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Title: Effect of Co-Administration of Bumetanide and Phenobarbital on Seizure Attacks and NKCC1/KCC2 Expression in Pilocarpine Model of Temporal Lobe Epilepsy

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Abstract:

Purpose: The resistance of temporal lobe epilepsy to classic drugs is thought to be due to a disruption in the excitation/inhibition of this pathway. Two chloride transporters, NKCC1 and KCC2, are expressed differently for the excitatory state of GABA. The present study explored the effect of bumetanide as a selective NKCC1 inhibitor either alone or in combination with the phenobarbital in the pilocarpine model of epilepsy.

Method: An animal model of status epilepticus (SE) was induced with pilocarpine in Wistar male rats followed by phenobarbital and/or bumetanide or saline administration for 45 days after the induction of SE by intraperitoneal (IP) injection. The rats were monitored, their behavior was recorded by researchers, and after 24 hours they were sacrificed to study the expression of NKCC1 and KCC2 using real time PCR.

Results: The data showed that the effects of a combination of bumetanide and phenobarbital on frequency rate and duration time of seizure attack were more than the effect of phenobarbital alone. In addition, in bumetanide and combined treatment group, NKCC1 expression decreased significantly, compared to untreated epileptic animals. A delayed decrement in NKCC1/KCC2 expression ratio after bumetanide application was also observed.

Conclusion: The combination of bumetanide and phenobarbital increased the inhibition of SE and maximized the GABA signaling pathway power, and can be considered as an effective treatment strategy in patients with epilepsy.

Key words: Bumetanide, KCC2, NKCC1, Phenobarbital, Temporal lobe epilepsy

Introduction

Temporal lobe epilepsy (TLE) is the common type of epilepsy in adults. TLE is characterized by seizures that emerge in the limbic system. TLE is often accompanied by initial precipitant damage including febrile seizure, perinatal hypoxia, head trauma and infection. The initial damage occasionally qualifies as status epilepticus (SE), a life-threatening neurologic

disorder accompanied by loss of consciousness. A cryptic and seizure-free period follows the accelerated damage and subsequently leads to recurrent seizures [1].

The mechanisms involved in TLE are somehow unknown, however some of the reported ones are disruption of blood–brain barrier, neurodegeneration, inflammation, changes in expression of diverse receptors and ion channels, and development of hyperexcitability of neurons. A wide variety of studies have investigated TLE, the mechanisms and risk factors but little is known [2, 3]. It has been suggested that the GABAergic signaling pathway plays a significant role in causing TLE [4].

Gamma-amino butyric acid (GABA) is an excitatory neurotransmitter and has an excitatory effect in the early stages of development, which is characterized by increased expression of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC1), and decreased expression of $\text{K}^+\text{-Cl}^-$ cotransporter (KCC2) in the brain. NKCC1 increases the intracellular Cl^- concentration, but KCC2 shows the opposite effect. Therefore, the neurons in which the expression of NKCC1 is elevated (due to lack of KCC2), the opening of Cl^- channel by GABA leads to the exit of Cl^- and depolarization of neuron [5].

Different proepileptogenic brain insults downregulate KCC2 and upregulate NKCC1, increasing intracellular Cl^- and hyperpolarization and causing development of neuronal excitation in some regions of brain [6-10].

The TLE pilocarpine model demonstrated that the change in GABA equilibrium potential (E_{GABA}) is limited to the epileptogenesis period and likely harbor an important mechanism associated with the appearance of epilepsy [11]. Mazarati et al. found that NKCC1 pharmacological blockade in the neonatal brain may show an antiepileptic effect [5].

In the present study rats were treated with bumetanide as a NKCC1 inhibitor after pilocarpine-induced epilepsy. Bumetanide is a very strong diuretic drug which selectively blocks NKCC1 in submicromolar concentrations, reducing intracellular chloride concentration [7, 12]. Bumetanide has neuro-protective effects in rat models with traumatic brain damage [13, 14][13, 14], however, its exact anti-epileptogenic function in TLE models with recurrent seizures (RS) is yet to be identified.

