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The effects of ATP sensitive potassium channel (K_{ATP}) opener and blockers on Bcl-2, Bax, and Cyt-c gene expression levels in epileptic rats

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Abstract

In this study, it was aimed to investigate the gene expression levels of Bcl-2, Bax, and cytochrome c (Cyt-c), in the cortex region of pinacidil as a K_{ATP} channel opener and glibenclamide as a blocker on penicillin model epilepsy. Male Wistar-Albino rats were used. A total of 4 main groups were formed: Control, Epilepsy, Epilepsy-opener, and Epilepsy-blocker groups, then three-time points were formed subgroups (1st day, 4th, and 8th). 48 rats were used in total. The epileptic focus was created by intracortical administration of penicillin at a dose of 500 IU/2 µl. Cortex is removed from all animals and cyt c, Bcl-2, and Bax gene expression levels were determined by qPCR. The SPSS 21 program was used for statistics. Bcl-2 and Bax gene expression levels were increased in the cortex regions of rats with epilepsy ($p<0.05$). Bcl-2, Bax gene expression levels, which increased due to epilepsy with the effect of K_{ATP} channels opened with pinacidil, returned to normal levels in the epilepsy opener group ($p<0.05$). Bcl-2 gene expression level, which was increased as a result of epilepsy due to the effect of K_{ATP} channels closed with glibenclamide, was higher than in the control and epilepsy-opener groups ($p<0.05$). Bcl-2 and Bax gene expression levels are increased in the cortex region due to epilepsy indicates that the apoptotic pathway could be activated. This study also It has been shown that the apoptotic pathway activated by epilepsy can be inactivated by pinacidil.

Keywords: epilepsy, Bcl-2, Bax, Cytochrome c, KATP channels

1. Introduction

Epilepsy is a common neurological disorder characterized by the temporary occurrence of abnormal, excessive neural activity in the brain (1). The role of neuronal death mechanisms in epilepsy has been debated for years. Neuron death from causes other than seizures generally does not involve a specific molecular mechanism and these cells cannot be rescued (2). In contrast, regulated cell death involves a cellular mechanism, and this mode of death can be manipulated by pharmacological and genetic means (3). Apoptosis is usually induced in two main ways. In the extrinsic pathway, apoptotic cell death is induced by death receptors, while in the intrinsic pathway, death signals directly or indirectly regulate mitochondria, leading to the release of cytochrome and the formation of the apoptosome complex (4).

Seizure-induced neuronal damage may involve both excitotoxic and apoptotic mechanisms. Bax/Bcl-2 expression rate increases in the hippocampus due to seizures, resulting in apoptotic cell death (5). Bcl-2 and Bcl-XL protein were increased in humans with seizure brains compared with controls (6).

The Bcl-2 gene family consists of pro-and antiapoptotic genes. This family performs its functions by forming dimers. The ratio of death antagonists (Bcl-2, Bcl-xL) to agonists (Bax) has been shown to have a critical role in determining the fate of cells (7,8). The Bcl-2 protein family determines the survival or death of a cell by controlling the release of mitochondrial apoptogenic factors, cytochrome, and apoptosis-inducing factors (AIFs), which activate activation (9,10).

There are two types of K_{ATP} channels in the cell: cytoplasmic and mitochondrial. The ones on the cytoplasmic membrane of K_{ATP} channels open outwards and are unidirectional. MitoK_{ATP} channels open into the mitochondria. During the seizure, the increased calcium in the cell passes to the mitochondria and the apoptotic pathway takes place here. Because calcium increases mitochondrial permeability, increases the release of cytochrome-c, and the pathway is stimulated (11).

Although it is not known for certain epilepsy, if the general characteristics of K_{ATP} channels are taken into account; cytoplasmic channels are opened and potassium is taken out of the cell, the cell becomes hyperpolarized (12). It is among our hypotheses that the apoptotic pathway may be inhibited by simultaneously opening the all K_{ATP} channels and allowing potassium to enter the mitochondria and that it may have a role

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in this pathway (13).

