

Article

Effect of Fingolimod on GABA, TNF α , SOD and glutathione peroxidase (Gsh px) level in PTZ induced generalized tonic clonic epilepsy in rats

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Abstract

Background: Epilepsy is one of the commonest serious brain disorders, with the highest risk in infants and older age groups. It is has multiple risk factors and a strong genetic predisposition. **Objectives:** Fingolimod (sphingosine-1-phosphate (S1P) analog) was tested for antiepileptogenic effects in rat model of pentenyle tetrazole (PTZ) induced epilepsy.

Methods: twenty four male Wistar albino rats were randomly divided into three groups; control (group A), epileptic non-treated (group B1) in which epilepsy was induced by sub-convulsive doses of PTZ and fingolimod-treated group (group B2) which received 1 mg/kg fingolimod orally once daily for three consecutive months. **Results:** spontaneous convulsions (SCs) of epileptic rats as regards the frequency, duration and severity have been reduced while GABA, SOD and glutathione peroxidase levels were elevated at the same time tumor necrosis factor alpha (TNF α) was reduced in fingolimod treated group. In addition a reduction of neuronal loss and preservation of the arrangement of pyramidal cells in the hippocampus was also observed in this group.

Conclusion: fingolimod administration demonstrated an antiepileptic effect through significant improvement of the chemical as well as the histological parameters.

Keywords: epilepsy, neuroinflammation, fingolimod, TNF α , GABA SOD, Gsh px

Introduction

Epilepsy is a severe chronic brain disease in which communication between neurons is altered. The main characteristic of epilepsy is the ability of the brain to generate seizures, which are paroxysmal clinical manifestations that indicate abnormal hypersynchronous discharges from the neurons.⁽¹⁾ Epilepsy can induce profound cognitive or memory problems⁽²⁾ and may be associated with bipolar disorder and psychosis⁽³⁾, in addition to depression and anxiety.⁽⁴⁾ The mechanism of seizures can be summarized as an increase in extracellular glutamate level which leads to excessive neuronal excitation, resulting in a rise of the intracellular calcium, which leads to generation of reactive oxygen species (ROS) and disturbance in the antioxidant system and finally neuronal injury and cell death.⁽⁵⁾ Epileptic people constantly have a low seizure threshold,⁽⁶⁾ which can be symptomatic or idiopathic.⁽⁷⁾ Symptomatic seizures indicate a clear-cut abnormality in the brain and about 30 % of the patients with epilepsy fall in this category. The remaining 70% of patients appears to be completely normal resulting in idiopathic epilepsy which is believed to have a genetic origin i.e.: mutations in one or several genes involved in brain excitation and / or inhibition.⁽⁸⁾ Unfortunately, many patients suffer from the side effect of the traditional AEDs, so efforts are being conducted to improve these patients' life quality through using the new well-tolerated AEDs that carry less side effects and less drug interaction (oxcarbazepine, lamotrigine, or Levetiracetam).⁽⁹⁾

Fingolimod is recently approved medication for treatment of relapsing remitting multiple sclerosis. It is the substrate of sphingosine kinases and after being phosphorylated it binds to G protein-coupled receptors named sphingosine-1 phosphate (S1P) receptors.⁽¹⁰⁾ (S1PRs) S1P 1-5.⁽¹¹⁾

Materials and methods

2.1. Experimental animals

Twenty four male Wistar albino rats were used of body weight ranging from 15 to 20 grams aged 15 days postnatal at the start of the study. Animals were kept under standard conditions of light and temperature, with free access to food and water. All experimental procedures were approved and performed in compliance with the guidelines of the Local Ethics Committee of Alexandria, Faculty of Medicine University of Alexandria.

