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Magnesium supplementation enhances the anticonvulsant potential of valproate in pentylenetetrazol-treated rats

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptor antagonists appear to enhance the anticonvulsant activity of antiepileptic drugs in several models of epilepsy. Therefore, the current study evaluates the modulatory effect of magnesium (Mg^{2+}), a non-competitive NMDA receptor antagonist, on a subprotective dose of valproate (VPA) against pentylenetetrazol (PTZ)-induced convulsions. Male Wister rats received either saline or PTZ (60 mg/kg, i.p.). The other three groups were pretreated with Mg^{2+} (40 mg/kg, p.o., 4 weeks), single subprotective dose of VPA (100 mg/kg, i.p.), or Mg^{2+} with VPA, before PTZ injection. PTZ provoked clonic convulsions, reduced GABA content, deranged brain redox status, and elevated nitric oxide (NO). Neither the subprotective dose of VPA nor Mg^{2+} alone guarded against clonic seizures invoked by PTZ, an effect that was achieved only by their combination and supported by a significant delay in seizure latency. Moreover, VPA leveled off glycine and aspartate, exerted no effect on glutamate, and unexpectedly reduced GABA and taurine levels. Mg^{2+} alone or in combination showed the same pattern on the aforementioned amino acids, except for taurine. All regimens restored glutathione (GSH) and total antioxidant capacity (TAC); however, only VPA normalized NO level. This study demonstrates that Mg^{2+} could enhance the antiepileptic efficacy of a subprotective dose of VPA, possibly by improving redox balance and modulation of some brain amino acids.

1. Introduction

Epilepsy continues to be a neurological disorder awaiting safer drugs with improved antiepileptic effectiveness. Despite progress in current antiepileptic therapy, neither are seizures adequately controlled nor medications free of untoward side effects (Bashkatova et al., 2003). Moreover, combinations of conventional antiepileptic drugs (AEDs) might fail to effectively control seizures (Kaminski et al., 2001). Actually, about 30% of epileptic patients do not respond to clinically established AEDs (Theodore and Fisher, 2007).

N-methyl-D-aspartate (NMDA) receptor antagonists were shown to possess anticonvulsant properties against several insults including PTZ-induced seizures and to enhance the

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Abbreviations: AED, antiepileptic drug; GABA, γ-aminobutyric acid; GSH, glutathione; Mg^{2+}, magnesium; MDA, malondialdehyde; NMDA, N-methyl-D-aspartate; NO, nitric oxide; PTZ, pentylenetetrazol; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; VPA, valproate

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effects of AEDs (Czechowska et al., 1993; Borowicz et al., 1996; Bikjdaouene et al., 2004; Feng et al., 2005). Magnesium (Mg^{2+}) is a non-competitive NMDA receptor antagonist, which modulates neurotransmission by blocking voltage-dependent NMDA receptor (Gathwala, 2001), hence antagonizing Ca^{2+} actions pre- and postsynaptically (Czeh and Somjen, 1989). In vitro, reduction of extracellular Mg^{2+} lowers the threshold level of excitatory amino acids necessary to activate NMDA receptor and induces spontaneous epileptiform activity in cortical neurons (Cao et al., 2003). Pronounced hypomagnesemia is documented in epileptic patients and those on certain AEDs (Sood et al., 1993; Dharnidharka and Carney, 2005) .

Several studies focus on the role of oxidative stress both as a consequence and a cause of epileptic seizures (Patsoukis et al., 2004a; Ilhan et al., 2006), a fact that is supported by the efficacy of antioxidants in the management of convulsive disorders (Sudha et al., 2001; Ilhan et al., 2005). In neuronal cell cultures, Mg^{2+} depletion increases oxidative cell death (Altura et al., 2003), while its elevation was shown to confer cytoprotection (Regan et al., 1998); similarly, in vivo dietary Mg^{2+} deficiency was documented to enhance oxidative stress (Bussiere et al., 2002b; Kuzniar et al., 2003). Indeed, magnesium's antioxidant potential is widely reported (Ariza et al., 2005; Turkoglu et al., 2008), thereby it could protect against free radicals surge linked with epileptic convulsions. Valproate (VPA) is one of the conventional AEDs that possess a broad spectrum of antiepileptic activity (Loscher, 1993). In an in vitro study by Fueta et al. (1995), Mg^{2+} was documented to improve VPA efficacy against 4-aminopyridine-induced ictal activity. However, the current study is the first to evaluate this finding in vivo, using an acute PTZ model in rats. For this purpose, seizure incidence, latency, as well as brain amino acid pattern, nitric oxide (NO), and oxidative stress biomarkers were targeted.

