Ivabradine Reduces Seizures in Pentylenetetrazol-Kindled Mice

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Background: Epilepsy is the second, after stroke, most common chronic neurological disorder with a serious socioeconomic impact. Hyperpolerization cyclic nucleotide gated (HCN) channels modulate neuronal excitability and their activation leads to development of a depolarizing (I_h) current that could provide a background setting for the development of epilepsy.

The aim of this study was to assess the potential anticonvulsant effect of ivabradine, HCN blocker, in comparison with diazepam in pentylenetetrazol (PTZ) kindled mice.

Methods: Forty Mice were randomly allocated into 4 equal groups; control, PTZ, diazepam (4mg/kg) and ivabradine (10 mg/kg) groups. Kindling model was produced by repeated i.p. administration of PTZ (40mg/kg), every other day for 9 days. Repeated PTZ treatment progressively increased the seizure score with the maximum score reached on day 9. Diazepam and ivabradine were administered intraperitoneally (i.p.), 10 minutes before each PTZ injection till the end of the schedule. Seizure score, latency and duration were recorded in all groups. Nitrite, malondialdehyde and reduced glutathione levels were assessed in brain homogenate at the end of schedule.

Results: Ivabradine significantly decreased kindling seizure score less than diazepam, while both drugs significantly decreased the duration of seizures and increased latency to seizures. The levels of nitrite and malondialdehyde were significantly increased in PTZ kindling group while, the reduced glutathione content was significantly decreased. Administration of either ivabradine or diazepam significantly decreased nitrite and malondialdehyde levels while increased the glutathione content of the brain.

Conclusion: Ivabradine has anticonvulsant effect, less than diazepam, and ameliorated the associated oxidative stress in PTZ kindled mice.

Key words: Epilepsy, Ivabradine, Diazepam, Pentylenetetrazole, HCN channels, I_h Current

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 available, suggesting the need to investigate new pharmacological treatments[1]. This chronic disorder 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 also as a very important model for complex partial epilepsy[3]. In this model, the initially subconvulsive stimuli become capable of evoking fully developed seizures due to lowering of seizure threshold[4]. The duration and 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 remains permanent[6]. Pentylenetetrazol (PTZ)–induced kindling model is among the first used models for antiepileptic drugs discovery. PTZ is a $GABA_A$ receptor antagonist. In addition, this chemoconvulsant induces alterations in glutamergic and antioxidant defense systems[7].

Hyperpolarization activated cyclic nucleotide gated (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 (I_h) 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 (I_f) current involved in the regulation of heart rate in the sinoatrial node[10]. In addition, the drug is considered to be the most specific blocker of central nervous system I_h 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]. Benzodiazepines, including diazepam, are widely used anticonvulsant drugs. These drugs are positive allosteric modulator of $GABA_A$ receptor increasing chloride influx and neuronal hyperpolerization[15].

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[16].
The present study was carried out to elucidate the anticonvulsant and antioxidant effects of ivabradine on PTZ-induced chemical kindling in mice in comparison with diazepam.

Materials and methods

Animals: Adult male albino mice (weighing 22–26 g) were obtained from National Research Laboratory, Cairo, Egypt and kept in colony cages with free access to food and tap water, under standardized housing conditions (temperature of 22 ± 1°C). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to 4 experimental groups. All experimental protocols were approved by the Ethics Committee of Zagazig University.

Drugs: Ivabradine (Servier, Courbevoie, France); pentylenetetrazol (PTZ) (Sigma (St Louis, MO, USA). Ivabradine and PTZ were supplied as white powder, freshly dissolved in normal saline at the day of the experiment. Diazepam ampoules (Memphis, Cairo, Egypt).

Kindling model: PTZ was injected, 40 mg/kg, i.p. on alternate days (day 1, 3, 5, 7 and 9) according to the standardized procedure[17]. After each PTZ injection, occurrence of seizure was evaluated using a scoring scale: 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 day 9 the maximum

seizure score was reached in PTZ-treated group. On day 10, mice were decapitated, the skull was opened, and cerebral cortex was dissected for biochemical estimations.

Experimental design: 40 mice were randomly divided into the following groups:

1- Control group: mice were injected saline i.p., every other day for nine days.

2- PTZ kindling group: mice were injected PTZ, every other day for nine days.

3- Diazepam-PTZ group: mice were injected diazepam 4mg/kg, i.p. [18] (Syapin 1983), 10 minutes before PTZ every other day for nine days.