2.2. Establishment of generalized tonic clonic (chronic epilepsy) model

Epilepsy was induced according to the following PTZ kindling protocol: regular intraperitoneal administration of PTZ at sub-convulsive doses (30 mg/kg i.p., 3 times a week, up to 10 weeks).⁽¹²⁾ Rats were observed for 30 minutes after each PTZ injections for assessment of seizures severity and scoring was as follows:

- Phase 0: No response
- Phase 1: Ear and facial twitching
- Phase 2: myoclonic body jerks
- Phase 3: clonic forelimb convulsions

- Phase 4: generalized clonic convulsions, turning onto one side position
- Phase 5: generalized clonic-tonic convulsions (or death within 30 minutes).⁽¹³⁾
- Phase 6: Mortality.⁽¹⁴⁾

The kindling status is reached when seizures stages 4 or higher occurred on two consecutive days.⁽¹⁵⁾

2.3. Experimental design

Rats were randomly divided into 3 groups, each group of 8 rats as follows:

- **Group A: (normal control):** rats in this group received 1 ml saline intraperitoneally (IP).
- **Group B: (epileptic):**

Group B 1: Epileptic non-treated.

Group B 2: Epileptic treated with Fingolimod:

Which was dissolved in 0.9% saline for a final concentration of 1 mg/ml. A dose of 1 mg/kg was administered orally through an orogastric lavage.⁽¹⁶⁾ Rats started treatment immediately after being fully kindled and continued for three months on daily basis. Rats were video monitored for 24h/day for the occurrence of any spontaneous convulsions.

2.4. Tissue preparation ⁽¹⁷⁾

At the end of the three months rats were scarified under ether anesthesia and the brain was rapidly removed and dissected to extract the hippocampus. Which was weighed and rinsed with 1ml phosphate-buffered saline (PBS), homogenized by Glascole homogenizer in 1 mL PBS and stored at -20° C. In order to breakdown the cell membrane: two freeze-thaw cycles were performed and the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernatant was separated by Rotofix 32A centrifuge of Hettitch at 4°C and 16 000 G rpm.

2.5. Measurement of GABA, TNF α , SOD and glutathione peroxidase (Gsh px) level:

Commercially available ELISA kits for GABA, TNF α , SOD and glutathione peroxidase (Gsh px) were used according to the manufacturer's instructions. (BioVision Inc., California, USA).

2.6. Histological examination

After processing into paraffin, RATs brain sections were sliced into transverse sections and were stained with toluidine blue dye and examined under light microscope for describing the histological changes in the control and epileptic brain.

3. Statistical analysis

Data was fed to the computer and analysed using IBM Statistical Package for the Social sciences (SPSS) software package version 20.0. The Shapiro –Wilk test was used to assess the normality of the parameters. All data were represented using range (minimum and maximum), mean and standard deviation (SD). One-way Analysis of Variance (ANOVA) followed by Tukey as a post-hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

4. A: Biochemical

Administration of fingolimod for three months duration exhibited a significant elevation in the level of GABA, SOD and Gsh px while there was reduction in TNF α level as shown in **figure (1)**.