Pharmacological studies have proven that K_{ATP} channels play an important role in controlling the seizure threshold (14). It has been determined that K_{ATP} channel agonists (diazoxide, cromakalim, pinacidil, levcromakalim, nicorandil, minoxidil, etc.) reduce seizures in the experimental model of epilepsy-induced by pentylenetetrazole (PTZ) and 4-aminopyridine (4a-AP: Aminopyridine) (15). K_{ATP} ion channel antagonists (glibenclamide etc.)(16) improve epileptic seizures. In other words, these agents alter neuronal excitability by modulating K_{ATP} ion channels (16).

Although the underlying mechanisms of K_{ATP} ion channel agonist and antagonist on epileptic seizures are not fully understood, few studies have investigated the effects of K_{ATP} ion channel agonist and antagonist in the penicillin-induced epilepsy model (17,18). It was determined that the K_{ATP} channel agonist cromakalim and the antagonist 5-HD have different effects in diabetic rats in a PTZ epilepsy model (18). Bepridil and P1075, which are selective K_{ATP} agonists, reduce seizures when administered before and after seizures. HMR1098 and 5HD, which are selective K_{ATP} antagonists, have been shown to increase seizures when administered before and after seizures (19).

Penicillin parenteral treatment has resulted in generalized seizures in cats and rats (20,21). It works by inhibiting the GABAergic inhibitory system (22). One of the other convulsive drugs, pilocarpine, has a PTZ-like action.

The penicillin model of epilepsy was used for this work because we previously explored the electrophysiological effects of KATP channel openers and blockers in prior studies. On the other hand, it is a readily applicable acute model. The focus of this research was to look at how the impacts discovered in prior studies alter gene expression. We wanted to look at this impact after a single dosage of penicillin was given for 8 days.

The most affected areas of the brain in epileptic seizures are the hippocampus and the cortex. This study, it was aimed to clarify the effect of non-selective K_{ATP} channel openers and closers on Bcl-2/Bax, cytochrome c gene expression, which is effective in the apoptotic process in the penicillin model epilepsy.

2. Materials and Methods

2.1. Experimental animals

The experimental animals used were obtained from BAIBU Experimental Animals Application and Research Center. All experimental animals have been treated based on the guiding principles approved by the animal ethical committee of Bolu Abant Izzet Baysal University as well as all the treatments comply with recommendations provided on the Declaration of Helsinki (Registration number:2018/36/A2).

The animals were kept for the 12 hours in a light/dark environment with a relative humidity of 60-70% and were fed ad libitum. Wistar albino male rats aged 2-4 months were used. Four groups were formed Control, Epilepsy, Epilepsy-Opener, Epilepsy-Blocker. Subgroups of each group were formed on 1stday, 4thday, and 8thday. Total of 48 animals were used. An epileptic focus was created as a result of intra-cortical 500IU/2 µl penicillin administration. Then, on the 1st, 4th, 8thdays after the seizure, the animals were decapitated and their cortex regions were removed, and Bcl-2, Bax, cyt c m RNA expressions were determined by qPCR. SPSS 21 program was used for statistics. ANOVA was used to determine the differences between the groups, and the LSD test was used as a post hoc test to determine which group this difference was from, $p<0.05$ values were considered significant (Fig. 1).

