Demonstration of PTZ-Induced Convulsive-Reducing Effect of Butamirate Citrate

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ABSTRACT

Butamirate has the possible effects on epileptic seizures, which is thought to perform central antitussive effect via medulla oblongata and nucleus tractus solitarius. In our study, these effects have been investigated on electrophysiological and clinical basis. 48 Sprague-Dawley rats were divided randomly into two groups for EEG recordings and behavioral assesment then these two groups divided to four groups: 6 for control, 6 for saline injection, 6 for relatively-low dose butamirate (5 mg/kg) and 6 for relatively-high dose butamirate (10 mg/kg) for each. Evaluation of the behavioral analyses after giving 70 mg/kg pentylenetetrazol (PTZ) first myoclonic jerk time and racine convulsion scales were analyzed, then in different rats for EEG recordings 35 mg/kg PTZ were given and spike percentages were evaluated in same doses of butamirate. In both 5 mg/kg and 10 mg/kg butamirate groups the FMJ onset times were statistically higher then the saline group, similarly both 5 and 10 mg/kg butamirate groups RCS scores were significantly lower than the saline group. In terms of spike percentages, 5 and 10 mg/kg butamirate were significantly lower than the saline group. As a result in our study, we showed that 5 and 10 mg/kg doses of butamirate have anticonvulsant effects on PTZ induced rats.

Key Words: Experimental Epilepsy Model, Butamirate Citrate, Pentylenetetrazol, Electroencephalogram

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Introduction

The epileptic seizure is a reversible phenomenon that develops as a secondary to synchronized neuronal activity as the neurons in the brain become overstimulated. Epileptogenesis is the process that occurs at the cellular and molecular level, which is necessary for seizure formation. There are many opinions about the mechanisms of epileptic seizures. γ-aminobutyric acid (GABA) is a major inhibitor neurotransmitter present in the brain and effective at the cellular level (Kang and Macdonald, 2009). During epileptogenesis; reduced GABA activity is known to cause neuronal hyperexcitability and agents that increase GABA activity have been shown to be antiepileptic (Olsen et al., 1999). It is also known that epileptic seizure is triggered by overexcitation of glutamate, which is also the most common excitatory stimulant (McNamara et al., 2006). Neuroprotective effects of both NMDA and non-NMDA receptor antagonists have been demonstrated in epileptic seizures (Penix et al., 1996). While it is known that there are genetic facilitating factors besides molecular and cellular mechanisms, the now accepted opinion is that epilepsy is in the polygenic basis. (Harden, 2002; Ferraro and Buono, 2006; Tan and Berkovic, 2010).

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Butamirate citrate (2- (2-diethylaminoethoxyl) ethyl 2-phenyl-butyrate-dihydrogen citrate) is a non-opioid agent that has been used for many years as an antitussive agent that shows its effect through the central nervous system (Klein and Musacchio, 1989). Central control of coughing; is provided via unmyelinated C fibers, mechanoreceptors and brain stem, especially the nucleus tractus solitarius (NTS), and the vagal afferent (Kubin *et al.*, 2006). NTS is known as the region where vagal afferents terminate in the medulla oblongata, and it contains GABA receptors, NMDA and AMPA / KA receptors (Accorsi-Mendonca *et al.*, 2015). In some studies; the relationship between epileptic seizures and NTS has been demonstrated. It has been found that epileptic activity, particularly the connections between NTS and forebrain, has spread throughout the brain from NTS by using glutamatergic (excitatory) pathways (Jhamandas and Harris, 1992; Rutecki, 1990).

In our study, the possible effects of butamirate on epileptic seizures, which is thought to perform central antitussive effect via medulla oblongata and NTS, has been investigated on the electrophysiological and clinical basis.

Methods
Animal and Laboratory
The experimental procedures employed in the present study were approved by Animal Ethics Committee. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, as confirmed by National Institutes of Health (U.S.). 48 male (24 of them for EEG recording and 24 of them are for behavioral studies) Sprague–Dawley rats, weighing 200–250 g each were utilized for this study. The rats were kept on a 12 hour –12 hour light–dark cycle (light from 07.00 to 19.00), in quiet rooms, with 22–24 ∘C ambient temperature. They were fed by standard laboratory food and tap water *ad libitum*.

