in RNA later™ (Qiagen, Germany) to inhibit ribonuclease and avoid RNA degradation. Then, it was stored in –20 °C.

Molecular procedures (RNA and cDNA)

RNA later™ was removed and hippocampus tissue was washed with PBS to eliminate inhibitors. Total RNA was extracted from 5 mg hippocampus tissue using High Pure RNA isolation kit (Roche, Germany). According to the handbook kit, the samples were treated with DNase-I enzyme to digest genomic DNA contamination. Lastly, the light absorbance at 260, 230 and 280 nm was measured using the Nano-photometer 2000c (ThermoScience, USA). The RNA samples with optimum A260/A280 and A260/A230 ratios (≥ 1.7) were selected to synthesize complementary DNA.

Reverse transcription reaction was implemented using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania). In according to kit handbook, reaction volume was 20 µL and components were used including; total RNA (1 µg ≈ 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 min at 25 °C, followed by 60 min at 42 °C and 5 min at 75 °C.

Primer design

In the study, *BAX* (pro-apoptotic) and *BCL-2* (anti-apoptotic) gene were selected as the target genes and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was carefully chosen as the internal reference gene. All genes sequence was got from NCBI database and primer sets were designed by *GeneRunner* and *PrimerExpress* software v.3.0 (Applied Biosystems, Foster City, USA). Finally, the primers were analyzed in basic local alignment search tool (BLAST) in order to avoid secondary structure and homology. Oligonucleotides sequences are shown in the Table 1.

Tab. 1. Primer sequences.

Oligo name	Sequence (5′→3′)	Amplicon (bp)
GAPDH-F	*AAGTTCAACGGCACAGTCAAGG*	22
GAPDH-R	*CATACTCAGCACCAGCATCACC*	22
BAX-F	*AGGGTGGCTGGGAAGGC*	17
BAX-R	*TGAGCGAGGCGGTGAGG*	17
BCL-2-F	*ATCGCTCTGTGGATGACTGAGTAC*	24
BCL-2-R	*AGAGACAGCCAGGAGAAATCAAAC*	24

Real-time PCR using free fluorescent dye

In the real-time PCR assay, SYBR Green-I was used as the reporter dye. All reactions were performed by Rotor-Gene Q apparatus (Qiagen, Germany). Total volume for PCR reaction was 25 µl and consisted of 12.5 µl of SYBR Green-I PCR Master Mix 2x (TaKaRa, Japan), 1 µl of forward and reverse oligonucleotide (0.4 µM), 5 µl of cDNA template (60 ng/µl) and ddH_2O (5.5 µl). Real-time PCR programme was performed for 15 min at 95 °C (Taq activation), following by 5 sec at 95 °C and 20 sec at 60 °C for 40 repeats and melting curve analysis ramping from 65 °C to 95 °C and rinsing 1 °C per each detection.

Amplification efficiency for interest genes was validated using 10-fold dilution series of control cDNA template as 2000, 200, 20, 2 ng. Standard curve was drawn by plotting the logarithmic input cDNA concentration versus mean CT and the slope was determined. PCR reaction efficiency was calculated using formula; $E\,\% = [(10^{-(1/\text{slope})} - 1] \times 100$.

Expression level of target genes was calculated using the comparative threshold cycle formula. The relative gene expression ratio (R) of target genes were calculated based on efficiency (*E*) and cross point (CP) of unknown samples versus controls using pffafle formula;

$$\text{Ratio} = \frac{\Delta\text{Cp target (control mean – sample mean)}\ (\text{E target})}{\Delta\text{Cp ref. index (control mean – sample mean)}\ (\text{E ref. index})} \quad (12).$$

Data processing and statistical analysis

All mathematical procedures were calculated using the Statistical Package for the Social Sciences software (SPSS Inc. v. 22). The statistical operations included Mean ratio (M), Standard Deviation (SD), Determination coefficient (R^2), Confidence Intervals (95% CI) and Standard Error of Mean (SEM). One-way ANOVA, non-parametric (independent sample Kruskal-Wallis test) and Tukey analysis (Post hoc test) were performed to determine significant differences between gene expressions of the interest groups. $p < 0.05$ was considered statistically significant.

Results

Real-time PCR validation

The slope of standard curves for *BCL-2*, *BAX* and *GAPDH* genes were obtained –3.41, –3.52 and –3.39, respectively (Fig. 1). PCR efficiency (*E*) was calculated 96% for *BCL*-2, 92 % for *BAX* and 97 % for *GAPDH* gene. Melting curve analysis shown the specific amplicon for *BCL-2*, *BAX*and *GAPDH* melted at 86.2 °C, 89.3 °C and 85.5 °C, respectively (Fig. 2). The results of melting curves were confirmed by gel electrophoresis of PCR product.