Although in neonatal seizure model by using either bumetanide alone or in combination with phenobarbital, GABA had an anticonvulsant effect, the combination of both drugs was more efficient [15]. Cleary et al. demonstrated that bumetanide increases phenobarbital efficacy in a rat model of hypoxic neonatal seizures [16].

In this study, we evaluate the effect of bumetanide, phenobarbital and combination of both drugs on the pilocarpine model of TLE in adult rats, separately.

Methods

Animals

Adult male Wistar rats weighing 250–270 g were housed in a controlled environment with light/dark cycle at $22\pm 1^{\circ}\text{C}$ for 2 weeks before beginning of experiment. Rats had free access to food and water. All animal experiments were executed according to the Helsinki declaration and the study was confirmed by the Ethics in Research Committee of Iran University of Medical Sciences.

Pilocarpine-induced epilepsy

To induce SE, pilocarpine hydrochloride, a muscarinic cholinergic agonist (Sigma; 360 mg/kg) were injected to animals intraperitoneally; IP. [15]. Animals were pretreated with cholinergic antagonist, scopolamine methyl nitrate (Sigma; 1 mg/kg IP) 30 min before pilocarpine injection to reduce the peripheral cholinergic effects [17]. The behavior of the rats was observed for several hours after injection and was scored using Racine's classification [18]. Only rats that displayed SE (stages 3–5) for 3–4 hours were selected in this study. For finishing seizures diazepam (7 mg/kg, IP) was injected to the rats. . Animals were hand fed after SE until they could eat and drink. After 2 weeks from the first spontaneous recurrent limbic seizures, the occurrence of spontaneous seizures was confirmed during 6–8 hours a day randomly.

Drug administration

At 45 d after induction of SE, rats received IP injections of phenobarbital (15 mg/kg), bumetanide (30 mg/kg) or a vehicle (Co). The phenobarbital was diluted in 0.9% saline and the bumetanide was dissolved in NaOH 0.1 M and 0.9% saline. In addition to the vehicle group, three treatment groups were tested: phenobarbital alone, bumetanide alone, and phenobarbital combined with bumetanide.

Rats were videotaped and their behavior was reviewed and scored by blinded investigator for severity, frequency and duration of tonic-clonic seizures. Origin 7.5 SR6 (Microcal Software; USA) was used for acquisition and data analysis.

Real-time RT-PCR detection of NKCC1 and KCC2 expression

Rats were sacrificed 24 h after drug administration and the hippocampal tissue was dissected by cold PBS. Total RNA was extracted from the tissue using RNXplus (Cinagen, Iran). By measuring of the optical density at 260 and 280 nm using a UV/VIS spectrophotometer (Ultrospec 2000; Pharmacia), the quantity of the isolated RNA were determined. The quality of it was examind by the agarose gel electrophoresis. The cDNA was generated from 1 µg of total RNA by reverse transcription using the CycleScript Reverse Transcription system (Bioneer, Korea). By quantitative RT-PCR using a real-time thermal cycler (Rotor-Gene 6000: Qiagen: Germany), the mRNA expression of NKCC1, KCC2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined The internal control was GAPDH

10 µl was the PCR volume reaction including: of 1 µl of cDNA, 5 µl AccuPower 2X GreenStarq PCR Master Mix (Bioneer, Korea) and 10 pM of each forward and reverse specific primer (Qiagen). The reaction conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Amplification specificity was checked by verifying a single peak on the melting curves. All samples and controls were normalized against reference gene. No template controls and reverse transcriptase control were included in each PCR run. All assays were carried out three times as independent PCR runs for each cDNA sample. The $\Delta\Delta CT$ method [19] was used to quantify the amplification-fold difference between groups; each gene expression was normalized with respect to GAPDH mRNA content.

To validate the use of the $\Delta\Delta CT$ method, 5-fold serial dilutions were performed on a cDNA sample over a 125-fold range. For each dilution sample, PCR were done twice

using target and reference genes primers. The average CT of all tests was calculated and the ΔCT of target (NKCC1 and KCC2) and reference (GAPDH) genes was determined. A plot of the log cDNA dilution versus ΔCT ($\Delta CT_{\text{target}} - \Delta CT_{\text{reference}}$) was made for each target and reference genes (Fig. 1) and the slope of fitted line was determined [19].