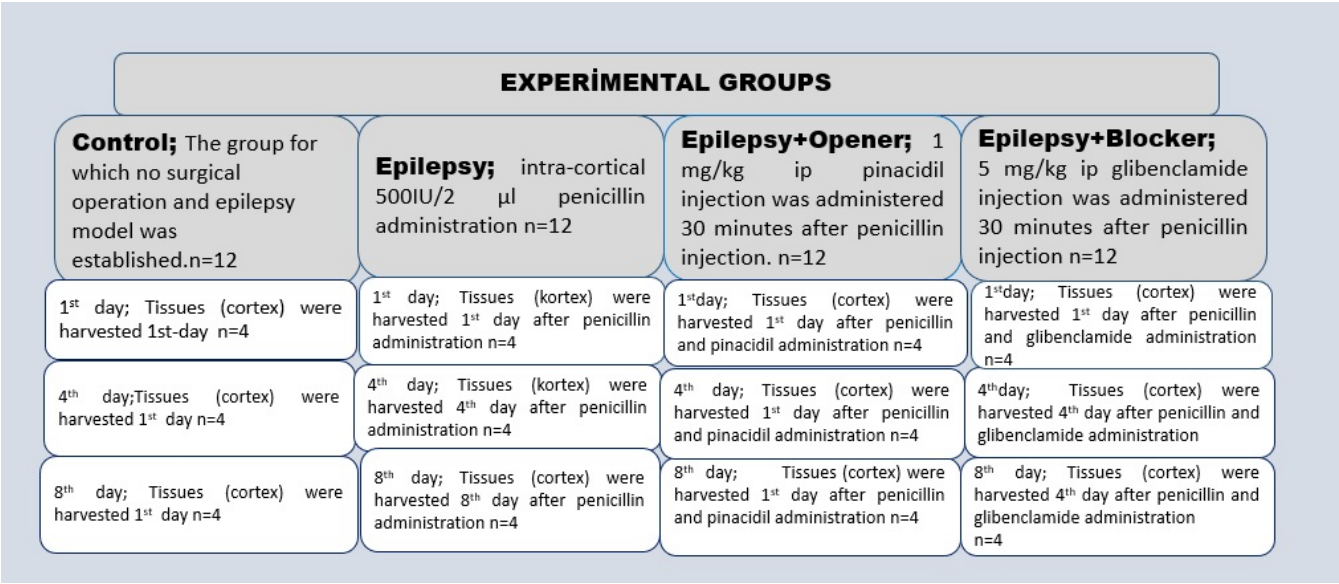

Fig. 1. Experimental groups

2.2. Surgical operation

All rats were anesthetized with 1.2 g/kg urethane intraperitoneally (i.p). and placed in a stereotaxic device. Left cerebral cortex 2mm posterior to bregma and 3 mm lateral to sagittal skull bone is removed, then dura matter is removed. To create epileptic focus 500IU with a Hamilton microinjector to a depth of 1.2 mm, 2µl of penicillin G was injected.

2.3. Drug administration

In this study, K_{ATP} channel opener Pinacidil (1mg/kg), and blocker Glibenclamide (5mg/kg) were given i.p. All drugs were applied 30 min after penicillin administration.

2.4. Q-PCR method

To detect changes in gene expression levels, total RNA was isolated, cDNA synthesis was performed, and qRT-PCR experiments were performed.

RNA isolation: For RNA isolation from tissue samples, 1 ml of Trizole solution was added to a 50 mg tissue sample and homogenized. The tubes were incubated at room temperature for 5 minutes, then 200µl chloroform was added, and manually shaken quickly for 15 seconds. The tubes were kept at room temperature for 3 minutes, centrifuged at 12,000g, and 4 °C for 15 minutes. The transparent colored upper phase was taken into a new tube and 500µl of 100% isopropanol was added. After incubation at room temperature for 10 minutes, the tubes were centrifuged for 10 minutes at 12,000g and 4 °C At this stage, the RNA in the sample formed a white precipitate at the bottom of the tube. The liquid in the tube was removed and the RNA precipitate was washed with 1ml of 75% ethanol and centrifuged at 7500g and 4 °C for 5 minutes. The resulting RNA was dissolved with 20-50µl of DEPC-ddH2O and its concentration was measured.

c DNA Synthesis: For each sample, 1µg of RNA, 2 µl of oligo dT, and DEPC-ddH2O were mixed with a final volume of 8µl and incubated for 5 minutes at 70 °C. After 10µl of 2X reaction buffer and 2µl of reverse transcriptase enzyme was added, the samples were incubated for 1 hour at 42 ˚C and 5 minutes at 80 ˚C. The cDNA samples were stored at -20˚C.