Relative quantification analysis

To determine the alteration of pro-apoptotic and anti-apoptotic gene expression in rat brain after ischemia/reperfusion and drug administration, the level of mRNA for *BCL-2* and *BAX* were measured. The *BAX* expression ratio increased approximately 3-times in the Ischemia/reperfusion (3.11 ± 0.28) group compared to the untreated (NR) and the drug group (Tab. 2, Fig. 3A). The statistical analysis, moreover, showed a significant difference for *BCL-2* expression between the experimental groups ($p < 0.001$). The mRNA level of *BCL-2* decreased in the ischemia/reperfusion group, while it was without alteration in the other groups (Tab. 2, Fig. 3). We excluded outlier data to achieve the accurate results during the statistical analysis.

The ratio of pro-apoptotic to anti-apoptotic gene (*BAX*/*BCL-2*) for experimental groups were calculated 2.00, 23.92 and 1.13 for the untreated, ischemia/reperfusion and Tacrolimus group, respectively (Tab. 2, Fig. 3).

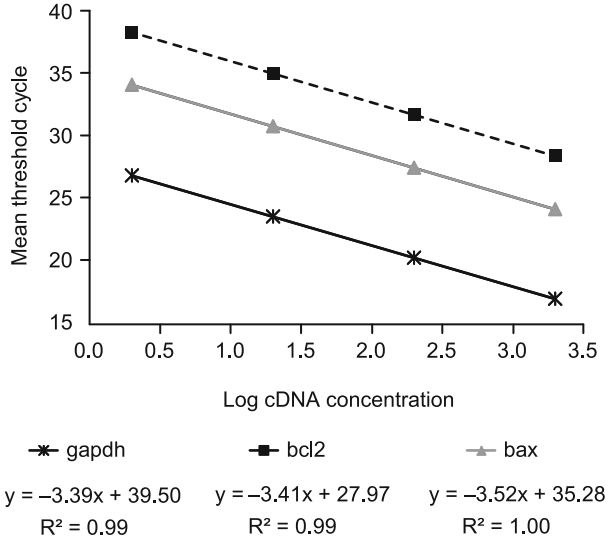

Fig. 1. Standard curves: Mean threshold cycle versus log cDNA concentration. Y-intercept and Determination coefficient for interest genes; bax (y = –3.52x + 35.28, R^2 = 1.00), bcl-2 (y = –3.41x + 27.97, R^2 = 0.99) and gapdh (y = –3.39x + 39.50, R^2 = 0.99).

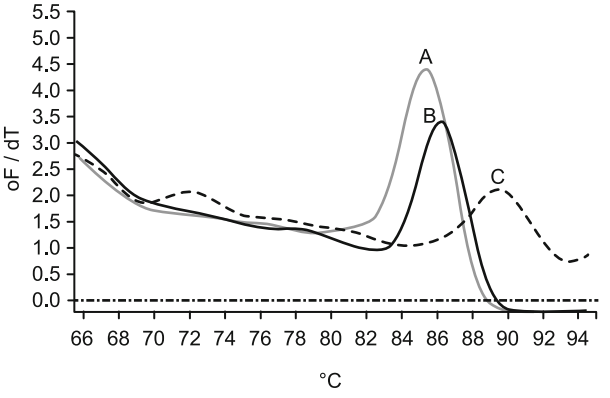

Fig. 2. Melting curve analysis on PCR products of the target and reference genes: A) gapdh (85.5 °C), B) bcl-2 (86.2 °C), C) bax (89.3 °C).

Discussion

It is well known that hippocampus has a pivotal role in learning, formation and consolidation of memory. Hippocampus is critically

Tab. 2. mRNA expression ratio for *BAX* and *BCL-2* in the experimental groups.

Experimental groups Target genes	Untreated (mean ± SD)	Ischemia/reperfusion (mean ± SD)	Ischemia/reperfusion+FK506 (mean ± SD)	p
BAX	0.90±0.08	3.11±0.28	0.68±0.06	<0.001
BCL-2	0.45±0.09	0.13±0.01	0.60±0.03	<0.001
BAX/ *BCL*-2	2.00	23.92	1.13	-

involved in the regulation of emotion, fear, anxiety, and stress. Most studies about hippocampus were performed on the regional specialization and organization of hippocampal functions in experimental models and in human neurological and psychiatric diseases (13).

Global cerebral ischemia (GCI) occurs commonly after a variety of clinical conditions including cardiac arrest (CA), shock and ischemia (14). The signalling pathways of cell death and survival were imbalanced during the cerebral ischemia/reperfusion injury. Over the past decade, many researches have revealed that apoptosis and necrosis are temporally distinct processes of neuronal cell death, which can occur during cerebral ischemia (15). Scientists investigate to find the molecular mechanism of apoptosis and to use this cell death for clinical treatment. There are two pathways, by which apoptosis can be initiated; the extrinsic and the intrinsic (or mitochondrial) pathways. Internal stimuli, such as severe DNA damage, hypoxia and oxidative stress are some triggers to initiate the intrinsic mitochondrial pathway (16).The intrinsic pathway induces a dysfunction of some organelles, in particular mitochondrial dysfunction, which results in the outer membrane permeabilization and the concomitant release of apoptogenic proteins into the cytosol allowing the execution of the apoptotic program (17).The intrinsic pathway is regulated by the group of proteins belonging to the Bcl-2 family. Bcl-2 proteins are functionally divided in two main groups namely the pro-apoptotic (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1)(16).

