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ORIGINAL ARTICLE

Potential Antiepileptic Effect of Ivabradine Against Pentylenetetrazole Induced-Kindleing in Male Mice

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ABSTRACT

Background: Ivabradine, Hyperpolarization activated cyclic nucleotide gated (HCN) channel blocker, is the most specific blocker of central nervous system Ih current. Valporate is one of the most commonly used antiepileptic drugs. **Objectives:** investigate the possible anticonvulsant effect of Ivabradine and its interaction with valporate in pentylenetetrazole (PTZ) induced- kindling in mice. ***Materials& Methods:*** mice were divided into four groups, “Group 1", "vehicle-treated group" “Group 2", "PTZ kindling Control group "Group 3**:** Ivabradine ", group 4 Valproate (VPA) group 5" Ivabradine and VPA. Kindling was produced by repeated intraperitoneally (i.p). administration of PTZ (40mg/kg), every other day for 9 doses. Both drugs were administered i.p., 30 minutes before each PTZ injection. Seizure score, latency were recorded. Their brains were removed for assessment of oxidant/antioxidant status and anti-inflammatory cascades. ***Results:*** Ivabradine and VPA individually significantly decreased seizure score and co administration of both drugs significantly decreased seizure score less than either vaporate or Ivabradine. Both drugs significantly increased latency to seizures. Ivabradine, VPA and their combined administration significantly elevated brain level of GSH, catalase and significantly decreased levels of nitrite, MDA, IL1β, and TNFα as compared to PTZ control group. Co-administration of both drugs resulted in a significant elevation in the brain level of GSH, catalase concomitant with a significant reduction in the brain levels of MDA, IL1β and TNFα as compared to either VPA or ivabradine groups. Conclusion: Ivabradine has anticonvulsant effect and potentiates the effect of VPA which may be attributes to HCN channel blockade, antioxidant and anti-inflammatory effects.

Key words: ivabradine; PTZ; Seizures; Valproate.

INTRODUCTION

Epilepsy is a neurological disorder that affects 1-2% of the population. A significant percentage of epileptic patients do not respond to anticonvulsant drugs, suggesting the need to investigate new pharmacological treatments [1]. It is characterized by recurrent seizures. Seizures are finite episodes of brain dysfunction resulting from abnormal discharge of cerebral neurons [2].

Kindling has been used as a chronic animal model for temporal lobe epilepsy and although there is a large number of models available for screening the anticonvulsant activity of a drug, the PTZ remain the‘gold standards’ in early stages of testing [3]. In this model, the initially subconvulsive stimuli become capable of evoking fully developed seizures due to lowering of seizure threshold[4]. The severity of induced seizures increases after seizures are induced repeatedly [5]. If the stimulus causes generalized convulsion in experimental animal, it is accepted that kindling is completed then this abnormal excitable status

remainspermanent [6]. PTZ is a GABAA receptor antagonist. Also, it induces alterations in glutamergic and antioxidant defense systems [7].

HCN channels contribute in modulation of rhythmic activity, transmission of synaptic potentials and plasticity phenomena [8]. The role of HCN channels activation results in neuronal membrane depolarizing (Ih) current that modulate neuronal excitability. Modification of the function of HCN channels can induce uncontrolled action potential firing and provide a background setting for the development of epilepsy[9].

Ivabradine, HCN channel blocker, is a pure heart rate lowering drug that specifically inhibits the inward funny (If) current involved in the regulation of heart rate in the sinoatrial node [10]. In addition, the drug is the most specific blocker of central nervous system Ih current [11]. Ivabradine exerts anti-anginal and anti-ischemic effects in patients with stable coronary disease [12]. Moreover, ivabradine has documented beneficial effect on nociception, inflammation and psychosis [13,14] .

In spite of huge funding for new antiepileptic drug development, many of them were withdrawn because their harmful adverse effects outweigh their beneficial actions. In addition, newly developed antiepileptic drugs are expensive to be afforded by patients of the third world countries [15].