Statistical analysis

All statistical analyses on PCR data were performed using SSPS 19.0 (SPSS; USA). One-way analysis of variance (ANOVA) with Dunnett's post hoc was used to examine significant differences between groups. All results were presented as mean values \pm standard deviation.

For monitoring the data, repeated measurement (SPSS 20) and the post hoc Tukey test were used for hour-to-hour and point-to-point analysis of seizure duration and for mean

seizure score of hour-to-hour severity for all groups of rats. Two-way ANOVA and post hoc Tukey were used to examine means for seizure attack frequency and duration in 20 h. All results were presented as mean values \pm standard error of mean. For all tests, $p < 0.05$ was considered to be statistically significant. Origin 7.5 SR6 (Microcal Software, USA) was used for data acquisition and analysis.

Results

Effects of phenobarbital and/or bumetanide on NKCC1 and KCC2 expression

The each and combination of phenobarbital (15 mg/kg) and bumetanide (30 mg/kg) were administered during the latent phase of pilocarpine-induced TLE. The animals were sacrificed 24 h post drug administration and the NKCC1 and KCC2 expression was quantified by RT-PCR.

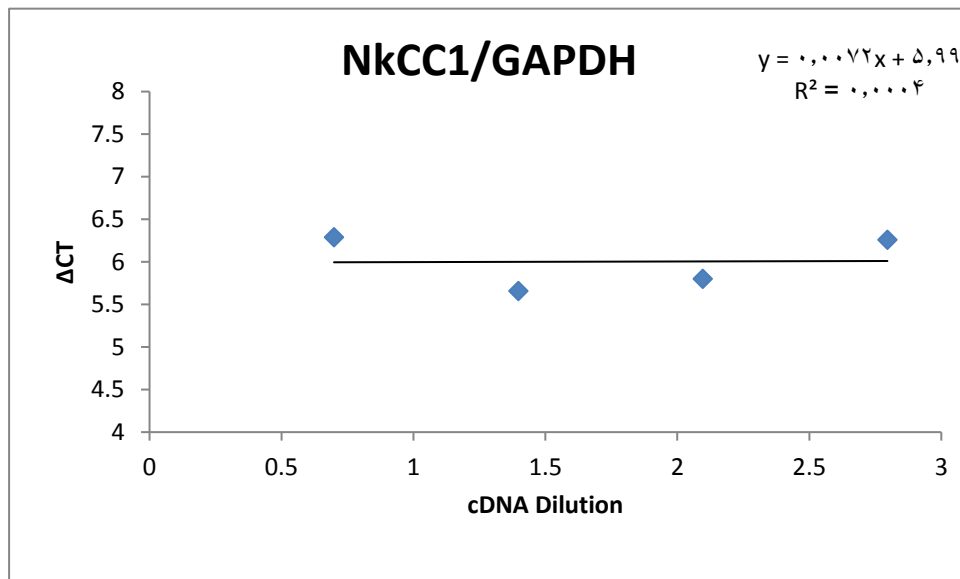
Compared to the unretailed epileptic animals, NKCC1 expression decreased significantly in the bumetanide and combined treatment groups.. KCC2 expression showed no significant alteration after drug administration (Fig 2a). The hippocampal NKCC1/KCC2 ratio, a good marker of GABA polarity, decreased significantly in the bumetanide ($P=0.013$) and combined groups ($p<0.001$) compared to the control group; this ratio was significantly lower for the combined group ($p=0.003$) than that of the phenobarbital group (Fig 2b).

Monitoring of animal for spontaneous recurrent seizures

To compare the efficacy of the drugs, the average severity, frequency and duration of spontaneous recurrent seizures during the chronic phase of the pilocarpine model of TLE were analyzed. All groups were monitored for 20 h following drug administration. The data revealed that the effects of combined bumetanide and phenobarbital significantly reduced the duration (Fig 3), severity (Fig 4), and frequency (Fig 5) of seizure attacks.