2. Results

Table 1 depicts that rats subjected to PTZ exhibited clonic convulsions with 2-min average seizure latency. Subeffective dose of VPA showed subtle non-significant tendency to protect against clonic convulsions. Though daily administration of Mg^{2+} for 4 weeks elevated serum Mg^{2+} level by 18% as compared to control group (data not shown), it failed to guard against PTZ-induced convulsions. On the other hand, in rats receiving both Mg^{2+} and VPA, the anticonvulsant potential of VPA was enhanced by the presence of the mineral. Those animals showed decreased convulsions' incidence and prolonged seizure latency, effects that were significant from the antiepileptic drug alone. Compared to control group, PTZ reduced only brain GABA content (21%), which was further decreased by Mg^{2+} (75%), VPA or their combination (62%) (Table 2). Taurine was inhibited only in the VPA-treated group by 25% and was reverted by Mg^{2+} pre-supplementation. Regarding the excitatory (aspartate) and co-excitatory (glycine) amino acids, all treatments reduced their levels significantly as compared to control and PTZ-treated rats, while the lowering effect of VPA on aspartate was augmented by the addition of Mg^{2+}. However, asparagine was altered only in VPA- and VPA/Mg^{2+}-treated groups (Table 2). PTZ induced a post-ictal increment of brain NO (61%; Fig. 1) and TAC (51%; Fig. 2) accompanied with depletion of GSH (31%; Fig. 3) content. The antioxidant parameters, however, were reinstated in all treated groups, while NO was reduced only by VPA. However, the level of lipid peroxides was not affected in all groups (data not shown).

Table 1 – Effect of acute administration of valproate (VPA; 100 mg/kg, i.p.) and/or magnesium aspartate (Mg^{2+}; 40 mg/kg, p.o. 4 weeks) supplementation on PTZ-induced clonic seizures in rats.

Groups	Clonic Seizures Incidence	Clonic Seizure Latency
	(%)	(min)
PTZ	100	2.01 ± 0.20
VPA	73	10.30 ± 3.83
Mg^{2+}	100	1.71 ± 0.08
VPA + Mg^{2+}	33*	22.03 ± 5.11*,**

Clonic seizure incidence is presented as percentage of 6–10 experiments; *$p<0.05$ compared to PTZ group using Fisher's Exact Test. Data for clonic seizure latency are expressed as means of 6–10 experiments ± SEM; *,**$p<0.05$ compared to PTZ and VPA-treated groups, respectively, using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test.

3. Discussion

In the current study, administration of Mg^{2+} supplement to the subeffective dose of VPA enhanced the anticonvulsant potential of the AED, though neither agent alone guarded against PTZ-induced clonic convulsions. These results extend and support the in vitro study of Fueta et al. (1995), who proposed that GABA-mediated potential plays a part in the potentiation of valproate's efficacy by Mg^{2+}. However, the results of the present work revealed a decline in brain GABA by VPA and/or Mg^{2+} treatments, as well as by PTZ pointing to a negligible role of GABA in seizure formation and protection. The correlation between PTZ and the inhibitory neurotransmitter is controversial, where PTZ administration either increased (Kondziella et al., 2002) or did not alter GABA levels (McCandless and Schwartzenburg, 1982). Moreover, Loscher (1981) stated that drugs that increase GABA did not necessarily protect against PTZ convulsions. Although PTZ did not alter taurine, yet VPA reduced it, an effect that was abolished by Mg^{2+} supplementation.

Additionally, the excitatory amino acid aspartate, and its precursor asparagine, along with the co-excitatory amino acid glycine were all decreased by VPA and/or Mg^{2+}. Notably, the further decrease in aspartate, among the interactions documented in Mg^{2+} supplemented/VPA rats, may account for its boosting anticonvulsive effect. Previously, Bikjdaouene et al. (2004) reported that other NMDA receptor antagonists mitigated the effects of PTZ on glycine. However, neither PTZ nor treatment regimens altered the other amino acids (glutamine, histidine, and serine) measured, suggesting thereby their negative influence on seizure induction or protection.