Experimental Procedures
48 rats were randomly divided into two groups: Group A for EEG recordings and Group B or behavioral assessment. In Group A; Rats were deeply anesthetized. Then, a small hole was opened with a drill under stereotaxically. The electrodes (Polyamide-coated stainless steel wires, 0.1 mm diameter and electrical resistance <1Ω/10 mm) were implanted on dura over left frontal cortex (2.0 mm lateral to the midline, 1.5 mm anterior to the bregma) and the reference electrode was implanted over cerebellum (1.5 mm posterior to the lambda, on midline) (Kubin *et al.*, 2006; Lemus *et al.*, 2011) for EEG recording. Then, the electrodes were fixed by using dental acrylic (Dental acrylic is a mixture of numerous alloys using for dental restoration). Rats were deeply anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) intraperitoneally (i.p.). 35 mg/kg is ideal for observing changes in EEG spikes but does not consistently produce observable behavioral changes while 70 mg/kg consistently produced observable behavioral changes but EEG readings have a small signal to noise ratio to see differences in drug concentrations. After 12 days from the electrodes were fixed, 24 rats were divided randomly into 4 groups (n=6): Group A1, A2, A3, A4.

Group A1 was defined as control and given no medication. Group A2 was administered saline i.p, Group A3 was administered 5 mg/kg butamirate citrate i.p. (Sinecod, 1.5 mg/ml, Novartis) and Group A4 was administered 10 mg/kg butamirate citrate i.p. The drugs were administered 30 minutes prior to pentylenetetrazol (PTZ) (35 mg/kg, i.p.) injection. All groups, except Group A1, were received 35 mg/kg PTZ and EEG were recorded. EEG recordings were taken in awake rats in a special container after 5 minutes from PTZ administration.

All EEG recordings and behavioral assessment protocols were performed as previously described (Erbaş *et al.*, 2015). In summary, the EEG recordings were taken for 60 minutes, the signals were amplified 10 thousand times and filtered within a range of 1-60 Hz. The EEG records were obtained by the BIOPAC MP150 Data Acquisition System (Biopac System Inc., Santa Barbara, CA, USA) and the spike percentage was evaluated. Two clinical neurophysiologists scored the EEG data for the spike percentage (which is a reproducible way of quantifying epileptiform activity to quantify the percentage of 1-second bins with at least one spike-wave, called "spike-wave percentage" (Erbaş *et al.*, 2015). The onset and cessation of this complex were identified by a higher amplitude (at least two-fold), compared with the baseline values. The cumulative duration of spike-wave was detected within 2-minute intervals.

Then the groups were rearranged with different 24 rats (Group B) and these rats were then divided into 4 groups (n = 6): Group B1, B2, B3 and B4. The first group (Group B1) was

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defined as control and given no medication. Group B2 was administered saline i.p, Group B3 5 mg/kg butamirate citrate i.p, Group B4 10 mg/kg butamirate citrate i.p. The drugs were administered 30 minutes prior to PTZ (70 mg/kg, i.p.) injection. Racine's Convulsion Scale (RCS) (Uyanıkgil *et al*., 2016) and onset times of 'first myoclonic jerk' (FMJ) was used to evaluate the seizures (for only PTZ 70 mg/kg) as follows: 0 = no convulsion; 1 = twitching of vibrassae and pinnae; 2 = motor arrest with more pronounced twitching; 3 = motor arrest with generalized myoclonic jerks (this time was recorded for evaluating FMJ onset time); 4 = tonic clonic seizure while the animal remained on its feed; 5 = tonic–clonic seizure with loss of the righting reflex; 6 = lethal seizure. Rats were observed for onset times of FMJ as previously described (Erbaş *et al*., 2015). The onset times were recorded as seconds. Almost all animals showing tonic generalized extension died. The observation period for PTZ-induced seizures was limited to 30 minutes duration (Erbaş *et al*., 2015). After this duration, the animals were euthanized.

Statistical Analysis
Results were expressed as a mean ± standard error of the mean (SEM). Data analyses were performed by utilizing SPSS version 23,0 for Windows. Shapiro-Wilk test is used to determine if a population of values has a normal distribution. The Racine convulsion scores were evaluated by Kruskal Wallis test and, first myoclonic jerk (FMJ) time was evaluated by one-way analysis of variance (ANOVA). Post-hoc Bonferroni test and Mann Whitney U test was utilized to identify differences between the experimental groups. The value of $p < 0.05$ was accepted as statistically significant.