The highest decrease in severity was seen 4, 14 and 17 h post drug injection. The frequency and duration of the seizures decreased significantly for all treated groups compared to the control group (Fig 3, 5). It was shown that the combined treatment was significantly more efficacious than the phenobarbital alone. The effects of the combined drugs improved the efficacy of the phenobarbital.

a



b

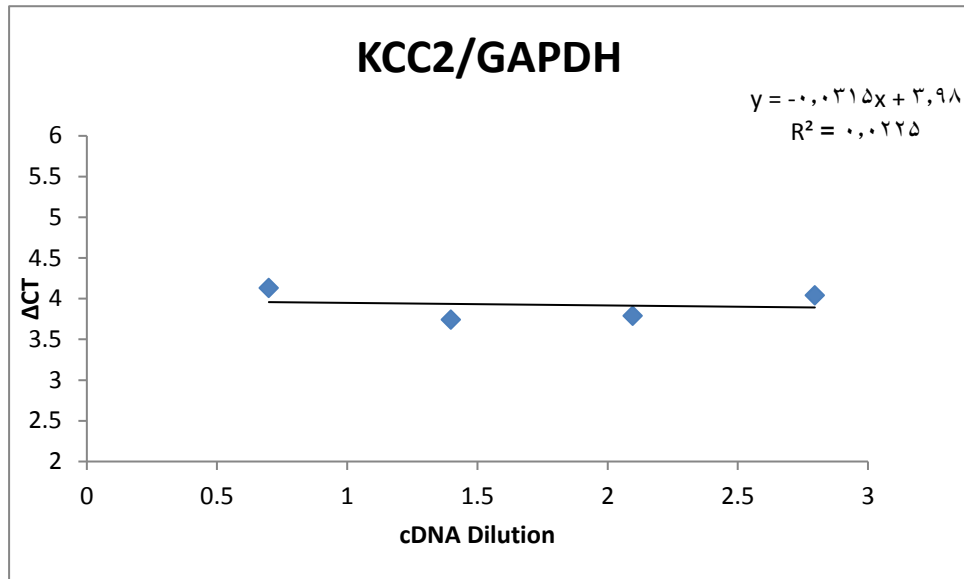


Figure 1(A&B): Validation of $\Delta\Delta CT$ method. cDNA sample was diluted four times. Serial dilutions were amplified by real-time PCR using reference and target gene primers in triplicates. The average CTs and ΔCT ($\Delta CT_{\text{target}} - \Delta CT_{\text{reference}}$) was calculated for each cDNA dilution. The line was fitted using linear regression analysis. **A:** NKCC1 and GAPDH, **B:** KCC2 and GAPDH. The slope of the both lines was less than 0.1; therefore, the $\Delta\Delta CT$ method can be used to analyze the data [19].