Albeit PTZ was reported previously (Rothman and Olney, 1995) to enhance the glutaminergic system, the present study showed no effect on glutamate level by PTZ treatment.

Table 2 – Effect of acute administration of valproate (VPA; 100 mg/kg, i.p.) and/or magnesium aspartate (Mg^{2+}; 40 mg/kg, p.o. 4 weeks) supplementation on brain amino acids in PTZ-treated rats.

Groups	Brain Amino Acid Contents (μmol/g Tissue)								
	GABA	Taurine	Histidine	Glycine	Glutamate	Glutamine	Aspartate	Asparagine	Serine
Control	3.65±0.23	3.93±0.12	0.24±0.008	2.25±0.09	7.02±0.06	1.62±0.11	5.71±0.14	1.70±0.10	1.32±0.05
PTZ	2.88±0.24*	4.27±0.32	0.26±0.039	2.03±0.21	7.76±0.06	1.72±0.22	6.18±0.51	1.43±0.18	1.58±0.11
VPA	1.38±0.10*,**	2.96±0.14*,**	0.21±0.022	1.25±0.16*,**	6.57±0.97	1.77±0.15	3.56±0.11*,**	1.07±0.09*	1.63±0.19
Mg^{2+}	0.92±0.01*,**	4.13±0.08	0.18±0.004	1.31±0.05*,**	7.10±0.09	1.74±0.03	2.21±0.02*,**	1.40±0.09	1.67±0.01
VPA + Mg^{2+}	1.37±0.06*,**	4.01±0.48***	0.27±0.002	1.25±0.19*,**	6.06±0.99	1.54±0.17	2.45±0.01*,**,***	1.08±0.13*	1.46±0.18

Data are expressed as means of 5–8 experiments ±SEM; *,**,***$p<0.05$ compared to control, PTZ, and VPA-treated groups, respectively, using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test.

However, its role in neuronal excitation could not be ruled out as a slight extracellular glutamate increase might be associated with reduced intracellular level leading to an overall constant one (Li et al., 2000). Overactivation of glutaminergic system was documented to provoke excessive Ca^{2+} influx generating reactive oxygen and nitrogen species (Rowbotham et al., 1998), which both jeopardize neuronal cell survival (Bloom, 2004).

Impaired brain redox status was shown herein by the post-ictal elevation of TAC and NO, as well as the decline of GSH; however, no change in lipid peroxidation was observed. The effect of PTZ on NO is consistent with other studies (Bashkatova et al., 2003; Bikjdaouene et al., 2003; Ilhan et al., 2005) and may be attributed to the PTZ-induced Ca^{2+} influx, thus activating Ca^{2+}/calmodulin-dependent neuronal nitric oxide synthase (nNOS) (Bikjdaouene et al., 2003). Depletion of GSH by PTZ supports previous work in striatum, hippocampus, and cerebral cortex (Patsoukis et al., 2004a,b, 2005) that might be attributed to cysteine deprivation caused by glutamate binding to the disulfide cystine transporter (Bannai and Kitamura, 1980). Moreover, high levels of NO, documented in the present work, could react with sulfyhydral groups, secondary to peroxynitrite formation (Lipton et al., 1993). This inverse correlation between GSH and PTZ was highlighted by Abe et al. (1999), who showed that PTZ convulsions were inhibited by the exogenous administration of this thiol.

Although the unchanged level of lipid peroxides was unpredictable, yet it goes in line with previous studies (Patsoukis et al., 2004a, 2005), where these authors concluded a time-dependent pattern for lipid peroxides formation in mouse cerebral cortex and striatum after PTZ administration, with an increment 15 min after PTZ, followed by normalization after 24 h. On the contrary, other studies declared enhanced generation of lipid peroxides in cerebral cortex (Bashkatova et al., 2003; Ilhan et al., 2004), hippocampus (Patsoukis et al., 2004b), and whole brain (Ilhan et al., 2005) after acute PTZ-induced seizures and in plasma of epileptic patients (Sudha et al., 2001). These discrepancies supposed different magnitude of oxidative stress involved in seizure formation or differences in residual antioxidant capacity of brain tissues.