The present study was carried out to elucidate the anticonvulsant, antioxidant and anti-inflammatory effects of ivabradine on PTZ-induced kindling in mice and its interaction with VPA.

MATERIALS & METHODS

Animals:

40 adult male Swiss albino mice weighing 15–35 g were used in the current study. Animals were purchased from the Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were randomly assigned to experimental groups. The experiment was performed in the pharmacology department laboratory, faculty of medicine, Zagazig University. Experimental design and animal handling were performed in accordance with protocols approved by the local experimental ethics committee guidelines of the Egyptian Society of Neuroscience. THhe study protocol was approved by the Ethical Committee of the Faculty of Medicine, Zagazig University

Experimental protocol

In PTZ-induced kindling": Gp1 (n:8) “Group 1", "vehicle-treated group" “Group 2""PTZ-kindled control group" received nine subconclusive doses of PTZ injections (40 mg/kg, i.p.) on alternate days for 17 days[15], Gp3 (n: 8) "ivabradine group" daily injected with ivabradine (20 mg/kg/d, i.p.) for 17 days 30 minutes before PTZ Gp4(n: 8) [16] " valproate group" daily injected with valproate (50 mg/kg, i.p.) for 17 days 30 minutes before PTZ and Gp5 (n: 8) " ivabradine and valproate group" injected with both ivabradine (20mg/kg, i.p.) and valproate (50 mg/kg, i.p.) daily for 17 days 30 minutes before PTZ. All drugs were dissolved in saline just before injection. After injections, the animals were observed for 20 minutes in Plexiglas cages. Occurrence of seizure was evaluated using a scoring scale [15]: 0, no effect; 1, jerks; 2, Straub’s tail; 3, clonus. The maximum kindling score is approached if the animal shows all the phases of convulsions (i.e. up to full-blown clonus) and equals 6 (sum of 0 + 1 + 2 + 3). On the last day, the maximum seizure score was reached in PTZ-treated group.

Biochemical assays

Mice from each group were euthanized by decapitation 24 h after last PTZ administration. Their brains were quickly removed in liquid nitrogen**.** The brain was homogenized in ice-cold saline for estimation of GSH, catalase, MDA, nitrite, TNF-α and IL-1β utilizing enzyme linked immunosorbant assay (ELISA) kits purchased from(blue gene biotec, USA), (eiaab, USA), (life spane bioscience, USA), (assay design, USA), (ray biotec, USA), and (CUSABIO, USA), respectively and results were expressed as mMol per gram tissue (µmol/g tissue)for nitrite, as Microgram/Gram(u/g) for catalase, picograms

pergram tissue (pg/g tissue) for GSH, IL-1β and TNF-α and as nanograms per gram tissue (ng/g tissue), for MDA.

StatisticalAnalysis

the obtained Continuous variables were tabulated as means± SD. Comparison between different groups were made using one way analysis of variances (one-way ANOVA) followed by Post-Hoc (least significant difference"LSD") tests as described by Petrie and Sabin[17]. The differences were considered to be significant when $p < 0.05$.

RESULTS

Pharmacological results

1- **Effects of ivabradine(20mg/kg), valproate(50 mg/kg),and their combination on seizures scores PTZ-induced kindling in mice(table 1):**

In the 1st, 2nd and 3rd injections there was no statistically significant differences in seizures scores among different groups of the study (P>0.05).

In the 4th injection, ivabradine significantly decreased (P<0.05) seizure score from (3.6±1.6) in the control group to (1.6±0.9), valporate significantly(P<0.05) decreased it from (3.6±1.6) in the control group to (2±0.9) and combination of both drugs significantly(P<0.05) decreased it from (3.6±1.6) in the control group to (1.3±0.8). Additionally, there was no statistically significant differences in seizures scores among ivabradine , valporate and combination groups.