Table 1. Primer list

Primers name	Primers	Tm (°C)
Bcl-2-F	ATGGGGTGAACTGGGGGEGGATTG	66
Bcl-2-R	TTTCATATTTGTTTGGGGCAGGTC	59
Bax-F	GAGAGGATGGCTGGGGAGAC	63
Bax-R	GGTGAGCGAGGCGGTGAGGACT	68
Sitokrom C-F	TGGACAGCCCCGATTTAAGT	57
Sitokrom C-R	TCAATAGGTTTGAGGCGACAC	58
GAPDH-F	ACCACCATGGAGAAGGCTGG	61
GAPDH-R	CTCAGTGATGCCCAGGATGC	61

Quantitative Real-Time PCR (qRT-PCR): Primers that bind with high specificity to the target gene regions to be tested for RT-PCR experiments were designed (Table 1). To investigate the level of mRNA expression, 1µl of cDNA, 1µl of primer mixture (10 µM, forward+reverse), 10µl of 2X SYBR Green, and 8µl of DEPC-ddH2O were added to each qRT-PCR reaction. The following program was used for the reaction:95˚C for 5 min, [95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 30 sec] x40, 72˚C for 5 min.

Analysis of the qRT-PCR results: Normalization with a housekeeping gene such as GAPDH was performed to prevent differences between samples and possible pipetting errors during the detection of gene expression levels. The analysis was performed using the ddCt method by the following equation.

ddCt = Ct (target gene) - Ct (housekeeping gene)

Target gene expression = 2^ (- ddCt)

3. Results

Bax gene expression level in the Epilepsy-8thday group; Control, Epilepsy-1stday, Epilepsy-4thdays, Epilepsy-opener-1stday, Epilepsy-opener-4thday, Epilepsy-opener-8thday, Epilepsy-blocker-1stday, Epilepsy-blocker-4thday, Epilepsy-blocker-8thday gene expression levels were statistically higher ($p<0.05$) (Fig. 2, Fig. 3). There was no statistically significant difference between the groups in cytochrome c, gene expression levels $p>0.05$ (Fig. 2).

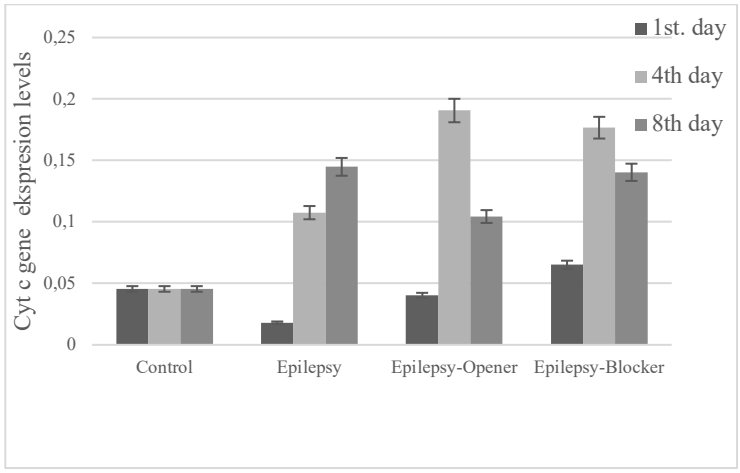

Fig. 2. Cyt C gene expression levels. There was no statistically significant difference between the groups

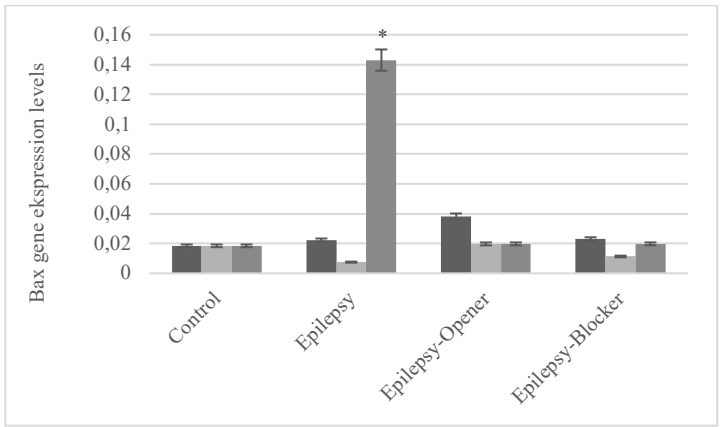

Fig. 3. Bax gene expression levels. * means significantly higher than the other groups $p<0.05$