Valproate and/or Mg^{2+} restored TAC and GSH reflecting, thus, their antioxidant potentials; however, VPA only lowered NO

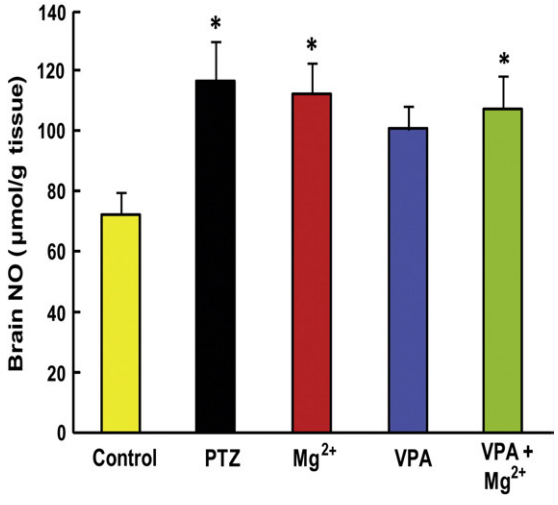

Fig. 1 – Effect of acute administration of valproate (VPA; 100 mg/kg, i.p.) alone and with magnesium aspartate (Mg^{2+}; 40 mg/kg, p.o., 4 weeks) supplementation on brain nitric oxide (NO) in PTZ-treated rats. Data represent the means of 6–10 experiments ±SEM; *$p<0.05$ compared to control group using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test.

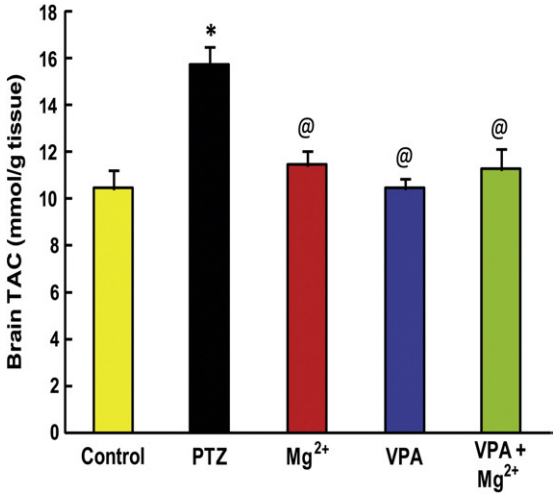

Fig. 2 – Effect of acute administration of valproate (VPA; 100 mg/kg, i.p.) alone and with magnesium aspartate (Mg^{2+}; 40 mg/kg, p.o., 4 weeks) supplementation on total antioxidant capacity (TAC) in PTZ-treated rats. Data represent the mean of 6–10 experiments ±SEM; *,@$p<0.05$ compared to control and PTZ groups, respectively, using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test.

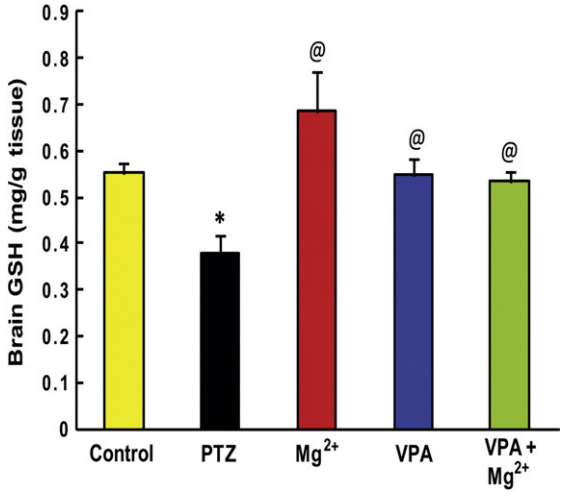

Fig. 3 – Effect of acute administration of valproate (VPA; 100 mg/kg, i.p.) alone and with magnesium aspartate (Mg^{2+}; 40 mg/kg, p.o., 4 weeks) supplementation on glutathione (GSH) in PTZ-treated rats. Data represent the mean of 6–10 experiments ±SEM; $^{*,@}p<0.05$ compared to control and PTZ groups, respectively, using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test.

level. Antioxidant activity of VPA is supported by Hamed et al. (2007), who showed a reduction in blood TAC in chronically treated epileptic patients. In agreement with our study, Cui et al. (2007) reported an increase in GSH level in cultured rat cerebral cortical cells after chronic VPA treatment, thereby conferring that GSH plays an important role in the neuroprotective effects of the drug. In a picrotoxin seizure model, anticonvulsive effect of some conventional AEDs is attributed partly to the inhibition nNOS activity (Rajasekaran et al., 2003; Rajasekaran, 2005), findings that could verify VPA reduction in NO in this study.

