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The impact of piracetam and octreotide on hippocampal expression of BAX and BCL2 genes in pentylenetetrazole-induced epileptic rats

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Abstract

Objective: Piracetam and octreotide as nootropic and neuroprotective medicines were used in the epileptic rats to evaluate their impacts on the hippocampal expression of BAX and BCL2 genes.

Materials and Methods: Six experimental groups of adult rats were pretreated for 7 days as follows: two piracetam groups (30 and 100 mg/kg/day), two octreotide groups (50 and 100 mg/kg/day), positive control group (injected with diazepam, 2 mg/kg, single dose before pentylenetetrazole injection) and negative control group (injected with normal saline). Seizures were induced with a single injection of pentylenetetrazole (PTZ, 60 mg/kg) after pretreatments. Signs of resulted seizures were classified according to the Racine scale. The BAX and BCL2 transcripts were measured in the hippocampus by quantitative real-time PCR.

Results: A delayed onset time of the seizures (stage 1 of Racine scale) and shorter time of the tonic-clonic seizures (stage 5 of Racine's scale) were observed in the piracetam and octreotide groups as compared to negative control group ($P<0.05$). The BAX transcript was higher in the positive control and octreotide (100 mg/kg) groups than other groups ($P<0.05$). The BCL2 transcript was higher in the positive control, piracetam (100 mg/kg) and octreotide (100 mg/kg) groups than the negative control group ($P<0.05$). The BAX/BCL2 ratio was lower in the positive control and piracetam (30 and 100 mg/kg) groups than other groups ($P<0.05$).

Conclusion: BAX/BCl2 ratio as an indicator of progressive apoptosis was attenuated by the piracetam in the hippocampus of epileptic rats while useful effects of the octreotide are independent of BAX/BCl2.

Keywords: Apoptosis, Gene expression, Pentylenetetrazole, Seizure

1. Introduction

Recurrent epileptic seizures characterize epilepsy, which is a category of neurological illnesses (1). Some cases of epilepsy are caused by brain damage, stroke, brain tumors, brain infections, or birth defects (2, 3). The possibility of repeated seizures is a long-term feature of epilepsy (4). The most common type (60%) is convulsive seizures. One-third of general seizures affects the both hemispheres of the brain, and two-thirds focal seizures attack one of the cerebral hemispheres (5). The remaining 40% of seizures are non-shocking seizures that cause a loss of consciousness and last about 10 seconds (6). Epilepsy can be caused by both inherited and acquired factors. Known acquired causes include the severe trauma to the brain, stroke, tumors, and other brain problems. Genetic epilepsy is more common in young people while brain tumors and strokes are more common in the elderly (7). In the majority of cases, genetics is assumed to be implicated either directly or indirectly (8). Some epilepsies are caused by hereditary gene abnormalities (1-2), while the majority of epilepsies are caused by the interplay of many genes and environmental variables (8, 9). Epilepsy is associated with the production of oxidative stress and failure in the mitochondrial function. These

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conditions play a critical role in the activation of caspases (e.g., caspase-9 and caspase-3) and induction of apoptosis in the experimental models of epilepsy (10).

Piracetam was first applied in Europe in the early 1970s and approved for the treatment of dizziness and age-related disorders. The potentiating effect of piracetam has been confirmed in the stroke/cerebral ischemia models of rat (11). Unlike most GABA mimics such as barbiturates, carbamazepines, and gabazine, which can cause amnesia in Alzheimer's patients, piracetam and other related medications are quite safe (12). The piracetam has important effects in the neurotransmission and influences a variety of neurotransmitters such as cholinergic transmitters. Clinical studies have shown that the piracetam has neuroprotective benefits. It also reduces lipophoshin, a marker of neuronal membrane damage in rats (13). In humans with acute cerebral ischemia, the piracetam improves cerebral blood flow (14), protects the cell, and prevents apoptosis (15). This medicine has antioxidant and antihypoxic properties and is able to inhibit lipid peroxidation (9).

Octreotide is a long-acting octapeptide under the brand name Sandostatin, which pharmacologically mimics the natural somatostatin. It was initially synthesized in 1979 by the chemist Wilfred Bauer and approved for use in the United States in 1988 (16). The octreotide plays an important role in regulating the inflammatory and apoptotic mechanisms as well as neuroprotective processes against ischemic stroke (17).

The octreotide, a synthetic somatostatin analogue, has many impacts on the somatostatin receptors, which are mostly found on neurons, inflammatory and vascular endothelial cells. This drug is routinely used to treat growth hormone-producing tumors (acromegaly and giant) (18).