In the 5th injection, ivabradine significantly (P<0.05) decreased it from (5.1±1.5) in the control group to (2±1), valporate significantly (P<0.05) decreased it from (5.1±1.5) in the control group to (1.3±0.8)and combination of both drugs significantly(P<0.05) decreased it from (5.1±1.5)in the control group to (1.2±0.4). Also there was no statistically significant difference in seizures scores among ivabradine, valporate and combination groups.

In the 6th injection, ivabradine significantly(P<0.05) decreased seizure score from (5.6±1.1)in the control group to (2.2±1), valporate significantly(P<0.05) decreased it from (5.6±1.1)in the control group to (1.2± 0.9)and combination of both drugs significantly (P<0.05)decreased seizure score from (5.6±1.1)in the control group to (1.2±0.4).Additionally there were no statistically significant differences in seizures scores among ivabradine, valporate and combination groups.

In the7th injection, ivabradine significantly (P<0.001) decreased seizure score from (5.6±1.3) in the control group to (4.3±1.5), valporate significantly (P<0.001) decreased it from (5.6±1.1) in the control group to (3±0.5) and combination of both drugs significantly decreased it from (5.6±1.1) in the control group to (1±0.2). Interestingly, valporate significantly (P<0.001) decreased seizure score (3±0.5) as compared to ivabradine (4.3±1.5). Regarding combined administration of both drugs (1±0.2),there was statistically significant(P<0.001) differences in seizures scores in relation to ivabradine(4.3±1.5) alone and valporate (3±0.5)alone.

In the 8 th injection, ivabradine significantly (P<0.001) decreased seizure score from (6±0) in the control group to (4.6±1.5), valporate significantly (P<0.001) decreased it from (6±0) in the control group to (3.2±0.3) and combination of both drugs significantly (P<0.001) decreased it from (6±0) in the control group to (1.2±0.1). valporate significantly (P<0.001) decreased it (3.2±0.3) as compared to ivabradine (4.6±1.5). As regard coadministration of both drugs, there was statistically significant (P<0.001) differences in seizures scores (1.2±0.1) as compared to either ivabradine alone (4.6±1.5) or valporate alone (3.2±0.3).

In the 9th injection, ivabradine significantly (P<0.001) decreased seizure score from (6±0) in the control group to (4.2±1), valporate significantly (P<0.001) decreased it from (6±0) in the control group to (2.9±0.4) and combination of both drugs significantly (P<0.001) decreased it from (6±0) in the control group to (1±0.3). Interestingly the seizure score in valporate group(2.9±0.4) was significantly lower than ivabradine group (4.2±1). Regarding combined administration of both drugs (1±0.3), there was statistically significant (P<0.001) differences in seizures scores as compared to either ivabradine

alone or valporate alone.

2-Effect of Ivabradine, Valproate and their combination on seizure onset in PTZ model in mice (table 2) :

In the1st, 2nd, 3rd, 5th and 6th injections, there were no statistically significant differences (P>0.05) in seizures onset among different groups of the study.

In the 4th injection, ivabradine significantly(P<0.05)increased latency from (7.9±3.9) in the control group to (8.7±3.3), valporate significantly (P<0.05)increased it from (7.9±3.9) in the control group to (12.1±1.9) and combination of both drugs significantly increased it from (7.9±3.9) in the control group to (11.8±3.3). The seizure onset in valporate (12.1±1.9) and combination (11.8±3.3) groups were significantly (P<0.001) higher than that of ivabradine group(8.7±3.3). Additionally, there was no statistically significant differences among valporate (12.1±1.9) and combination (11.8±3.3) groups.

In the 7th injection, ivabradine significantly (P<0.001) increased latency from (4.8±2.1) in the control group to (9.6±2.8), valporate significantly (P<0.001)increased it from (4.8±2.1) in the control group to (11.2±2.8) and combination of both drugs significantly increased it from (4.8±2.1) in the control group to (13.5±5.3). the mean values of latency in the combination group(13.5±5.3) were significantly(P<0.001) higher than that of ivabradine group(9.6±2.8). Moreover, there was no significant difference regarding it in the valporate group (11.2±2.8) as compared to either the ivabradine (9.6±2.8) or the combination (13.5±5.3) groups.