4- Ivabradine-PTZ group: mice were injected ivabradine 10mg/kg, i.p. [19], 10 minutes before PTZ every other day for nine days.

Dissection and homogenization: On day 10 of the study, animals were decapitation. Brains were removed, rinsed in isotonic saline and weighed. Tissues were stored at -80 ^{0}C until assays. For assay, a 10% (w/v) tissue homogenate was prepared with 0.1 mol/L phosphate buffer (pH 7.4).

Nitrite estimation

Nitrite was measured in samples of brain homogenate using a spectrophotometric assay based on the Greiss reaction according to Raghvendra et al[20]. The nitrite concentration was calculated from a standard curve and expressed as µmol/mL.

Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the whole brain was performed according to the method of Wills[21]. In this assay, the concentration of malondialdehyde (MDA) was estimated. The results are expressed as nmol MDA/mg protein using the molar extinction coefficient of chromophore ($1.56 \cdot 10$ L/mol/cm).

Estimation of reduced glutathione (GSH) content

GSH in the brain was estimated according to the method of Ellman[22]. A 0.75 mL of homogenate was precipitated with 0.75 mL of 4% sulfosalicylic acid by keeping the mixture at 4 ^{0}C for 1 h and the samples were immediately centrifuged at 1200 g for 15 min at 4 ^{0}C. The assay mixture contains 0.5 mL of supernatant and 4.5 mL of 0.01 M dithiobisnitrobenzoic acid. The yellow color developed was read immediately at 412 nm. The results were expressed as nmol GSH per mg protein

Statistical analysis

Statistical analysis of data in all models was performed with one-way ANOVA followed by the *post hoc* Tukey-Kramer test for multiple comparisons. Differences among values were considered statistically significant if $p < 0.05$.

Results

Effect of diazepam and ivabradine on seizure score, latency and duration:
Diazepam significantly decreased seizure score from 5.12±0.66 in PTZ kindling group to 1.33±0.21. Administration of ivabradine induced significant decrease in seizure score to 3.22± 0.49, in respect to PTZ kindling group while, was significantly increased in respect to

diazepam-PTZ group. Administration of either diazepam or ivabradine induced significant increase in latency to seizure from 4.85±0.54 minutes in PTZ kindling group to 13.93±2.13 and 12.42±1.98 minutes respectively. There were no significant differences between diazepam-PTZ and ivabradine-PTZ groups. Diazepam and ivabradine, significantly decreased duration of seizure from 6.11±0.75 minutes in PTZ kindling group to 2.13±0.31 and 2.55±0.36 minutes respectively, while there were no significant differences between diazepam-PTZ and ivabradine-PTZ groups. (Table 1)

Table 1: Effect of ivabradine and diazepam on seizure score, latency and duration in PTZ kindling seizure model in mice.

Groups	Saline	PTZ kindling	Diazepam-PTZ	Ivabradine-PTZ
Seizure score	0	5.12±0.66*	1.33±0.21#	3.22± 0.49$
Latency to seizures	0	4.85±0.54*	13.93±2.13#	12.42±1.98#
Duration of seizure	0	6.11±0.75*	2.13±0.31#	2.55±0.36 #

Data represent mean± standard error of mean. *Significant compared to saline group. #Significant compared to saline and PTZ kindling group. $Significant compared to saline, PTZ and diazepam-PTZ groups. PTZ: pentylenetetrazole, SE: standard error.

Effect of diazepam and ivabradine on MDA, GSH and nitrite levels:

PTZ administration caused a significant increase in MDA and nitrite levels from 0.35±0.04 nmol/mg and 144±15 µg/ml in the saline group to 0.98±0.06 nmol/mg and 295± 33 µg/ml respectively, while decreased GSH from 34±0.23 nmol/mg in saline group to 3.57±0.43 nmol/mg. Diazepam significantly decreased MDA and nitrite levels to 0.41±0.05 nmol/mg and 165± 21 µg/ml respectively, while increased GSH level to 7.19±0.73 nmol/mg in relation to PTZ group. Administration of ivabradine significantly decreased MDA and nitrite levels to 0.44±0.03 nmol/mg and 143± 11 µg/ml respectively, while increased glutathione level to 7.22±0.77 nmol/mg in relation to PTZ group, while were non-significantly different in respect to diazepam-PTZ group. (Table 2)

Table 2: Effect of ivabradine and diazepam on MDA, GSH and nitrite levels in PTZ kindling seizure model in mice.