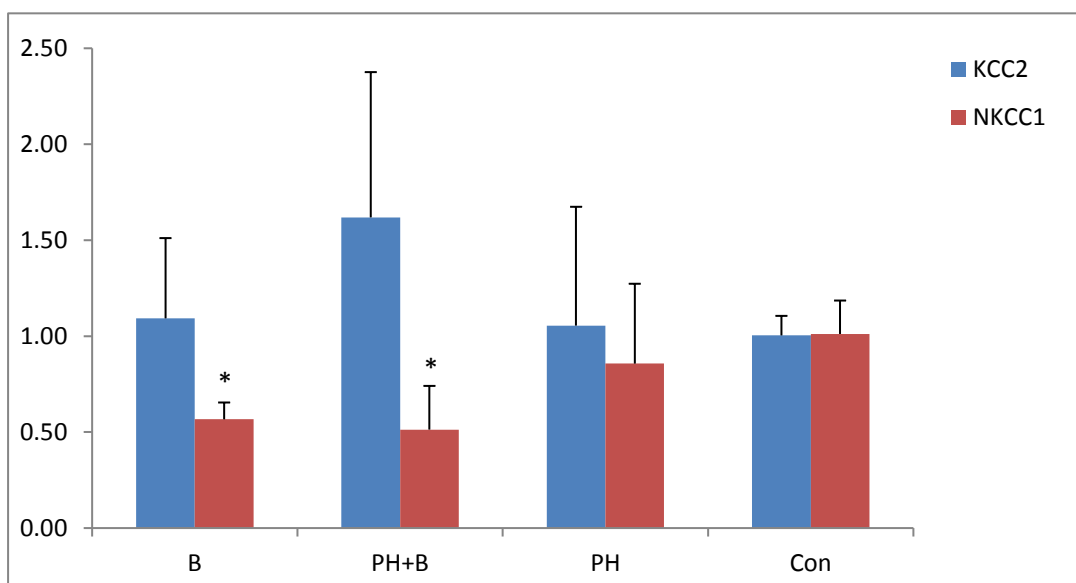


Figure 2a. KCC2 and NKCC1 expression in the hippocampus. NKCC1 and KCC2 expression was quantified by real-time PCR. NKCC1 expression was significantly decreased in bumetanide and combination treatment group compared to untreated epileptic animals. KCC2 expression showed no significant alteration after drug administration. * $p < 0.05$ compared to control group. Results were shown using the mean \pm standard deviation (SD).

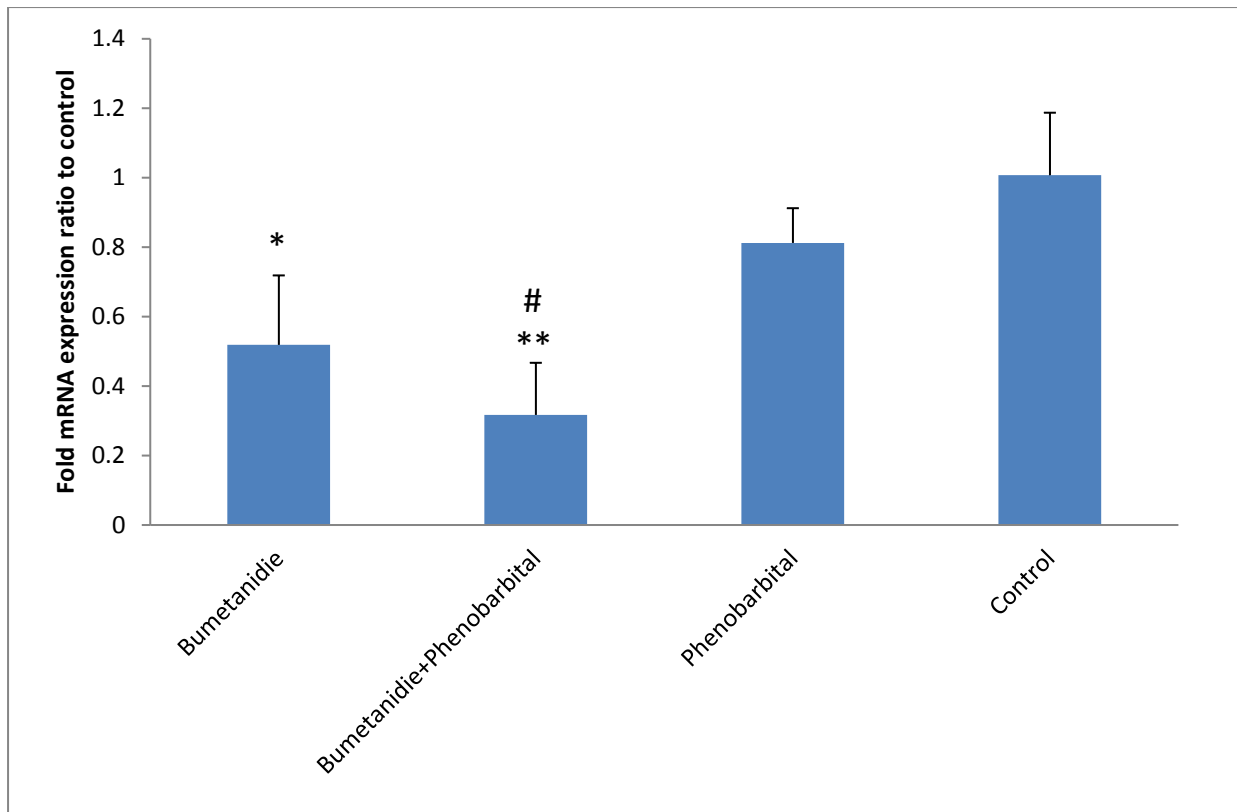


Figure 2b. NKCC1/KCC2 mRNA expression ratio in the hippocampus. NKCC1/KCC2 ratio was significantly decreased in bumetanide (*P=0.013) and combination groups (**p<0.001) compared to control group and this ratio in combination group was significantly lower (#p=0.003) than that of phenobarbital group. Results were shown using the mean \pm standard deviation (SD).