The octreotide has antiproliferative and antioxidant properties via suppressing the inflammatory mediators (e.g., proinflammatory cytokines), scavenging the free radicals and inhibiting transforming growth factor. It has been shown that the octreotide reduces the oxidative and inflammatory damage in a variety of diseases (19).

It also increases myeloperoxidase (MPO) activity. MPO is a heme enzyme of mammalian phagocytes and usually plays an important role in host defense and inflammatory tissue damage (20).

The aim of this study was to investigate involving of the piracetam and octreotide in the improvement of apoptosis in the epileptic seizers through estimating the relative level of mRNA expression of two genes, BAX (BCL2 associated X protein) and BCL2 (B-cell lymphoma 2) and their ratio. These genes are known as apoptosis regulators, and their ratio determine the susceptibility of a cell to apoptosis (21).

2. Materials and Methods

2.1. Animals

A total of thirty adult male Wistar rats (180–220 g) was purchased, divided into six groups and used as experimental animals in this study. The rats were housed in a regular laboratory setting (23°C, 12 hours of light and 12 hours of darkness, 75% relative humidity) and fed a standard diet ad libitum. The studies were carried out between the hours of 8 a.m. and 12 p.m. Each treatment group consisted of five rats.

2.2. Experimental groups and treatments

All drugs were purchased from Sigma Aldrich (St. Louis, MO, USA) and administrated as intraperitoneal (ip) injection. The six experimental groups of adult rats were prepared and pretreated as followed: two piracetam groups (30 and 100 mg/kg/day, ip, for 7 days), two octreotide groups (50 and 100 mg/kg/day, ip, for 7 days), positive control group (injected with diazepam, 2 mg/kg, single dose before pentylenetetrazole injection) and negative control group (injected with normal saline). Seizures was induced by a single injection of pentylenetetrazole (PTZ, 60 mg/kg, ip) after pretreatments. After PTZ injection, the convulsive behavior was recorded by a video camera for 30 min, and the signs of seizures according to the Racine scale were classified. Then, the animals were sacrificed, brain was removed, and the hippocampus was isolated. The hippocampal samples were immediately frozen in liquid nitrogen for 24 h, and then stored in −70°C to be analyzed later.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from homogenized hippocampal samples (50 mg) using RNX-Plus (Sinaclon Bioscience, Karaj, Iran). According to Pirany et al. (2020), 500 µl RNX-Plus and 200 µl chloroform were added to samples (22). The upper phase of mixture was separated after centrifugation at 16,000 × g, 4°C for 15 min and then added to an equal volume of isopropanol (100%) in a new tube. The mixture was again centrifuged at 16,000 × g, 4°C for 15 min. The supernatant was discarded, the pellet was washed with 500 µl ethanol (75%), and centrifuged for 8 min at 5000 × g. At the end, the RNA pellet was resuspended in 50 µl distilled water. All extracted RNA samples were treated by DNase (Sinaclon Bioscience) according to manufacture method. The DNase treatment of samples removes contaminating genomic DNA. To evaluate the purity of samples, their absorbance ratio (A260/280) was estimated that should be between 1.8 and 2.2 in spectrophotometry (23). The cDNA was synthesized with a reverse transcriptase reagent kit (Takara Bio Inc., Japan). The reverse transcription was done at 37°C for 15min and then the reverse transcriptase was inactivated with

heat treatment (85°C for 15s). The produced cDNA was kept at -20°C for real-time PCR (24).

2.4. Real-time quantitative PCR

To measure the relative expression of the BAX and BCL2 genes in the hippocampal samples, PCR reactions were done using a Syber green premix kit (Takara Bio Inc., Japan) and a real-time PCR thermocycler (Rotor Gene Q 6000, Qiagen, USA) (24). The online software of Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the specific primer sequences of the target and control genes. Details of the primers are given in Table 1.

Table 1. Primers used in the quantitative real-time PCR analysis

Target	Primers	PCR product	Accession no.
GAPDH	5′-CCCACTAAAGGGCATCCTGGG- 3′ 3′ -GAGGCCATGTAGGCCATGAGG-5′	195	NM_017008.4
BAX	5′-CTTTCCTACTTCGGGACCCCC- 3′ 3′- CCGTTCCCCATTCATCCCAGG-5′	169	NM_017059.2
BCL2	5′-AGCCGGGAGAACAGGGTATG- 3′ 3′ -CAGTATCCCACTCGTAGCCC-5′	90	NM_016993.2

GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; BAX, BCL2 associated X protein; BCL2, B-cell lymphoma 2.