Groups	Saline	PTZ kindling	Diazepam-PTZ kindling	Ivabradine-PTZ kindling
MDA (nmol/mg protein)	0.35±0.04	0.98±0.06*	0.41±0.05#	0.44±0.03#
GSH (nmol/mg protein)	34±0.23	3.57±0.43*	7.19±0.73#	7.22±0.77#
Nitrite (µg/ml)	144±15	295± 33*	165± 21#	143± 11#

Data represent mean± standard error of mean. *Significant compared to saline group. #Significant compared to saline and PTZ kindling group. PTZ: pentylenetetrazole, MDA: malondialdehyde, GSH: reduced glutathione, SE: standard error.

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 (Na^+, Ca^{2+}, and K^+) or ligand-gated ($GABA_A$ and cholinergic nicotinic receptor) channels[23]. HCN channels are unique in that they are dually activated by voltage hyperpolarization and intracellular cAMP. These 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.

The results of the present work revealed that, seizure score and duration are decreased by ivabradine while increased the latency to seizures. While, diazepam decreased seizure score more than ivabradine, the effect of both drugs on latency and duration of seizure were insignificantly different. These results cope with Luszczki et al.[19] 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 therefore, the drug elevated the threshold for electroconvulsions in mice. In addition, Mansour and Ibrahim[24] reported that ivabradine increased latency and decreased duration of seizure in kainite model of epilepsy.

In support of these finding, Bender et al. [25] postulated that the resected hippocampi from patients with temporal-lobe epilepsy, showed enhancement in the levels of HCN1 channel expression with dendritic localization in granule cells of the dentate gyrus. In addition, another work has identified a mutation in the HCN2 gene with augmentation of I_h current in patients with genetic epilepsy and had febrile seizures[26]. However, the results of the present study don't cope with Poolos et al.[27] who reported that lamotrigine, a widely used antiepileptic drug in clinical practice, increased I_h current. In addition, the antiepileptic drug gabapentin was found to increase I_h current in CA1 pyramidal neurons[28]. The reported increase in I_h current with the previous two drugs might be attributed to enhancement of neuronal hyperpolerization due activation of $GABA_A$ receptor. The resulting membrane hyperpolerization is a well documented stimulus for opening HCN channels and I_h 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[29].

Oxidative stress has a major role in most of the epilepsy models. There is a strong correlation between PTZ administration and oxidative stress[30]. PTZ kindling causes alterations in antioxidant defense systems of the brain[31]. The oxidative stress imposed during seizures is an outcome of free radicals produced from the abnormal structural alterations of cellular proteins, membrane lipids, DNA and RNA [32]. These free radicals attack membrane lipids and result in lipid peroxidation[33]. Of all the free radicals that can occur in vitro, the hydroxyl free radicals are considered to be the most reactive and hazardous[34]. One source of hydroxyl radicals is peroxynitrite, which is generated by the spontaneous reaction of superoxide anion and nitric oxide. When nitric oxide synthesis is increased, hydroxyl radical formation is enhanced. This initiates process of lipid peroxidation and the formation of protein adducts resulting in cell damage[35].

In the present study, PTZ kindling induced increases in brain MDA (marker for lipid peroxidation due to free radicals) and nitrite levels (the stable end-product of nitric oxide in the in vitro system) in addition to a decrease in the reduced GSH content of the brain. Administration of ivabradine or diazepam decreased MDA and nitrite levels while increased the glutathione content in the brain. These effects could be related to attenuation of kindling process as kindling per se was reported to involve generation of free radicals[36], as well as due direct enhancement of antioxidant machinery in the brain.

Indeed, previous studies reported that both ivabradine and diazepam have antioxidant effects. In this context, Beytur et al. [37] reported that ivabradine directly ameliorates nitric oxide and lipid peroxidation induced renal inflammatory response in ischemic reperfusion injury in rats by inhibiting oxidative stress. In addition, Colak et al. [38] 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 reactive oxygen species production due to decrement in NADPH oxidase activity as well as prevention of eNOS uncoupling[39]. Consequently, NADPH with glutathione reductase could increase GSH production[40] and increase its content of the brain.

The diazepam-induced antioxidant effects have already been discussed in the literature. Previous reports showed that modulation of $GABA_A$ receptors through different benzodiazepine receptor agonists can reduce the oxidative damage produced by acute immobilization[41] and psychological stress[42].

In conclusion: Ivabradine had anticonvulsant effect, less than diazepam, in PTZ kindled mice and ameliorated the associated oxidative stress.