In the 8th injection, ivabradine significantly (P<0.05) increased latency from (5.2±1.7) in the control group to (9.2±3.7), valporate significantly (P<0.05) increased it from (5.2±1.7) in the control group to (9.1±1.6) and combination of both drugs significantly (P<0.05) increased it from (5.2±1.7) in the control group to (9.5±2.8).Additionally there were no statistically significant differences among ivabradine, valporate or combination groups.

In the 9th injection, ivabradine significantly(P<0.001)increased latency from(5.9±1.7) in the control group to (9.2±3.7), valporate administration significantly(P<0.001) increased it from (5.9±1.7) in the control group to (11.5±1.6) and coadministration of both drugs significantly(P<0.001) increased it from (5.9±1.7) in the control group to (13.2±2.8). Seizure onset in the combination group(13.2±2.8) were significantly (P<0.001) higher than that of ivabradine group(9.2±3.7). Moreover, there was no significant difference regarding latency in the valporate group as compared to either the ivabradine or combination groups.

Biochemical results

Compared to normal animals, PTZ-kindling caused a significant reduction (p<0.05) in brain GSH and catalase by 60.8% and 59% respectively. Moreover, PTZ-kindling significantly (p<0.05) increased brain MDA, nitrite, IL-1β and TNFα by 970%,52.9%,793 and 944% respectively. Ivabradine, valproate, or their combination significantly (p <0.05) decreased brain nitrite by 53.2%, 67.9%, and 81%, respectively, as compared to PTZ control group. Moreover, the used drugs significantly (p <0.05) reduced the brain TNFα by 48%, 63%, and 76.3%, respectively, as well as IL-1β by 49.6%, 65.3% and 75.6%, respectively Compared to PTZ control group, and decreased brain MDA by 51%, 68.9% and 80.4%, respectively Compared to PTZ control group. Compared to chronic PTZ control group, Ivabradine, valproate, or their combination caused significant (p <0.05) elevation of brain GSH by 80%, 163.8%, and 366.6%, respectively, Moreover, the used drugs caused significant elevation of catalase level by 102% ,194.6% and343.6respectively (figure1).

Table (1): Effect of ivabradine, Valproate and their combination on seizure scores in PTZ model in mice:

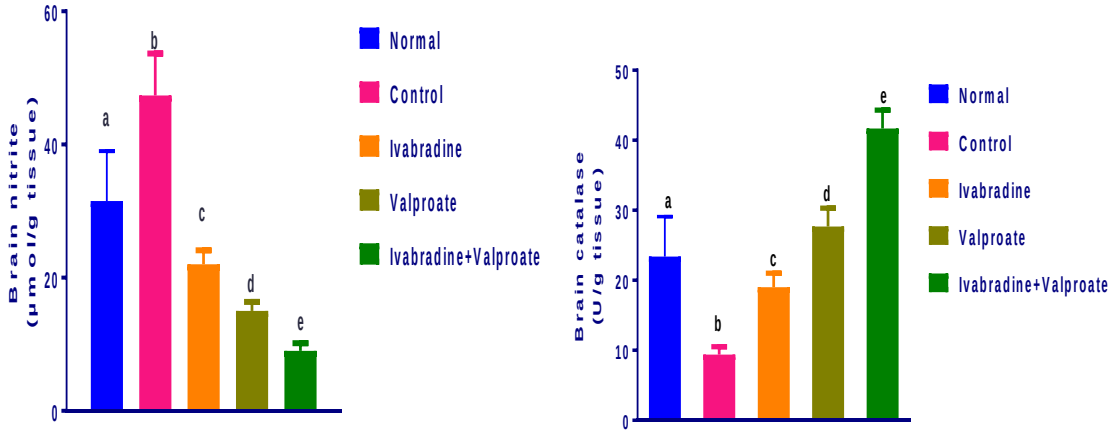
Groups PTZ Dose		Control	Ivabradine	Valproate	Ivabradine + Valproate	*P-value*
1st	*n*	8	8	8	8	**0.99**
	Mean±SD	1.5±0.3	0.8±0.3	0.9±0.4	0.7±0.2	
2nd	*n*	8	8	8	8	**0.46**
	Mean±SD	1.1±0.3	0.9±0.3	0.9±0.3	0.9±0.3	
3rd	*n*	8	7	8	8	**0.26**
	Mean±SD	1.5±0.6	1±0.5	1.2±0.5	1.1±0.4	
4th	*n*	8	7	6	6	**0.004**
	Mean±SD	3.6±1.6[a]	1.6±0.9[b]	2±0.9[b]	1.3±0.8[b]	
5th	*n*	7	7	6	6	**0.001**
	Mean±SD	5.1±1.5[a]	2±1[b]	1.3±0.8[b]	1.2±0.4[b]	
6th	*n*	7	7	6	6	**<0.001**
	Mean±SD	5.6±1.1[a]	2.2±1[b]	1.2± 0.9 [b]	1.2 ±0.4[b]	
7th	*n*	7	7	6	6	**<0.001**
	Mean±SD	5.6±1.3 [a]	4.3±1.5 [b]	3±0.5 [c]	1±0.2 [d]	
8th	*n*	6	7	6	6	**<0.001**
	Mean±SD	6±0[a]	4.6±1.5 [b]	3.2±0.3[c]	1.2± 0.1[d]	
9th	*n*	6	7	6	6	**<0.001**
	Mean±SD	6±0[a]	4.2±1 [b]	2.9±0.4[c]	1± 0.3[d]	

Values are means of mice ± SD **Values are means of mice± SD. Values with different superscript small letters are significantly different (p <0.05), *n* = Number of mice in each group.**

Table (2): Effect of ivabradine, valproate and their combination on seizure onset in PTZ model in mice:

Latency (minutes)	Groups	Control	Ivabradine	Valproate	Ivabradine + Valproate	*P-value*
1st	***n***	8	8	8	8	0.77
	Mean±SD	9.3±3.2	7.9±5.3	7.7±4.8	6.9±4.9	
2nd	***n***	8	8	8	8	0.51
	Mean±SD	10.5±3.7	7.7±4.1	8.8±4.4	8±3.8	
3rd	***n***	8	7	8	8	0.21
	Mean±SD	9.5±3.3	10.3±4.7	8.2±2.3	8±3.6	
4th	***n***	8	7	6	6	**<0.001**
	Mean±SD	7.9±3.9 a	8.7±3.3^{b}	12.1±1.9^{c}	11.8±3.3^{c}	
5th	***n***	7	7	6	6	0.13
	Mean±SD	8±2.5	11.3±2.5	11±3.4	10.7±2.6	
6th	***n***	7	7	6	6	0.55
	Mean±SD	7.6±3.1	10.4±2.6	9.8±3.7	9.4±5.3	
7th	***n***	7	7	6	6	**<0.001**
	Mean±SD	4.8±2.1^{a}	9.6±2.8^{b}	11.2±2.8^{bc}	13.5±5.3^{c}	
8th	***n***	6	7	6	6	**0.028**
	Mean±SD	5.2±1.7^{a}	9.2±3.7^{b}	9.1±1.6^{b}	9.5±2.8^{b}	
9th	***n***	6	7	6	6	**<0.001**
	Mean±SD	5.9±1.7^{a}	9.2 ±3.7^{b}	11.5±1.6^{bc}	13.2±2.8^{c}	

- **Values are means of mice± SD. Values with different superscript small letters are significantly different (p <0.05), *n* = number of mice in each group. Values are means of mice± SD, n = Number of mice in each group.**