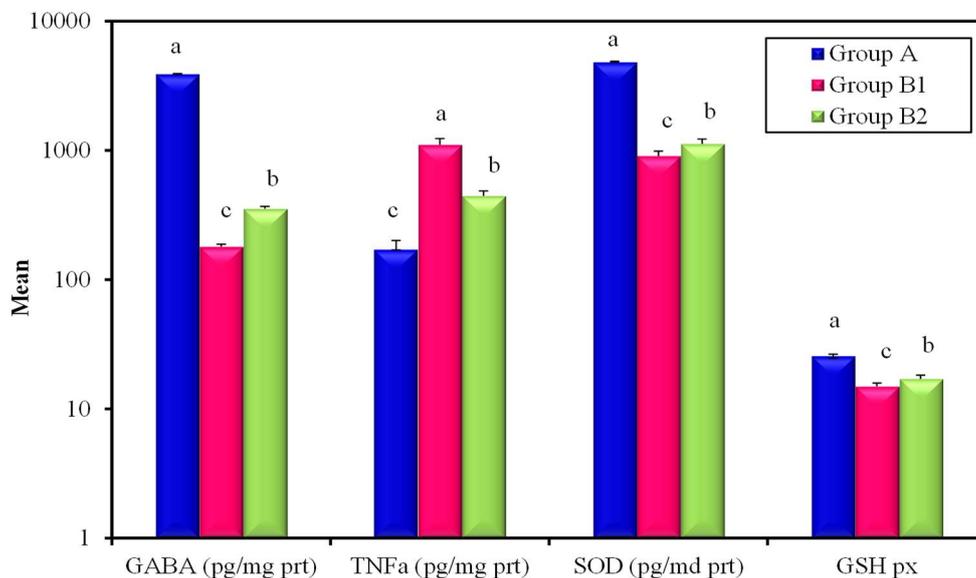


Figure (1): Effect of the Fingolimod on GABA, SOD, GSH px and TNF α level.

Table (I). Comparison between the three studied groups according to different parameters

	Group A	Group B1	Group B2	F	P
GABA (pg/mg prt)					
Min.	3843.0	170.0	329.0		
Max.	3945.0	194.0	374.4		
Mean	3890.9 ^a	179.8 ^c	353.1 ^b	70928.9*	<0.001*
±SD.	34.33	7.78	15.66		
TNFa (pg/mg prt)					
Min.	120.0	889.0	389.0		
Max.	210.0	1250.0	501.0		
Mean	170.9 ^c	1104.9 ^a	443.5 ^b	276.646*	<0.001*
±SD.	30.40	131.61	42.04		
SOD (pg/md prt)					
Min.	4716.7	758.0	943.0		
Max.	4920.0	1021.0	1219.0		
Mean	4804.6 ^a	903.3 ^c	1121.9 ^b	5044.01*	<0.001*
±SD.	80.52	80.93	99.15		
GSH px					
Min.	24.63	13.52	15.32		
Max.	26.67	16.30	18.79		
Mean	25.67 ^a	14.93 ^c	17.07 ^b	267.938*	<0.001*
±SD.	0.78	0.91	1.20		

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the studied groups

*: Statistically significant at $p \leq 0.05$

Means with Common letters are not significant (i.e. Means with Different letters are significant)

Group A: normal control, Group B1: epileptic non- treated, Group B2: Epileptic treated

4.B: Histological results

Fingolimod exerted considerable therapeutic effect that was reflected by reorganization of hippocampal amnios layers, preservation of classical neural structure of most pyramidal cells and reappearance of densely stained Nissl granules. **Figure (2)**

