**Artículo de Investigación**

# Ethanol inhibits cooling-induced spinal seizures

Etanol inhibe convulsiones espinales inducidas por enfriamiento

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**ABSTRACT**

The isolated spinal cord can generate Paroxysmal seizure-like activity similar to those observed in intact animals. Patterns of tonic-clonic seizures can be induced by sudden cooling the isolated spinal cord-hindleg preparation, an experimental model of seizures that depends on release of excitatory amino acids (EAA)**.** We examine whether clinically relevant doses of ethanol can prevent the onset and severity of spinal seizures. The characteristic phases of seizures and their intensity were assessed by recording muscle contractions. The onset and duration of seizures were measured after intralymphatic (i.l.) administration of ethanol at doses of 1.5, 2.5 and 5 g/kg diluted to 10% with Ringer's solution. The tonic phase of seizures was effectively shortened or eliminated in a dose dependent manner when ethanol was given at 1.5 and 2.5 g/kg. At doses of 5 g/kg ethanol abolished all phases of seizures while producing moderated motor impairment. The latency of seizure onset was enhanced by 71% and 145% at ethanol doses of 1.5 and 2.5 g/kg, respectively. The effect of ethanol on the pattern of seizure activity was compared with that of known antagonists of EAA receptors. We concluded that ethanol inhibition of the tonic phase was linked to inhibition of N-methyl-D-aspartate (NMDA) receptors, while depression of the clonic phase of seizure was due to its blocking action on α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors; on the other hand, its effect on the latency of seizure onset resembled that of drugs acting by enhancing γ-amino-butyric acid ($GABA_A$) receptor activity.

**Key words**: Alcohol, spinal cord, seizures, cooling, excitatory amino acids.

**RESUMEN**

La medula espinal aislada es capaz generar patrones de convulsiones tónico-clónicas similares a aquellas observadas en animales intactos. Los patrones de convulsiones tónico-clónicas pueden inducirse mediante el enfriamiento brusco de la preparación aislada de la médula espinal-extremidades posteriores, un modelo experimental de convulsiones que depende de la liberación de aminoácidos excitatorios (EAA). Evaluamos si dosis de etanol clínicamente relevantes pueden prevenir el inicio y severidad de las convulsiones espinales. Las características de las convulsiones fueron estudiadas usando registros de las contracciones musculares. Evaluamos el inicio y duración de las convulsiones a dosis de etanol de 1,5, 2,5 y 5 g/kg. Dosis de etanol de 1,5 y 2,5 g/kg redujeron o eliminaron, de una manera dosis dependiente, la fase tónica de las convulsiones. Dosis de etanol de 5 g/kg eliminaron todas las fases de las convulsiones; aunque las mismas produjeron signos de incoordinación motora moderada. La latencia del inicio de las convulsiones incrementó en un 71% and 145%, a dosis de etanol de 1,5 y 2,5 g/kg, respectivamente. Cuando el efecto del etanol sobre el patrón de las convulsiones fue comparado con el efecto de antagonistas AAE, concluimos que la inhibición del etanol sobre la fase tónica está asociada a la inhibición de los receptores NMDA, mientras que la depresión de la fase clónica fue similar a la acción antagonista de receptores AMPA. Por otra parte, su efecto sobre la latencia del inicio es similar a las drogas que actúan aumentando la actividad de los receptores $GABA_A$.

**Palabras Clave**: Alcohol, medula espinal, convulsiones, enfriamiento, aminoácidos excitatorios.

Recibido: 01-07-2019 Aceptado: 10-07-2019

## INTRODUCTION

Ethanol complex actions on the central nervous system (CNS) are the result of its interaction with multiple neurotransmitter systems [1]. While acute exposure to high concentrations of ethanol can enhance the function of serotoninergic (5-$HT_3$) [2], gabaergic ($GABA_A$) [3], glycinergic (GlyRs) [4], and cholinergic receptors [5]; low, clinically relevant, concentrations of ethanol can effectively inhibit α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartate (NMDA) type ionotropic glutamate receptors (iGluRs) [6]. In the CNS, NMDA receptors (NMDARs) play a central role in neuro transmission and synaptic plasticity; however, their excessive activation can lead to neuronal cell death when abnormally high levels of glutamate accumulate in the extracellular space during both acute and chronic neurological conditions [7]. Pathological activation of NMDARs has been implicated in excitotoxic neuronal death during seizures [8], traumatic brain injury (TBI), stroke and cerebral hypoxia/ischemia [9]. Currently, there are no effective neuroprotective therapies to prevent brain damage associated with these neurological conditions [10]. In preclinical studies, acute ethanol treatment has shown to be neuroprotective against glutamate neurotoxicity *in vitro* (11] and *in vivo* animal models of brain ischemia and traumatic brain injury [12]; however, evidence for a neuroprotective effect of ethanol in humans remains controversial [13]. While a significant body of evidence shows high blood alcohol levels during TBI correlates with both, poor cognitive recovery [14] and higher mortality [15]; recent evidence show day-of-injury alcohol intoxication may be neuroprotective [12, 16]; and reduce mortality [17]; especially in TBI patients without a history of pre-injury alcohol abuse [16].