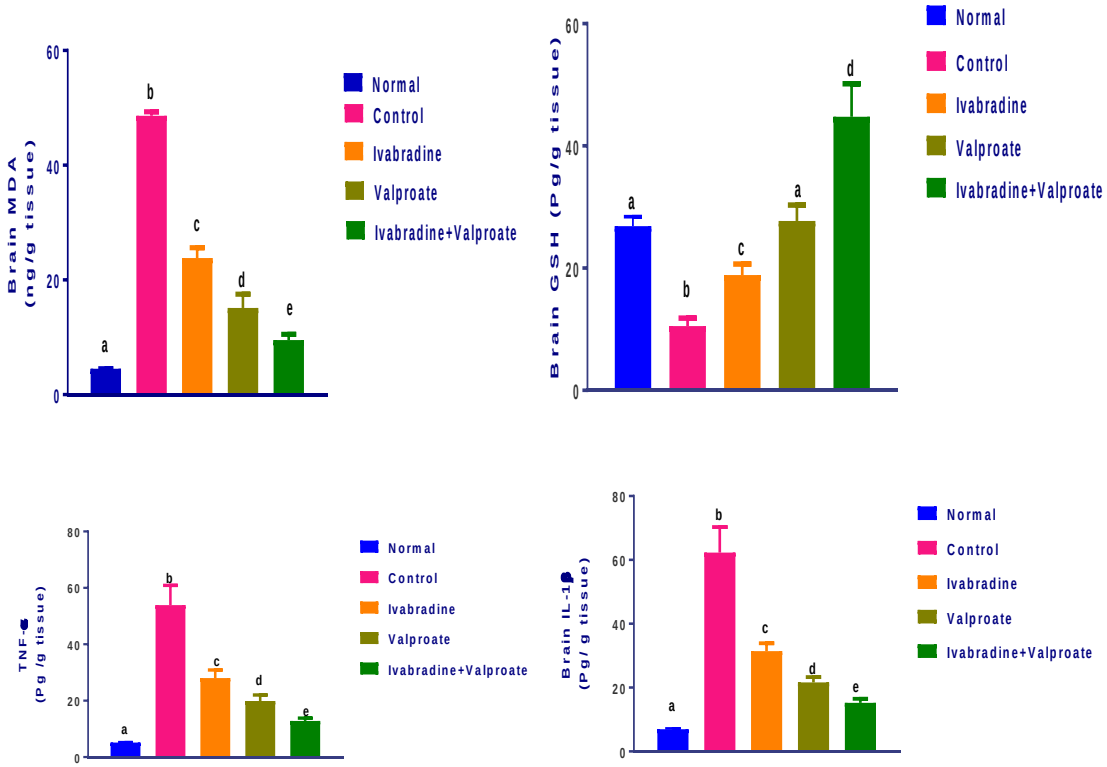


Fig. 1: showing comparison between different groups regarding brain nitrite, catalase, GSH, MDA, IL-1β, TNF-*α*. Values are means of mice ±SEM. Bars not sharing the common small superscript letters differ significantly at *P*<0.05.

DISCUSSION

The etiology of epilepsy remains unknown in about 60% of cases. Several types of epilepsy have a genetic component mainly linked to mutations in genes encoding voltage-gated or ligand-gated (GABAA) channels [18].

HCN channels have established role in modulating neuronal excitability [9]. The present study assessed the anticonvulsant effect of ivabradine, HCN blocker, in PTZ-induced kindling in mice as well as on the parameters of oxidative stress and inflammation. The results of the present work revealed that, ivabradine decreased seizure score while increased the latency to seizures. Valporate decreased seizure scores more than ivabradine, the effect of both drugs on latency was insignificantly different difference in seizure score among the treated groups. The difference in seizures score and latency found in the last three injections as kindling was achieved.There were no statistically significant differences (P>0.05) in seizures onset among different groups in the1st, 2^{nd,} 3rd injections of the study and no difference regarding latency in the1st, 2^{nd,} 3rd, 5th and 6th injections as animals aren't fully kindled. These results cope with Luszczki et al. [16] who found that ivabradine increased the threshold for maximal electroshock-induced tonic seizures in mice. They supposed that ivabradine, due to its HCN channel blocking properties, is able to support the ionic homeostasis in the brain, and elevated the threshold for electroconvulsions in mice. In addition, Mansour and Ibrahim [19] reported that ivabradine increased latency of seizure in kainite model of epilepsy.