The current investigation pointed to the antioxidant capacity of Mg^{2+} that could be relevant to its NMDA receptors blocking ability with the subsequent decline in Ca^{2+} influx, stabilizing nerve cell membrane (Lang-Lazdunski et al., 2000; Ustun et al., 2001), and inhibiting NADPH oxidase (Bussiere et al., 2002a), which lowers superoxide radical formation (Afanas'ev et al., 1995). Magnesium could also enhance GSH through its pivotal role in its synthesis, where both glutamyl cysteine and glutathione synthetases are Mg^{2+}-dependent enzymes (Minnich et al., 1971; Hans et al., 2003). In fact, hypomagnesemia was found to deplete GSH, which was reversed by Mg^{2+} supplementation (Mills et al., 1986; Kuzniar et al., 2003).

In conclusion, the anticonvulsant effect of the subprotective dose of VPA was enhanced by Mg^{2+} supplementation, an effect that may be related to the modulation of amino acids and the redox status.

4. Experimental procedures

4.1. Animals

Adult male Wistar rats (180±20 g), obtained from El Nile Pharmaceutical Company (Cairo, Egypt), were allowed 1 week acclimatization in the animal house at the Faculty of Pharmacy, Cairo University (Cairo, Egypt), before carrying out any experimentation. Rats were housed in groups at constant temperature (23±2 °C), humidity (60±10%), and a light/dark (12 h/12 h) cycle with lights on at 5:00 am. They were allowed free access to food and water throughout the experimental period. The study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy Cairo University (Cairo, Egypt). Seizure induction was done between 9:00 am and 12:00 pm to minimize circadian influences on seizure susceptibility.

4.2. Treatments

Rats were allocated into 5 groups, group I received saline and served as control. Group II received single intraperitoneal PTZ (Sigma-Aldrich, MO, USA) injection in convulsive dose of 60 mg/kg (Uma Devi et al., 2006). Group III was injected intraperitoneally with sodium valproate (VPA; Sanofi-aventis, Paris, France) in an equivalent dose of 100 mg/kg valproic acid 30 min before PTZ administration (Ilhan et al., 2006). Groups IV and V were supplemented orally with magnesium aspartate (MP, Ohio, USA) in an equivalent dose of 40 mg/kg day Mg^{2+} for 4 weeks (Djukic-Cosic et al., 2006). Twenty-four hours after the last dose of Mg^{2+} supplementation, group IV was intraperitoneally injected with PTZ, while group V received PTZ 30 min following VPA. PTZ and all treatments were dissolved in 0.9% saline.

4.3. Seizures assessment and tissue sampling

After PTZ injection, rats were placed singly in Plexiglas cages and were observed for 30 min. Incidences and latency of clonic convulsive attacks, which lasts over 3 s with an accompanying loss of righting reflex were recorded. Seizure latency for rats showing no convulsive attacks within the observation period was taken as 30 min (Uma Devi et al., 2006).

Rats were euthanized by decapitation after seizures assessment; brains were removed and dissected bilaterally. One brain half was homogenized in 75% (v/v) aqueous methanol (HPLC grade, Sigma-Aldrich, MO, USA), homogenates were centrifuged at 4000×*g*, 20 min, 4 °C, and supernatants were dried under vacuum. The residues were employed for the determination of brain amino acids contents. The second brain half was homogenized in ice-cold saline and was used for the estimation of brain redox status and NO. Besides, blood samples were collected for serum Mg^{2+} estimation.

4.4. Determination of serum magnesium

Serum Mg^{2+} level was assessed according to the method described by Chauhan and Sarkar (1969) using a test reagent kit (Biodiagnostic, Giza, Egypt). In brief, to serum samples, EGTA was added to complex interfering Ca^{2+} prior to the incubation with metallochrome dye calmagite to form a chromophore that was measured at 520 nm.