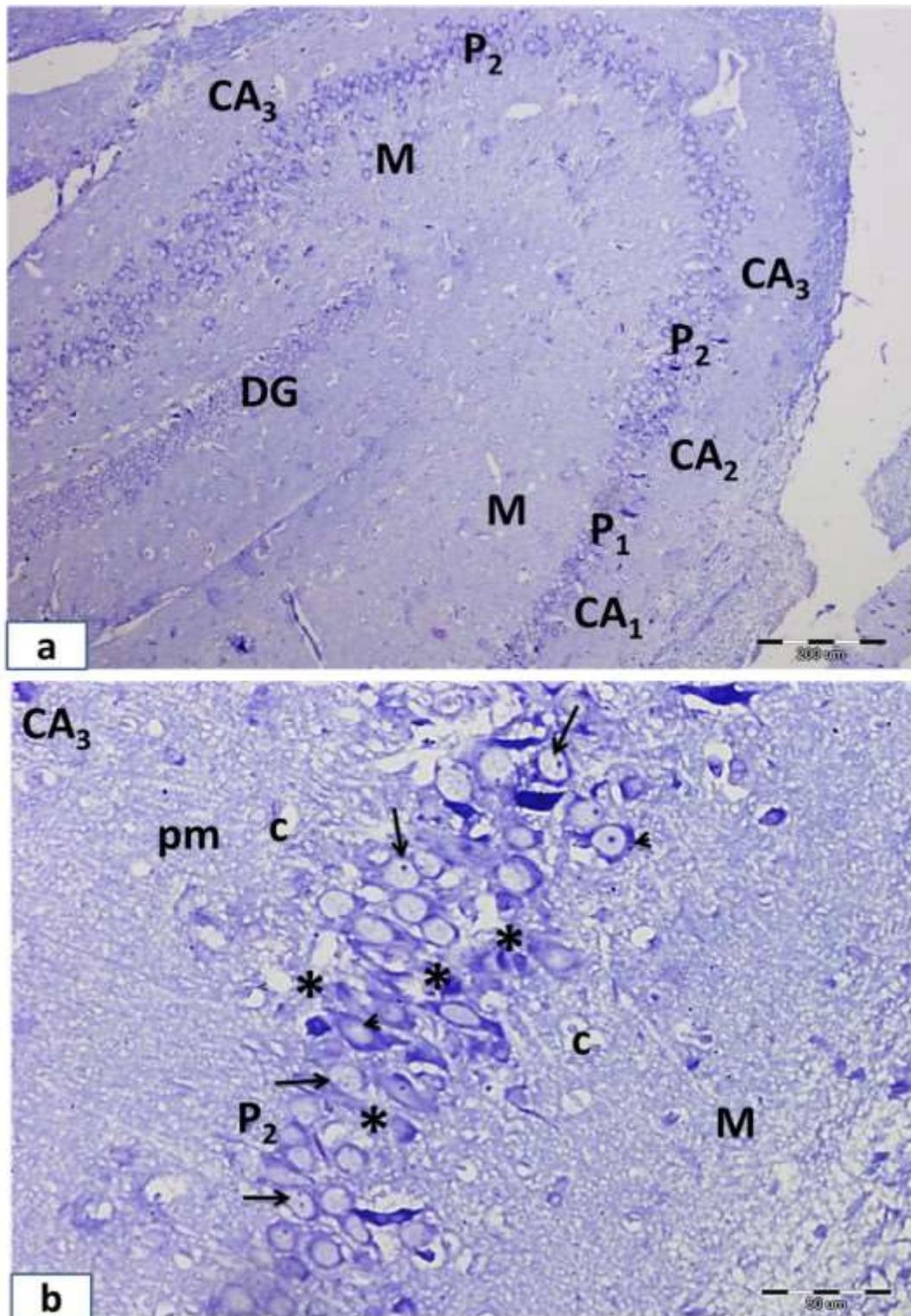


Fig.2: Photomicrographs of Fingolimod treated epileptic rats' brain (GB2III) revealing coronal section of hippocampus proper (CA) and dentate gyrus (DG) . The hippocampus proper is composed of an outer pleomorphic layer (pm), middle pyramidal (p) and inner molecular layers(M). The pyramidal layer show disorganized arrangement especially in CA₃ zone. Most large pyramidal cells appear vacuolated, pale stained with diminished Nissl granules (↑).Others depict classical structure (Δ). Few apoptotic cells (*) are also seen. Dilated capillaries (c) are occasionally depicted in outer and inner layers.Toluidine blue stain, MicMag a *100 b*400

Discussion

In this study fingolimod was administered at daily dose of 1 mg/kg for three successive months and this result in an antiepileptic effect which may be attributed to in part by decreased level of TNF α an important inflammatory mediator. This finding is supported by a study carried out by Fei Gao et al at 2012, in which the antiepileptic effect of fingolimod was tested in an adult rat model of lithium-pilocarpine induced epilepsy fingolimod was administered at dose of 1 mg /kg for 14 successive days and it was found that there were dramatic suppression of glial activation, and reduced TNF α level.⁽¹⁶⁾ During inflammation, there is enhanced production of cytokines by the endothelial lining of the BBB, circulating immune cells and brain microglia and astrocytes.⁽¹⁸⁾ Inflammatory mediators, includes interleukin (IL)-6 and IL-1b, transforming growth factor (TGF), and tumor necrosis factor alpha (TNF α)⁽¹⁹⁾ play a critical role in the occurrence of seizures,⁽²⁰⁾ by inducing disruption in BBB permeability , also TNF α induces AMPA dependent excitatory mechanism and reduces GABA-A inhibitory effect while IL1b enhances the Ca⁺² influx through phosphorylation of AMPA receptors.⁽²¹⁾