In support of these finding, Bender et al. [20] postulated that the resected hippocampi from patients with temporal-lobe epilepsy, showed

enhancement in the levels of HCN1 channel expression. In addition, another work has identified a mutation in the HCN2 gene with augmentation of Ih current in patients with genetic epilepsy and had febrile seizures [21]. However, the results of the present study don't cope with Poolos et al. [22] who reported that lamotrigine increased Ih current. In addition, gabapentin was found to increase Ih current in CA1 pyramidal neurons [23]. The reported increase in Ih current with the previous two drugs might be attributed to enhancement of neuronal hyperpolerization due activation of GABAA receptor. The resulting membrane hyperpolerization is a well documented stimulus for opening HCN channels and Ih current occurrence. Indeed, in various types of neurons the voltage-activated Na+/K+ "Ih" current has been identified as depolarizing current and activated by membrane hyperpolarization facilitated by cAMP[24].

It is postulated that oxidative stress resulting from excessive free-radical release is likely implicated in the initiation and progression of epilepsy Oxidative stress occurs during epileptogenesis due to seizures-associated glutamate excitotoxicity and NMDA receptor overactivation leading to increase in oxidation of macromolecules of the neurons just before the neuronal loss [25]. Also, ROS may activate NFκB, leading to the production of pro-inflammatory cytokines, which in turn enhance inflammation and, therefore, the generation of more reactive species [26].

In the current work, administration of ivabradine or valporate resulted in significant increase of the brain levels GSH , catalase in addition to decrease of MDA (marker for lipid peroxidation due to free radicals), nitrite levels (the stable end-product of nitric oxide in the in vitro system) and inflamatory cytokines (IL1β and TNF-α). These effects could be related to attenuation of kindling process as kindling was reported to involve generation of free radicals [27], as well as due direct enhancement of antioxidant machinery in the brain.

Indeed, previous studies reported that both ivabradine and valporate have antioxidant and anti-inflammatory effects. In this context, Beytur et al. [28] reported that ivabradine directly ameliorates nitric oxide and lipid peroxidation and renal inflammatory response by inhibiting oxidative stress. In addition, Colak et al. [29] postulated that ivabradine mitigated the oxidative stress and improved the hemodynamic parameters in doxorubicin-induced cardiotoxicity in rats. Moreover, ivabradine was found to decrease the ROS production due to decrement in NADPH oxidase activity as well as prevention of eNOS uncoupling [30]. Consequently, NADPH with glutathione reductase could increase GSH production. Yue-Chun et al. [31] reported that ivabradine reduce the expression of TNF-α, IL-1β in dilated cardiomyopathy model in mice.

In addition to antioxidant and anti-inflammatory effects of ivabradine, it could reduce apoptosis in hippocampal neurons and this could be related to its ability to reduce TNF-αand caspase3[32] .

The Present study shows, VPA administration protects against PTZ induced seizures with reducing brain oxidative stress and inflammatory mediators According to Rajeshwari et al. [33], the antioxidant effect of VPA is attributed to its free radical scavenging ability, enhancement of enzymatic antioxidant network (catalase and GSH). Himmerich et al. [34], reported that the cytokine production is due to elevation in ROS generation. Thus, the anti-inflammatory effect of valproate may be due to antioxidant effect.

CONCLUSION

Ivabradine had anticonvulsant effect, less than VPA, but potentiated the effect of VPA in PTZ kindled mice and ameliorated the associated oxidative stress and inflammation. Further experimental studies are needed to confirm the anticonvulsant effect of the drug in other models of seizure.

Declaration of interest :
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
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