4.5. Determination of brain amino acids

Brain amino acids (μmol/g tissue), viz GABA, glycine, taurine, histidine, glutamate, glutamine, aspartate, asparagine, and serine contents were estimated using a fully automated high-pressure liquid chromatography system (HPLC; Perkin- Elmer, MA, USA) according to the precolumn phenylisothiocyanate derivatization technique described by Heinrikson and Meredith (1984). Brain residues were reconstituted in 2:2:1 mixture (v) of methanol:1 M sodium acetate trihydrate:triethylamine then re-dried under vacuum. The reaction of derivatization was performed for 20 min at room temperature using a 7:1:1:1 mixture (v) of methanol:triethylamine:double-distilled deionized water:phenylisothiocyanate, then subjected again to vacuum until dryness. Derivatized amino acids were reconstituted with sample diluent consisting of 5:95 mixture (v) of acetonitrile:5 mM phosphate buffer (pH=7.2). After sonication, samples were filtered (0,45 μm; Millipore). A Pico-Tag physiological free amino acid analysis C18 (300 mm×3.9 mm i.d.) column from Waters (MA, USA) and a binary gradient of Eluents 1 and 2 (Waters) were used. The column temperature was set at 46± 1 °C and a constant flow rate of 1 ml/min was maintained throughout the experiment. Samples were injected in volumes of 20 μl, and the absorbance of the derivatized amino acids was measured at 254 nm. Amino acid standards were prepared in double-distilled deionized water, and GABA standards were prepared in polyethylene vials to prevent adhesion to glass.

4.6. Determination of nitric oxide

Nitric oxide content (nmol/g tissue) was quantified indirectly as nitrite/nitrate concentration using Griess reaction-dependent method (Miranda et al., 2001). Brain homogenates were deproteinated with absolute ethanol for 48 h at 4 °C and then centrifuged at 12,000×*g* for 15 min at 4 °C. To an aliquot of the supernatant vanadium trichloride 0.8% (w/v) in 1 M HCl was added for the reduction of nitrate to nitrite, followed by the rapid addition Griess reagent consisting of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride and 2% (w/v) sulfanilamide in 5% (v) HCl and incubated for 30 min at 37 °C. Mixtures were cooled, and the absorbance at 540 nm was measured.

4.7. Determination of total antioxidant capacity

Alterations in redox system were assessed by measuring total serum TAC using commercial kit supplied by Biodiagnostic Co. (Giza, Egypt) based on the method described by Koracevic et al. (2001), where antioxidants present in the sample eliminate hydrogen peroxide (H_2O_2), and its residual level is determined by an enzymatic reaction at 505 nm.

4.8. Determination of glutathione

Glutathione (mg/g tissue) was assessed using Ellman's reagent as described by Beutler et al. (1963). Brain homogenates were deproteinated with 10% (w/v) 5-sulfuosalicylic acid for 30 min at 4 °C and then centrifuged at 3000×*g* for 15 min at 4 °C. An aliquot of the acid soluble supernatant was diluted with phosphate buffer (0.3 M, pH 7.7), 5,5′-dithiobis-2-nitrobenzoic acid (1 mM) was added to the samples, and the optical density was determined at 412 nm.

4.9. Determination of lipid peroxides

The thiobarbituric acid reaction of Mihara and Uchiyama (1978) was adopted for estimation of lipid peroxides level, using malondialdehyde as a standard. To brain homogenates, 1% (v) orthophosphoric acid and 0.6% (w/v) thiobarbituric acid were added, mixtures were then boiled for 45 min at 100 °C. After cooling, the colored product was extracted by *n*-butanol, vortexed, and centrifuged at 3000×*g* for 15 min. The absorbance of the upper layer was read at 535 and 520 nm. The difference in absorbance was expressed as nmol/g tissue thiobarbituric acid reactive substances (TBARS).

4.10. Statistical analysis

Data were expressed as means of 5–10 animals ±SEM, and comparisons between means were carried out using one-way ANOVA followed by Student–Newman–Keuls Multiple Comparisons Test. Incidence of convulsion was analyzed using Fisher's Exact Test. A probability level of less than 0.05 was accepted as statistically significant.

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