Fingolimod can suppress the inflammatory reaction by different ways : preventing the migration of lymphocytes into the CNS⁽²²⁾ reducing the release of different inflammatory cytokines by activated astrocytes and microglial cells which express S1P receptors leading to decreased neuronal hyperexcitability and attenuating spontaneous convulsions (SCs) in the chronic epilepsy state.⁽²³⁾ In addition to that Fingolimod was found to increase GABA level which is the main inhibitory neurotransmitter. This finding is supported by a study that was done at 2019 to assess the effect of fingolimode on neurochemicals of the brain in Alzheimer's disease and it was concluded that administration of fingolimod at 0.03 mg/kg /day enhances neuronal GABA level⁽²⁴⁾. Reduced levels of extracellular GABA are found in the white matter of patients with neurodegenerative diseases⁽¹⁸⁾ this reduction in GABA level is mainly attributed to the effects of secreted interleukin (IL) 1b from infiltrating T lymphocytes.⁽¹⁹⁾ And as fingolimod inhibit T lymphocyte infiltration to the CNS it end by increased in GABA level .This inhibitory mediator is formed by transamination of α -ketoglutarate to glutamic acid, which is then decarboxylated by glutamic acid decarboxylase (GAD) to GABA.⁽²⁵⁾

It acts on both types of GABA receptors first GABA (A) receptor which are ligand-gated ion channels that produce hyperpolarization in the neuron by increasing inflow of chloride conductance ending by rapid inhibitory effect. And GABA (B) receptors which are G protein-coupled receptors that increases the potassium conductance through the neurons leading to hyperpolarization, it is also responsible for decreasing the calcium entry leading to slow inhibitory effect.⁽²⁶⁾ So increased level of GABA end by hyperpolarization and stabilization of excited neurons and this may explain in part the antiepileptic effect of fingolimod. In the current work and after three months duration of treatment with fingolimod at dose of 1mg /kg daily, an elevation in both SOD and glutathione peroxidase levels was significantly found. These findings are in accordance with study that was carried out by Hongmei Wu et al in 2017 who concluded that treatment with fingolimod resulted in an obvious reduction in malondialdehyde (MDA) levels and increase in SOD and GSH-Px activity.⁽²⁷⁾ On the other hand previous studies on SOD activity have been conducted by Bożena Adamczyk et al in 2018 on patients with remitting relapsing multiple sclerosis reported that patients treated with fingolimod at dose of (0.5 mg/daily) showed lower SOD activity when compared to the control group.⁽²⁸⁾ This

discrepancy between the two results may be due to lower dose used in their study and different disease pattern between epilepsy and multiple sclerosis.

Oxidative stress plays a major role in epilepsy as prolonged excitation of neurons can lead to generation of chemical by-product e.g.: malondyaldehyde (MAD) & nitric oxide (NO) that results in damage to neuronal cells and induction of cell death ⁽²⁹⁾, through protein nitrosylation and mitochondrial damage.⁽²⁰⁾ Fingolimod restored nitrosylation to almost normal levels, due to its ability to inhibit the inducible NO ⁽³⁰⁾ an effects mediated by the impact of fingolimod on SIP receptors in addition to non-receptor mechanisms which include an increase in expression of Nrf2 and its translocation to the nucleus ,beside that there is also increased production of heme oxygenase 1 (OH1), and thioredoxin, with these factors, especially Nrf2 and OH1, being important to increasing antioxidant enzymes such as GPX.⁽³¹⁾ Finally it could be concluded that fingolimod demonstrates considerable antiepileptic effect and this was approved chemically and histologically.

The limitation of this study is its short duration.

Conflict of interest: None

Funding: None

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