Results
Electrophysiological findings
In group B (n=24) electrophysiological findings were evaluated, EEG was recorded and spike percentages were evaluated. Subgroups were created as B1 (naive), B2 (PTZ (35 mg/kg) and saline-treated), B3 (PTZ (35 mg/kg) and 5 mg/kg butamirate citrate treated) and B4 (PTZ (35 mg/kg) and 10 mg/kg butamirate citrate treated). When group B3 (PTZ (35 mg/kg) and 5 mg/kg butyrate citrate) compared to the saline-treated B2 group in terms of percentage of spike, the percentage of spikes in the B2 group was found to be 65.5 ± 2.5 and in the B3 group it was found to

be 21.5 ± 4.1, and the difference was statistically significant ($p < 0.0001$). In the B4 group, 10 mg butamirate was given, the percentage of spikes was significantly reduced compared to the control group (12.8 ± 2.3, $p < 0.0001$) (Figure 1). Spike percentages of the study group are given in Table 1.
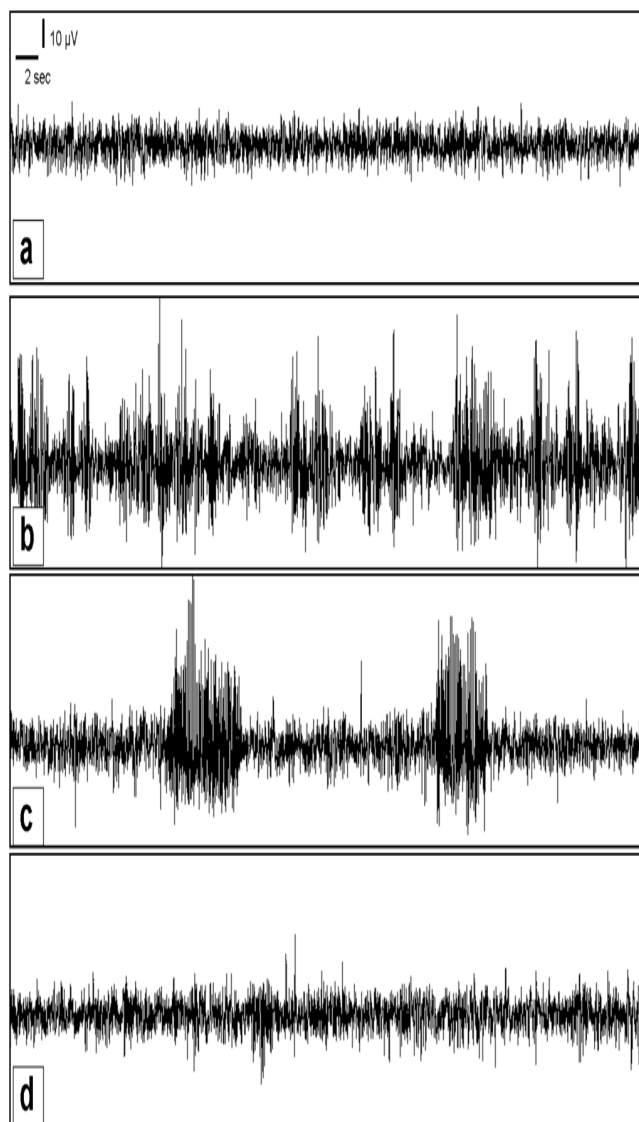

Figure 1. EEG recording of study groups; (a) Control group, (b) PTZ and saline group, (c) PTZ and 5 mg/kg butamirate citrate group, (d) PTZ and 10 mg/kg butamirate citrate group

Table 1. Spike Percentages of study group in EEG. PTZ: pentylentetrazol, * $p < 0.0001$, PTZ (35 mg/kg) and 5 mg/kg or 10 mg/kg butamirate citrate group compared with PTZ (35 mg/kg) and saline group. * $p < 0.0001$ (different from saline-treated PTZ group)

Drugs Group	Spike Percentage
1-Control (B1 group)	% 0
2-PTZ (35 mg/kg) and saline (B2 group)	% 71.8 ± 3.7
3-PTZ (35 mg/kg) and 5 mg/kg butamirate citrate (B3 group)	% 21.5 ± 4.1*
4-PTZ (35 mg/kg) and 10 mg/kg butamirate citrate (B4 group)	% 12.8 ± 2.3*

Racine Score and first myoclonic jerk time evaluation

In group A (n= 24), subgroups divided like group B, respectively. Seizure activity of the study group was assessed by RCS. In the epileptic seizure A2 group with PTZ, Racine score average was detected as 5.6 ± 0.2. In the A3 group given 5 mg/kg Butamirate, the Racine score average was 2.5 ± 0.4 and in the A4 group given 10 mg/kg, the average was detected 1.8 ± 0.5. The difference was statistically significant (respectively, $p<0.0001$, $p<0.0001$), when we compared the two groups with the A2 saline group. The average FMJ onset time, which is another clinical assessment, has prolonged statistically significant in the A3 group given 5 mg/kg when we compared with the saline group (161.6 ± 14.7 sec, 65.5 ± 2.5 sec, $p<0.0001$). In the A4 group given 10 mg/kg butamirate, it was also found that it was statistically significantly longer than the A2 saline group (241.3 ± 34.2 sec, $p<0.0001$) (**Table 2**).