Bcl-2 gene expression levels were statistically higher in the Epilepsy 8thDay group than in the Control, Epilepsy 4thDay group, Epilepsy-blocker 4thDay groups ($p<0.05$). Bcl-2 gene expression levels were statistically higher in the Epilepsy-Blocker 8thDay group than in the Control, Epilepsy 4thDay groups, Epilepsy-Blocker 4thDay groups ($p<0.05$) (Fig. 4).

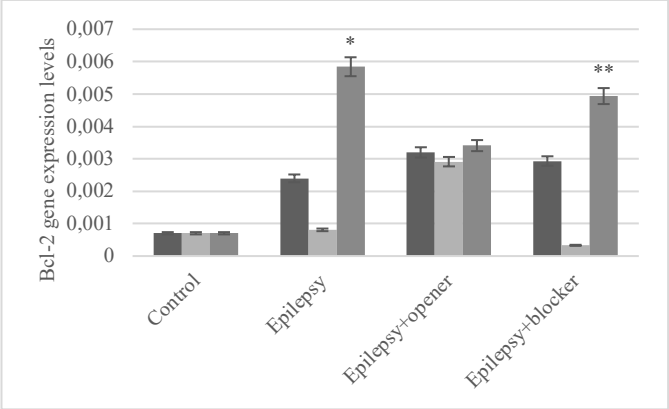

Fig. 4. Bcl-2 gene expression levels. *epilepsy-8thday group was significantly higher than the control, epilepsy-4thday, epilepsy-blocker-4thday groups $p<0.05$. ** Epilepsy-blocker-8thday group was significantly higher than the control, epilepsy-4thday, and Epilepsy-blocker 4thday groups

4. Discussion

Recurrent brief seizures lead to progressive loss of hippocampal neurons (23). It is unclear whether the role of the Bcl/Bax gene family and neuronal death is direct in the epileptic phase of seizure formation, but it occurs secondary to the effects of severe and prolonged seizures in epileptogenesis. It has been shown that the Bcl-2 gene family and apoptosis have a role in epileptogenesis (23).

Overactivation of glutamate receptors, accompanied by Ca^{+2} overload, is thought to be responsible for the death of neurons in various conditions such as stroke and epilepsy. In addition to such an excitotoxic mechanism, neurons die if deprived of important growth factors and trophic effects, which are conditions sensitive to certain oncogene products such as the Bax protein (5).

Opening and blocking of K_{ATP} channels are known to affect contractility: cell adhesion, gap, and tight junction regulation, protection against metabolic ischemia and hypoxia, cell health, and cellular adaptation to stress (24). In this study, a neuronal loss that may occur in the cortex region of the non-selective K_{ATP} opener pinacidil and its blocker glibenclamide, which is used in penicillin-induced epilepsy, and Bcl-2, Bax and cyt c gene expression levels on the 1st, 4th and 8th days after the seizure were determined. As a result, Bcl-2 and Bax gene expression levels increased in the Epilepsy 8thday group. It was shown that the expression level of Bcl-2 and Bax gene expression levels increased 8thday after seizure induction.

Mitochondria play an important role in the apoptosis of mammalian cells by releasing various apoptogenic proteins, including cytochrome c (cyt c), into the cytoplasm (9,10). The Bcl-2 protein family regulates these mitochondrial changes during apoptosis (9,10).

In this study, cytochrome c levels were determined, but no significant difference was found between the groups. According to this result, it may be that a single seizure does not cause a neuronal loss in the cortex and that the increase in gene expression of Bcl-2/Bax genes may indicate that neuron loss is controlled.