The internal control gene (i.e., GAPDH) was used to assess relative quantification, to control the variability between samples, and to normalize the input load of cDNA. The PCR solution was consisted of 0.5 µl cDNA (40 ng), 5 µl Syber green premix and 0.3 µM of each specific primer, to give a total volume of 10 µl. The thermal program of PCR was 95°C for 2 min (as initial denaturation) and 40 thermal cycles (95°C for 15s, 61°C to 63°C for 30s, 72°C for 20s). The melt curve analysis of PCR findings was noticed to check if PCR products had a specific attribute for each gene (Figure 1).

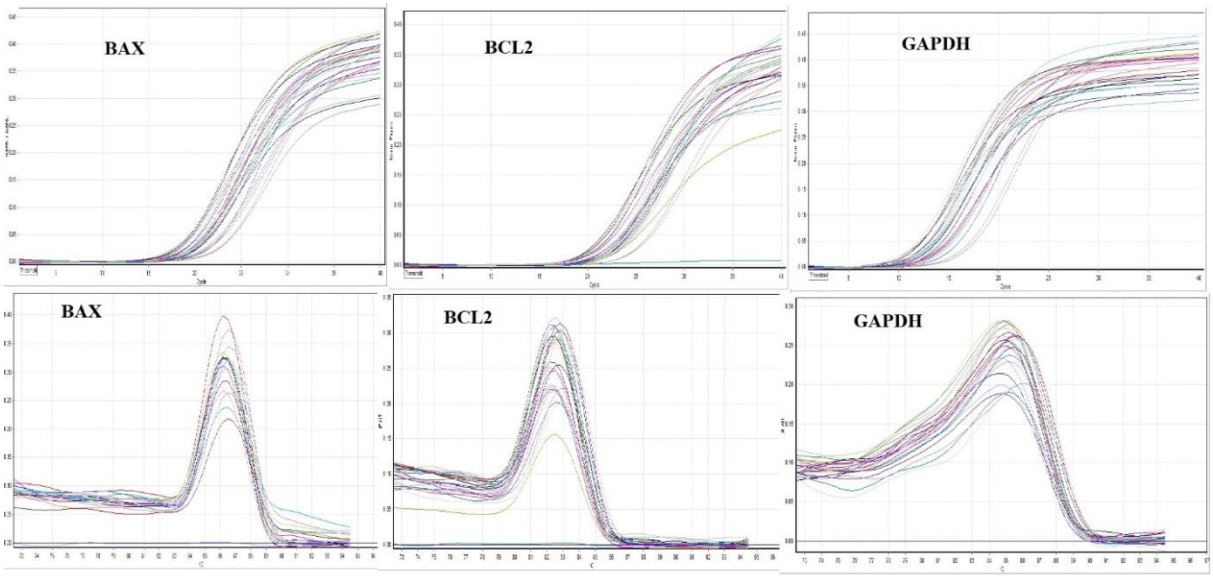

Fig. 1. Amplification (up row) and melting (down row) curves of the two target genes and one reference gene (GAPDH) amplicons after the real time PCR reactions, all melting curves are showing one peak. X-axis (horizontal): temperature (C); Y-axis (vertical): negative derivative of fluorescence over temperature (dF/dT).

3. Results

3.1. Effects of drugs on the stages of seizures

The recorded findings indicated a delayed onset time of the seizures (stage 1 of Racine scale) and shorter time of the tonic-clonic seizures (stage 5 of Racine's scale) in the piracetam and octreotide groups as compared to negative control group (P<0.05).

3.2. Expression of BAX and BCL2 genes and their ratio

Table 2 indicates relative expression of two BAX and BCL2 genes and their ratio in the PTZ-induced epileptic groups. The relative expression of BAX gene was higher in the positive control and octreotide (100 mg/kg) groups than other groups (P<0.05). The relative expression of this gene did not change in the piracetam (30 and 100 mg/kg) and octreotide (50 mg/kg) groups as compared to the negative control group (P>0.05).

The relative expression of BCL2 gene was higher in the positive control, piracetam (100 mg/kg) and octreotide (100 mg/kg) groups than the negative control group (P<0.05). The relative expression of this gene was also higher in the octreotide (100 mg/kg) group while was lower in the piracetam (100 mg/kg) group than the positive control group (P<0.05).

The BAX/BCL2 ratio was lower in the positive control and piracetam (30 and 100 mg/kg) groups than other groups (P<0.05). This ratio did not change in the octreotide (50 and 100 mg/kg) groups as compared to the negative control group (P>0.05).