Further experimental studies are needed to confirm the anticonvulsant effect of the drug in other models of seizure.

References

1. Vieira V, Glassmann D, Marafon P, Pereira P, Gomez R, Coitinho AS Effect of diclofenac sodium on seizures and inflammatory profile induced by kindling seizure model. Epilepsy Res 2016; Nov, 127:107-113
2. Porter RJ, Meldrum B S Antiseizure Drugs. In: Katzung BG, Masters SB, Trevor AJ (Eds), Basic& Clinical Pharmacology. 12th ed., Mc Graw Hill Medical, New York, USA . 2012; PP: 403-426
3. Morimoto K, Fahnestock M, Racine RJ Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 2004; 73(1):1-60
4. Kücker S, Töllner K, Piechotta M, Gernert M Kindling as a model of temporal lobe epilepsy induces bilateral changes in spontaneous striatal activity. Neurobiol Dis 2010; Mar;37(3):661-72
5. Bertram E The relevance of kindling for human epilepsy. Epilepsia. 2007; 48 (2): 65–74
6. Erdoğan F, Kucuk A, Golgeli A The assesment of the features of seizures and EEG in pentylenetetrazol-induced kindling. Journal of Neurological Sciences 2006; 23(2):84-92
7. Loscher W Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure 2011; 20(5):359-68

8. DiFrancesco JC, DiFrancesco D Dysfunctional HCN ion channels in neurological diseases. Front Cell Neurosci 2015; 9:71
9. Baruscotti M, Bottelli G, Milanesi R, DiFrancesco JC, DiFrancesco, D HCN-related channelopathies. Pflugers Arch 2010; 460: 405–415
10. Vilaine JP The discovery of the selective *If* current inhibitor ivabradine. A new therapeutic approach to ischemic heart disease. Pharmacol Res 2006; 53: 424–434
11. Bucchi A, Barbuti A, Difrancesco D, Baruscotti M Funny current and cardiac rhythm: insights from HCN knockout and transgenic mouse models. Front Physiol 2012; 3: 240-251
12. Tardif JC, Ford I, Tendera M, Bourassa MG, Fox K Efficacy of ivabradine, a new selective I(f) inhibitor, compared with atenolol in patients with chronic stable angina. Eur Heart J 2005; 26:2529 –2536
13. Roubille F, Lattuca B, Busseuil D, Leclercq F, Davy JM, Rhéaume E, Tardif JC Is ivabradine suitable to control undesirable tachycardia induced by dobutamine in cardiogenic shock treatment? Med Hypotheses 2013; Aug, 81(2): 202-6
14. Lally J, Brook J, Dixon T, Gaughran F, Shergill S, Melikian N Ivabradine, a novel treatment for clozapine-induced sinus tachycardia: a case series. J Cell Physiol 2014; Jun, 229(6): 813-23
15. Divljakovic J, Milic M, Timic T, Savic M Tolerance liability of diazepam is dependent on the dose used for protracted treatment. Pharmacological Reports 2012; 64: 1116-1125
16. Wahab A Difficulties in treatment and management of epilepsy and challenges in new drug development. Pharmaceuticals 2010; 3: 2090-2110
17. Dhir A, Naidu PS, Kulkarni SK Effect of naproxen, a non-selective cyclo-oxygenase inhibitor, on pentylenetetrazole-induced kindling in mice. Clin Exp Pharmacol Physiol 2005; 32 579–584
18. Syapin PJ Inhibition of pentylenetetrazol induced genetically-determined stereotypic convulsions in tottering mutant mice by diazepam. Pharmacol Biochem Behav 1983; Mar;18(3):389-94
19. Luszczki JJ, Prystupa A, Andres-Mach M, Marzęda E, Florek-Łuszczki M Ivabradine (ahyperpolarization activated cyclic nucleotide-gated channel blocker) elevates the threshold for maximal electroshock-induced tonic seizures in mice. Pharmacol Rep 2013; 65(5): 1407-14
20. Raghvendra V, Agrewal JN, Kulkarni SK Melatonin reversal of lipopolysaccharide-induced thermal and behavioral hyperalgesia in mice. Eur J Pharmacol 2000; 395: 15–21
21. Wills ED (1966) Mechanism of lipid peroxide formation in animal tissues. Biochem. J 99: 667–676
22. Ellman GL Tissue sulfhydryl groups. Arch. Biochem. Biophys 82 70–7723- Thomas RH, Berkovic SF (2014) The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol 1959; 10 283–292
23. Thomas RH, Berkovic SF The hidden genetics of epilepsy-a clinically important new paradigm. Nat Rev Neurol 2014; 10 283–292