a

b

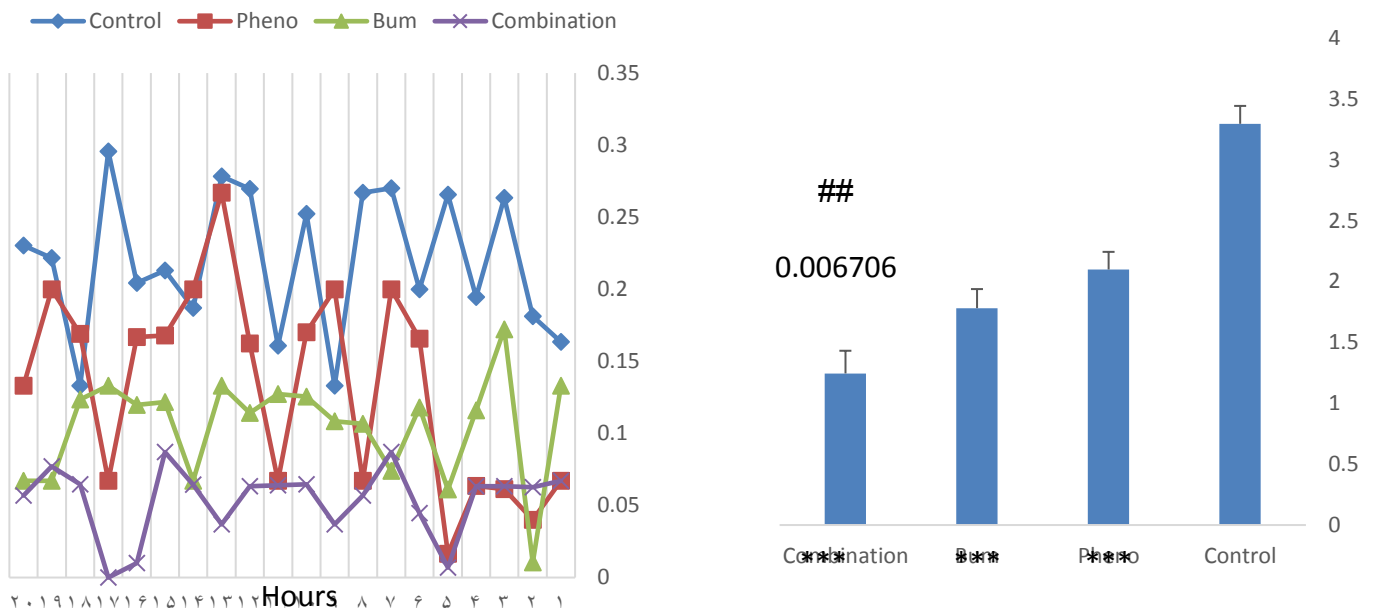


Figure 3 a&b. Mean duration of recurrent seizures in 20 hours after drug injection. All of the groups were monitored for 20 hours following drug administration. Duration of seizure attacks were significantly decreased in all treated groups compared to control group. In addition,, combination therapy was significantly more effective than phenobarbital alone. *** $p < 0.00001$ compared to control group. ## $p < 0.006706$ compared to phenobarbital group. Results were shown using the mean \pm standard deviation (SD).