Table 2. Racine scores and FMJ times of study groups. PTZ: pentylentetrazol.* $p<0.0001$, PTZ (35 mg/kg) and 5 mg/kg or 10 mg/kg butamirate citrate group compared with PTZ (35 mg/kg) and saline group. * $p<0.0001$ (different from saline-treated PTZ group)

Drugs Group	Convulsion Stage (Racine)	FMJ onset time (sec)
1-Control (A1 group)	0	0
2-PTZ (35 mg/kg) and saline (A2 group)	5.6 ± 0.2	65.5 ± 2.5
3-PTZ (35 mg/kg) and 5 mg/kg butamirate citrate (A3 group)	2.5 ± 0.4*	161.6 ± 14.7*
4-PTZ (35 mg/kg) and 10 mg/kg butamirate citrate (A4 group)	1.8± 0.5*	241.3 ± 34.2*

Discussion

Epilepsy is a chronic neurological disease that affects 6 million people in Europe that is significantly important socially and for both patients and their relatives (Baulac *et al.*, 2015). Although mechanisms of epilepsy formation are not fully understood, many neurotransmitter and cell level mechanisms play a role in the etiopathogenesis. In addition, any mutation that occurs in the ion channels causes an increase or inhibition in the excitatory neurotransmission by changing the activity of the excitatory and inhibitory neurotransmission (Kang and Macdonald, 2009). It is precise that, in Epileptogenesis, there is the presence of increased neuronal excitability and synchronization. GABA, the main inhibitory agent of the brain, the glutamate activity of its excitatory and its receptors have become the common target of many antiepileptic agents. Cough is controlled by both peripheral and central interactions. Bronchopulmonary vagal afferents are mostly composed of unmyelinated C fibers, and the cell bodies of C fibers are in the jugular ganglion (Canning and Mori, 2011; Coleridge and Coleridge, 1984).

Butamirate has been used effectively and safely for centuries as a centrally acting antitussive agent. Cough is controlled by both peripheral and central interactions. Bronchopulmonary vagal afferents are mostly composed of unmyelinated C fibers, and the cell bodies of C fibers are in the jugular ganglion (Canning and Mori, 2011; Coleridge and Coleridge, 1984). Vagal afferent fibers enter the brainstem with NTS and the ventrolateral region of the medulla oblongata can be considered as the main control center for coughing (Shannon *et al.*, 1998; Shannon *et al.*, 2000). The brainstem is responsible for the processing and controlling of the afferent information; for the conscious part of the coughing, the cortical and subcortical structures are responsible (Canning *et al.*, 2014). In a study which has been conducted, antiepileptic activity has been demonstrated in media-caudal in NTS by increasing GABA neurotransmission or reducing glutamate neurotransmission (Walker *et al.*, 1999). In line with this information; the possible effect of butamirate citrate on epileptic seizures has been investigated in our study, which has been used effectively and safely antitussive in both children and adults for many years. In our study; in rats with epileptic seizures with PTZ, the percentage of spikes recorded in EEG in both doses of butamirate was significantly lower than in the control group. Previously, another antitussive dextromethorphan has been shown to have antiepileptic activity (Chien *et al.*, 2012) but no association has been reported between butamirate and epileptic seizures. In addition, when we evaluate the study group clinically; butamirate group showed significant improvement in Racine score compared to the control group and significant prolongation in butamirate at the onset of FMJ. These findings demonstrate the antiepileptic activity of butamirate both clinically and electrophysiologically.

In the group given butamirate, there was no slowing down or dizziness compared to the control group and this finding suggests that the effect of butamirate is shown not via GABA but via glutamate pathway. The sedation and drowsiness

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in the high doses observed in the majority of the antiepileptic agents were not observed in both doses of butamirate in the rats in our study group. Besides the antiepileptic activity of butamirate, when it is combined with the knowledge that no side effects are observed at high doses, in daily clinical practice, it has been also shown that it has been tolerated safely and easily.

It is also known that the afferent fibers of cough are medullated through vagal nerve and project to the media-caudal part of the NTS, and the suppression of afferent fibers and the anticonvulsant activity have been shown previously (Jhamandas and Harris, 1992). It is known that inhibition of NTS, particularly by vagal nerve stimulation, reduces epileptic activity, possibly by reducing the glucose metabolism in NTS (Takaya *et al*., 1996).

As a result; control of cough is provided mainly via the vagal nerve and NTS, and the antiepileptic effect of butamirate, which is a central antitussive effect, has been shown for the first time in our study. Although the antiepileptic activity of butamirate is shown both clinically and electrophysiologically, there is a need for further work in the future that will show the effect of the butamirate on epileptogenesis, possibly through glutamate neurotransmission and the vagal nerve.

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