Diazoxide, a selective mitochondrial K_{ATP} channel opener, affected the increase of Bcl-2 levels in the cortex of rats exposed to cerebral ischemia-reperfusion injury. Non-selective K_{ATP} channel blockers have been shown to have the opposite effect (25). Bepridil and P1075, which are selective K_{ATP} agonists, reduce seizures when administered before and after seizures. The selective K_{ATP} antagonists HMR1098 and 5HD have been shown to increase seizures when administered before and after seizures (19). Drugs that have an impact on specific mitochondrial K_{ATP} channels or cytoplasmic channels have been investigated (19).

In this study, pinacidil showed neuronal loss caused by epilepsy by opening both channels. Glibenclamide, on the other hand, closed both channels in the opposite way to pinacidil. Bcl-2 and Bax, which were increased with epilepsy and decreased with pinacidil, were found to be the same with glibenclamide in the epilepsy group. It has been observed that the closure of K_{ATP} channels does not affect neuronal loss. Pinacidil has been shown to have a protective effect against neuronal loss caused by epilepsy.

Epileptogenesis and status epilepticus are the stages in which epilepsy becomes chronic, treatment resistance develops, spontaneous and tonic-clonic seizures occur, and the seizure threshold falls. Neuronal loss increased as a result of the high electrical activity throughout this phase. Many processes have been attributed to this process, one of which is ion channel alterations. We hypothesized that the main source of the decrease in seizure threshold and the occurrence of very severe seizures is at the cellular level, that ion channels may have an effect on the apoptotic process at the cellular level, and that we wanted to investigate the levels of Bcl-2, Bax, and cyt c gene expression. We selected K_{ATP} channels as ion channels since we previously demonstrated their impacts in electrophysiology. Many investigations have revealed K_{ATP} channels in the mitochondrial membrane, and it has been suggested that they protect the cell from apoptosis. With this study, we demonstrated that a single seizure can activate an apoptotic pathway at the cellular level, that it may play a role in epileptogenesis, that opening of K_{ATP} channels may play a role in this process, and that it should be taken seriously in the first and only seizure in the treatment process. We demonstrated that therapies that promote antiapoptotic gene expression can be recommended in this process. In prior research, we have established electrophysiologically that K_{ATP} channel openers lessen seizures. In this study, we demonstrated

the antiapoptotic effects of K_{ATP} channel openers. This study implies that the decrease in seizures caused by the impact of K_{ATP} channels could be due to a shift in antiapoptotic gene expression. The mechanism of the seizure decrease could be attributed to increased expression of antiapoptotic genes and decreased expression of the Cytochrome c gene. Many researchers have attempted to prevent and treat seizures in the chronic period. In contrast to these researchers, we wanted to look into the effects of a single seizure on gene expression during the acute phase of epilepsy. In the future, we intend to assess gene expression levels in chronic models. This study is strong in the mentioned aspects.

In these studies, the effects of KATP channel opener and blockers on penicillin model epilepsy, which is an acute model, were investigated, and bcl2, bax and cyt c gene expression levels in the cortex, the region most affected by epilepsy, were investigated.

We were unable to include procedures such as immunohistochemistry or Elisa to validate gene expression data due to a limited budget when organizing the study. We were only able to determine their gene expression due to funding constraints. As a result, we isolated RNA from all cortical tissues from rats and used Q PCR to evaluate gene expression.

In conclusion, it has been shown in this study that K_{ATP} channel openers, which have been shown to reduce seizures on epilepsy in previous studies, can reduce neuronal loss in the epilepsy-related cortex region. At the same time, it was shown in this study that opening K_{ATP} channels during seizures may be effective in reducing neuronal loss.

Conflict of interest

The authors declared no conflict of interest.

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Acknowledgments

None to declare.

Ethical Committee Approval

All experimental animals have been treated based on the guiding principles approved by the animal ethical committee of Bolu Abant Izzet Baysal University as well as all the treatments comply with recommendations provided on the Declaration of Helsinki (Registration number:2018/36/A2).

Authors' contributions

Concept: Ü.K, H.S., Design: Ü.K, H.S., Data Collection or Processing: Ü.K, H.S., Analysis or Interpretation: Ü.K, H.S., Literature Search: Ü.K, H.S., Writing: Ü.K, H.S.

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