Tacrolimus is a neutral macrolide antibiotic that is extracted from the fungus Streptomyces tsukubaensis. This drug is a potent anti-T cell agent and has been shown to be effective in preventing the rejection of transplanted organs (18, 19). Published research about tacrolimus has focused on effects associated with therapeutic use. Many previous investigations have indicated a neuroprotective effect of Tacrolimus during cerebral ischemia, although there are conflicting reports regarding their efficacy. Zhou et al showed that FK-506 attenuated neuronal necrosis through increased expression of GAP-43 gene in the FK-506 intervention group (6). Sakasi et al investigated the effect of FK-506 on ischemia-induced NO production in association with the pathogenesis of delayed neuronal death (20). He reported that FK506 was a neuroprotective drug and inhibited NO production during global ischemia. Another research published that rats, administered tacrolimus acutely after a traumatic brain injury, showed significantly fewer non-convulsive seizures than untreated rats (21).

In the present study, we evaluated the molecular mechanism of FK-506 in apoptosis by quantification of pro-apoptotic (*BAX*) and anti-apoptotic (*BCL*-2) gene expression in hippocampus following a global ischemic/reperfusion. Quantitative results showed *BAX* gene up-regulated 3-times in the ischemia/reperfusion group ($p<0.001$) compared to other experimental groups, while the expression of *BCL*-2 gene down-regulated ($p < 0.001$). In the experimental group, there was no change in *BAX* and *BCL*-2 gene expression. Moreover, the ratio of pro-apoptotic/anti-apoptotic gene expression (*BAX*/*BCL*-2) increased approximately 12-fold in the ischemia/reperfusion group compared to the other groups. The results showed that global cerebral ischemia/reperfusion induced *BAX* as pro-apoptotic gene and tacrolimus as neuroprotective drug inhibited *BAX* gene expression and induced *BCL*-2 gene expression as an anti-apoptotic gene.

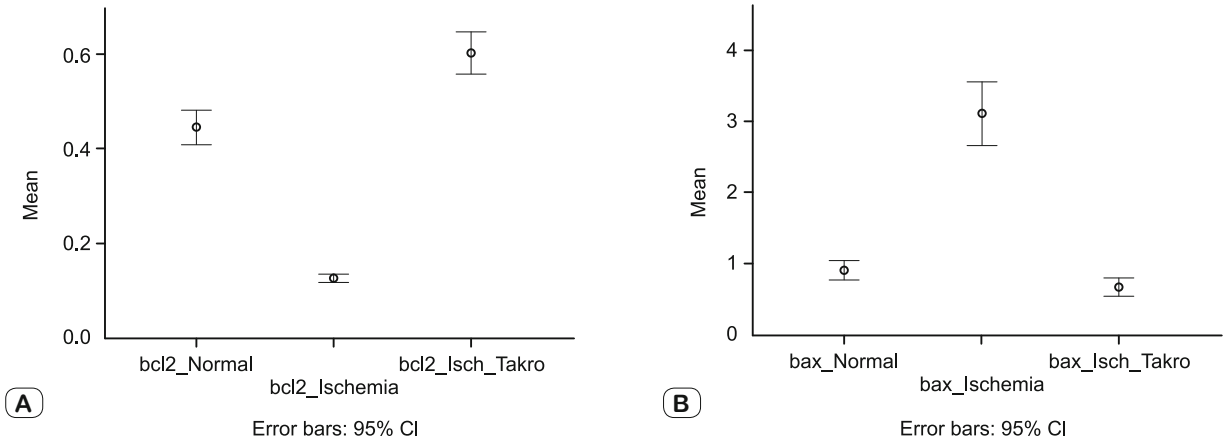

Fig. 3. Gene expression ratio (median ± IQR) of bcl2 and bax in experimental groups compared to normal control.

In the parallel research, TUNEL (Terminal deoxynuclotidyl transfrased UTP nick-end labelling) assay was performed to detect apoptotic bodies. The number of TUNEL-positive cells was significantly increased in the CA1 region of the hippocampus after ischemia in ischemia/reperfusion group. There was a significant difference in the apoptotic body numbers in the ischemia/reperfusion group ($p = 0.001$), while there was no significant differences between the control group and the drug group ($p = 0.023$). This showed that apoptotic cells were significantly decreased by repeated injection of FK-506 in CA1 region of hippocampus.

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