24. Mansour ME, Ibrahim AN Possible anticonvulsant effect of ivabradine in kainite-induced epilepsy in rats: Amiloration of oxidative stress. World J of Pharmaceut Res 2015; 4(12); 247-257
25. Bender RA, Soleymani SV, Brewster AL, Nguyen ST, Beck H, Mathern GW, Baram TZ Enhanced expression of a specific hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) in surviving dentate gyrus granule cells of human and experimental epileptic hippocampus. J Neurosci 2003; 23:6826–6836
26. Dibbens LM, Reid CA, Hodgson B, Thomas EA, Phillips AM, Gazina E, Cromer BA, Clarke AL, Baram TZ, Scheffer IE Augmented currents of an HCN2 variant in patients with febrile seizure syndromes. Ann Neurol 2010; 67:542–546
27. Poolos NP, Migliore M, Johnston D Pharmacological upregulation of I_h channels reduces the excitability of pyramidal neuron dendrites. 2002; Nat Neuro sci 5: 767–774
28. Surges R, Freiman TM, Feuerstein TJ Gabapentin increases the hyperpolarization-activated cation current I_h in rat CA1 pyramidal cells. Epilepsia 2003; 44:150–156
29. Accili EA, Proenza C, Baruscotti M, DiFrancesco D From funny current to HCN channels: 20 years of excitation. News Physiol Sci 2002; 17: 32–37
30. Patsoukis N, Zervoudakis G, Panagopoulos NT, Georgiou CD, Angelatou F, Matsokis NA Thiol redox state (TRS) and oxidative stress in the mouse hippocampus after pentylenetetrazole induced epileptic seizure. Neurosci Lett 2004; 357(2):83-6
31. Dhir A Pentylenetetrazole kindling model of epilepsy. Curr Protoc Neurosci 2012; 9:9-37
32. Ilhan A, Iraz M, Kamisli S, Yigitoglu R Pentylenetetrazole-induced kindling seizure attenuated by Ginkgo biloba extract (EGb 761) in mice. Prog Neuropsychopharmacol Biol 2006; 30: 1504–1510
33. Dhir A, Padi SS, Naidu PS, Kulkarni SK Protective effect of naproxen (non-selective COX-inhibitor) or rofecoxib (selective COX-2 inhibitor) on immobilization stress-induced behavioral and biochemical alterations in mice. Eur J Pharmacol 2006; 535: 192–198
34. Haliwell B Reactive oxygen species and the central nervous system. J Neurochem 1992; 59 1609–1623
35. Singh A, Kumar G, Naidu PS, Kulkarni SK Protective effect of FK-506 (tacrolimus) in pentylenetetrazol-induced kindling in mice. Pharmacol. Biochem. Behav 2003; 75;853–860
36. Coyle JT Oxidative stress, glutamate, neurodegeneration disorders. Science 1993; 262: 689–69
37. Beytur A, Binbay M, Sarihan ME, Parlakpinar H, Polat A, Gunaydin MO Dose dependent protective effect of ivabradine against ischemia-reperfusion-induced renal injury in rats. Kidney Blood Press Res 2012; 35(2): 114-9
38. Colak MC, Parlakpinar H, Tasdemir S, Samdanci E, Kose E, Polat A, Sarihan E Therapeutic effects of ivabradine on hemodynamic parameters and cardiotoxicity induced by doxorubicin treatment in rat. Hum Exp Toxicol 2012; Sep 31(9): 945-54
39. Kroller-Schon S, Schulz E, Wenzel P, Kleschyov AL, Hortmann M Differential effects of heart rate reduction with ivabradine in two models of endothelial dysfunction andoxidative stress. Basic Res Cardiol 2011; 106: 1147–1158

40. Winkler BS, De Santis N, Solomon F Multiple NADPH producing pathways control glutathione (GSH) content in retina. Exp Eye Res 1986; 43(5):829–847
41. Kumar A, Goyal R Possible involvement of GABAergic modulation in the protective effect of gabapentin against immobilization stress induced behavior alterations and oxidative damage in mice. Fundam Clin Pharmacol 2007; 21:575–81
42. Singh A, Kumar A Protective effect of alprazolam against sleep deprivation-induced behavior alterations and oxidative damage in mice. Neurosci Res 2008; 60:372–9