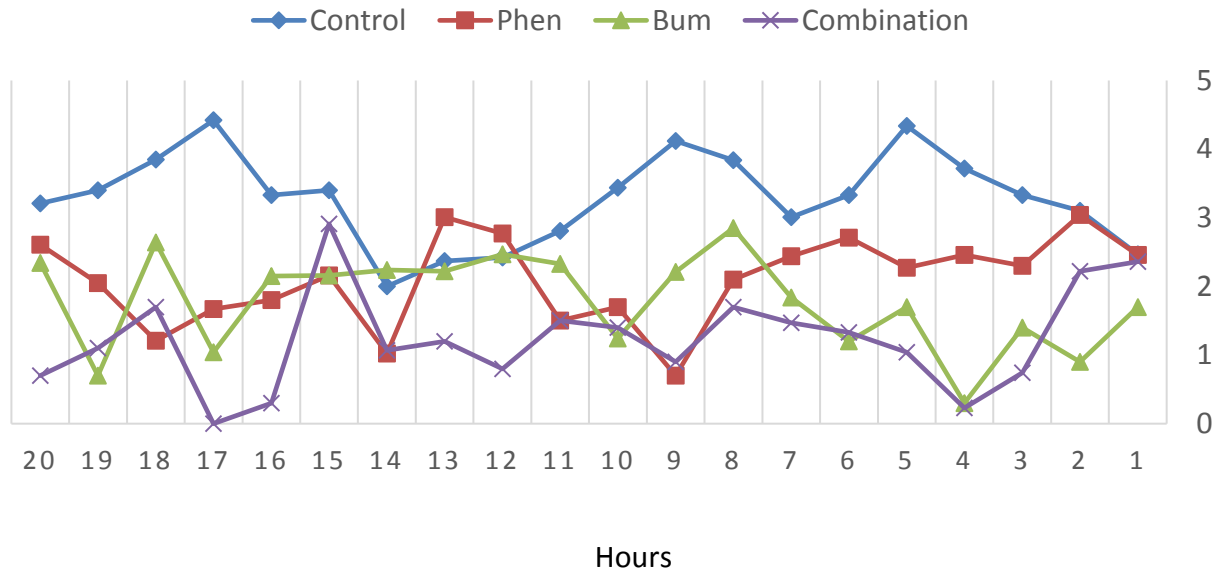


Figure 4. Mean severity of recurrent seizures in 20 hours after drug injection. All the groups were monitored for 20 hours after drug administration. The severity of seizure attacks was significantly decreased in combination group. The most reduction in severity was observed 4, 14 and 17 hours after drug injection.