Table 2. Relative expression of genes (gene/GAPDH) and their ratio in the PTZ-induced epileptic groups

Groups	BAX	BCL2	BAX/BCL2
Negative control (normal saline)	0.017 ± 0.006^{a}	0.002 ± 0.000^{a}	8.142 ± 0.605^{a}
Positive control (diazepam, 2 mg/kg)	0.031 ± 0.004^{b}	0.049 ± 0.024^{b}	5.501 ± 0.764^{b}
Piracetam (30 mg/kg)	0.019 ± 0.002^{a}	0.003 ± 0.000^{a}	6.112 ± 0.707^{b}
Piracetam (100 mg/kg)	0.022 ± 0.003^{a}	0.006 ± 0.001^{c}	6.081 ± 0.900^{b}
Octreotide (50 mg/kg)	0.017 ± 0.001^{a}	0.002 ± 0.000^{a}	8.433 ± 0.893^{a}
Octreotide (100 mg/kg)	0.241 ± 0.098^{c}	0.061 ± 0.259^{d}	8.030 ± 1.076^{a}
P value	0.001	0.000	0.038

a,b,c,d Significant difference between groups for each gene within columns (Kruskal-Wallis, P < 0.05). A P-value less than 0.05 is considered to be statistically significant (Mann–Whitney). PTZ, pentylenetetrazole.

4. Discussion

It has been determined that the apoptotic degeneration occurs in the hippocampal neurons during early and prolonged epileptic seizures. The BAX has been reported to be increased in the regions where apoptotic neuronal death occurs while the intensity of BCL-2 decreased. The down-regulation of BCL2 that develops cell survival, and the up-regulation of BAX, that progresses the apoptosis, may have functional importance in the epileptic seizures (25). In apoptosis, the BAX protein stimulates the cascade of reactions by releasing cytochrome C from the mitochondria leading to whole activation of caspases and finally causes the cell death. BCL2 is supposed to prevent BAX from releasing cytochrome C, hence confining downstream activation of apoptotic cascade (26). In fact, the balance between BAX and BCL2 determines the progress of apoptosis. Concomitant increasing or decreasing of these genes don't change the severity of apoptosis. On the other word, The ratio of BAX/BCL2 could be more accurate indicator indicating the cell apoptotic degeneration than each of these genes individually (21). As our data showed, in many groups the expression of both BAX and BCL2 genes was up- or down-regulated that may not influence the progression of apoptosis, while in the piracetam-pretreated groups, the BAX/BCL2 ratio was considerably decreased that could be evidence for the lower apoptosis of hippocampal neurons during epileptic seizures.

The piracetam is a neuroprotective drug, but little known about its effects in the apoptosis. It was revealed that the piracetam has neuroprotective benefits through preventing the epigenetic changes in the astrocytes (25). Previous studies have also reported the antioxidant effect of the piracetam during the neuronal degradation (27, 28). In a Parkinson model, chronic treatment with the piracetam exhibited the neuroprotective effect via its anti-inflammatory properties (29). However, our data are in agreement with the previous studies for the neuroprotective effect of piracetam, and it may be useful in the treatment of epileptic seizures.

The previous findings recommended that octreotide administration delays epileptogensis via the reduction of cortical dopamine as well as the modulation of inflammatory and apoptotic reactions. The octreotide

improved PTZ-induced epilepsy via modulating myelope roxidase, tumor necrosis factor-α, normalizing interleukin-10, decreasing inducible nitric oxide synthase activity, nitric oxide, and caspase-3 levels (30). On the other hands, there are controversy studies suggesting that the octreotide could induce apoptosis in different cells. It has been suggested that in the cultured cells obtained from the human somatotroph tumor, hepatoma, and meningioma cells, octreotide may promote apoptosis by increase of caspase-3 activity while it could not change the mRNA levels of apoptotic components such as p53, p63, p73, BCL-2, BAX, BID, BIK,TNFSF8, and FADD (31-33), or increase apoptotic cells (evaluated by Tunel assay). However, our results indicated that the octreotide administration could not change BAX/BCL2 ratio in the epileptic seizers. Probably, the effects of this drug on the apoptosis are independent of BAX/BCL2.

Conclusion

In this study, the pentylenetetrazole-induced epileptic rats were pretreated by two neuroprotective drugs, piracetam and octreotide. Estimation of BAX/BCl2 ratio (gene expression) as indicator of progressive apoptosis showed that the piracetam attenuates the apoptosis in the hippocampus of epileptic rats while useful effects of the octreotide are independent of BAX/BCl2.

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Compliance with ethical standards

All procedures performed in this study were in accordance with the ethical standards of the Ethics committee of the Board of Research of Mohaghegh Ardabili University (approved code: IR.UMA.REC.1400.086) and with 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declare that they have no competing interest.

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