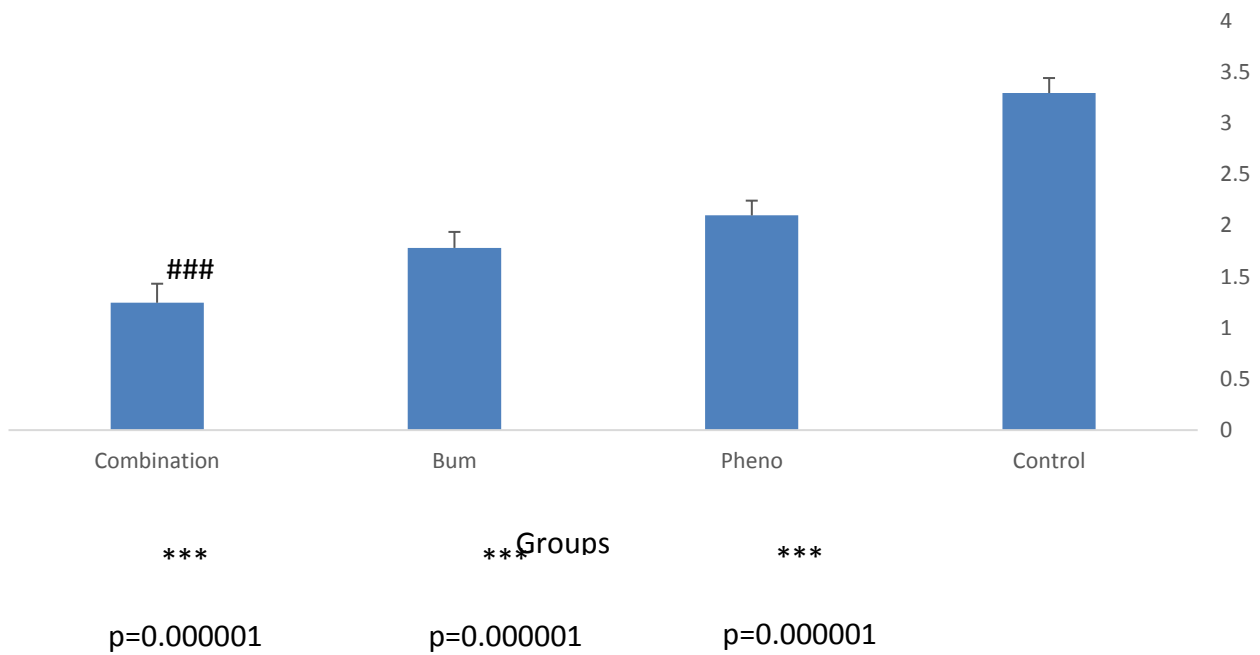


Figure 5. Mean frequency of recurrent seizures in 20 hours after drug injection. All the groups were monitored for 20 hours after drug administration. Frequency of seizure attacks was significantly decreased in all treated groups compared to control group. Also, combination therapy was significantly more effective than phenobarbital alone. *** $p < 0.000001$ compared to control group. ### $p < 0.000804$ compared to phenobarbital group. Results were shown using the mean \pm standard deviation (SD).

Discussion

The present study was performed to investigate whether administration of NKCC1 inhibitor bumetanide alone or with phenobarbital prevents or modifies the development of epilepsy. The results showed that the combination of bumetanide and phenobarbital treatment decreased the score, frequency and duration of seizures, and showed increased efficacy over the phenobarbital alone treatment.

The combination of drugs targeting NKCC1 and anticonvulsants increase GABA receptor-mediated conductance exemplify rational anticonvulsant polypharmacy, significantly benefiting some forms of intractable seizures.

Recent studies have shown the role of excitatory GABAergic signaling in the pathogenesis of TLE that occurs after ischemia in adults [2, 20]. Kahle et al showed that bumetanide could be effective for treatment of seizures in adults [21].

Bumetanide could also be helpful for adult seizures and ischemic encephalopathy by upregulating NKCC1 and decreasing the excitability that follows these injuries [21]. For instance, in the models of cerebral ischemic injury, it has been shown that a prolonged increase in $[Cl^-]_i$ lead to hyper-excitability of GABAergic neurons [22, 23]. This elevation in $[Cl^-]_i$, which would be prevented by bumetanide, is associated with an increased expression of NKCC1 [21].

For example, neurons in adult TLE accumulate Cl [24], probably due to a high ratio of NKCC1 to KCC2 [21].

KCC2 can be down-regulated by a variety of insults, including brain lesions, spinal cord transections, traumatic insults, and seizures. The evidence of KCC2 internalization after seizures in mice was supported by producing tyrosine phosphorylation of KCC2 and internalization of the cotransporter [25-27].

bumetanide is able to counteract the depolarizing action of GABA which recorded in patients with TLE [3, 28]. Cleary et al showed that bumetanide in human neonates decreased risk of seizures [16].

During the first postnatal week, NKCC1 expression in rats is highest in cortical neurons, and then decreases until next 14 days. Then, it drops to the low levels as adults [29, 30]. Conversely,, at birth, the expression of KCC2 is minimum in cortical neurons of rat. During the first postnatal week, the expression of KCC2 is low, and at 14 d post-natal is similar to that of adults [31].

The higher levels of NKCC1 early in development is accompanied by increasing $[Cl^-]_i$ [32, 33] and excitatory GABA [34-36]. Dzhala et al. [37] demonstrated similar pattern in the human cortex. This data supports the hypothesis that GABA is excitatory in immature human cortical neurons and neonates, and maybe susceptible to seizures [21].

It therefore seems logical to combine bumetanide (which decreases $[Cl^-]_i$, and subsequently blocks the excitatory effect of GABA) with phenobarbital (that opens GABA_A receptor Cl^- channels),

The efficiency of bumetanide with phenobarbital was tested for treatment of recurrent epileptic activity in vitro [15]. Although phenobarbital is not able to diminish the recurrent seizures in 70% of cases, phenobarbital in combination with bumetanide abrogates seizures and significantly reduces the frequency, duration, and power of seizures.

The results of this study indicate the high anticonvulsant efficacy of the combined bumetanide and phenobarbital treatment. In conclusion, combined treatment with bumetanide and phenobarbital after SE increases inhibition and maximizes the anticonvulsant power of the GABA system and can be considered useful for treatment strategy of TLE.

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