In spite of these negative results, recent evidence indicate that acute and low doses of ethanol have neuroprotective activities [12] and one of the most surprising effects is the protection against convulsions demonstrated in different animal model of seizures. Thus, ethanol can effectively inhibit seizures induced by pentylenetetrazol (PTZ) [18], nicotine [19], bicuculline [20], isoniazid [21] and ammonium acetate [22]. In addition, ethanol can raise the threshold for tonic seizures in the maximal electroshock seizure (MES) model [23]. It alters electrographic seizures on slices of rat hippocampus [24] and inhibits NMDA-evoked electrophysiologic activity in medial septal neurons [25]. Ethanol also raises the focal stimulation threshold in the hippocampal after discharge model [23]. Despite its neuroprotective activity, at clinically relevant doses (0.4 to 2.5 g/kg), the beneficial effect of ethanol is often overlooked when compared to the toxic effect of chronic alcohol abuse. Indeed, chronic blockade of NMDARs by ethanol abuse causes up-regulation of NMDARs, especially NR2B-containing NMDARs [26]; a compensatory mechanism thought to underlie seizures and neurotoxicity during ethanol withdrawal [12].

Almost a century ago, it was serendipitously discovered by Ozório de Almeida, in Brazil, that rapid cooling of the frog isolated spinal cord-induced a seizure like activity easily observed in the isolated hind legs [27]. It is now known that this type of rapid and profound cooling has a deleterious effect on spinal motoneurons [28]. Rapid cooling of the isolated spinal cord produces large depolarization of motoneurons accompanied of paroxysmal activity partially due to release of excitatory amino acids (EAAs) [29, 30]. The seizure can be recorded electrophysiologically directly in the spinal cord [30] or as muscles contractions using the isolated spinal cord–hindlimb preparation [29]. Recent evidence shows activation of NMDA, AMPA/Kainate and GABA receptors is a key event in this spinal seizure paradigm [30,31]. Here we show that ethanol can prevent the development of spinal seizures induced by sudden cooling.

## METHODS

Experiments were performed using cane toads, also known as giant neotropical toads (*Rhinella marina*) weighting between 42 and 73 g. Before each experiment, amphibians were killed by pithing followed by decapitation in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the 2000 Report of the American Veterinary Medical Association (AVMA), Panel on Euthanasia. All protocols and experimental procedures used during the study were previously approved by the Research Council (CDCHT) of the University Centroccidental Lisandro Alvarado, Barquisimeto, Venezuela. After the animal was pithed, the spinal cord was separated at the C1 level from the rest of the body, maintaining the sciatic nerves intact at both central and hind leg connection as previously described [29]. Seizures were induced by gently placing the isolated spinal cord into a tissue organ bath containing Ringer's solution with the following composition (mmol/ l): NaCl, 114; CaCl2, 1.9; KCl, 2.0; NaHCO3, 10; glucose, 5.5. Ringer's solution was maintained at pH 7.4and kept at 7 ºC. The intensity of the seizure was assessed by recording the contractions of the gastrocnemius muscle using a type B myograph connected to a physiograph (Narco Biosystems, Austin, TX). The latency of seizure onset was defined as the time elapsed between the immersion of the spinal cord into the cold bath and the visualization of the first group of muscle contractions. The duration of seizure episode was determined on the recording paper by measuring the time from the appearance of the first muscle contraction until all muscle activity ceased.

The dose of ethanol expressed in g/kg necessary for each animal, was diluted to the final concentration of 10 % using Ringer's solution and it was injected into the ventral lymphatic sac, 45 min before the preparation was mounted. Control animals were injected with Ringer's solution. As

shown in the results section, a minimum number of animals (4 to 8) were used at each dose. Data was analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's F test for multiple comparison.

## RESULTS

Rapid cooling of the isolated spinal cord evoked seizure activity that was recorded as muscle contractions characterized by three well defined phases: initially brief fibrillary muscle twitchings (tremors), followed by a group of disordered clonic muscle contractions (clonic phase) that increased its intensity until the isolated hind legs reached an extended positions (tonic phase) and then a second group of irregular muscle contractions appeared until all the muscles activities ceased (Fig 1A). Mean latency of seizure onset was 65.3 $\pm$ 4.5 sec (n = 8) and mean seizure duration was 12.7 $\pm$ 1 sec.

Ethanol shortened or inhibited the tonic phase of seizures at doses of 1.5 and 2.5 g/kg with concomitant depression of the clonic phase (Fig 1A). At doses of 5 g/kg, ethanol abolished all the seizure activity and no muscle contractions were observed in the hind legs. At this high dose, motor coordination was impaired, and animals were not able to jump normally. The anti-seizure activity of ethanol was dose dependent (Fig 1B).

Ethanol at doses of 1.5 and 2.5 g/kg increased the latency of seizure onset by 71%, n = 6, ($p < 0.05$) and 145%, n=4, ($p < 0.0001$), F=22.35, of control, respectively (Fig 1C) without affecting the duration of the clonic phase (Fig 1D). As occur when selective antagonists of NMDA receptors were used, ethanol blocked the tonic phase of the spinal seizures [29]. This effect of ethanol was unique when compared to other anticonvulsant [31], but similar to the pattern of seizure observed with the anesthetic urethane, an ethanol derivative [32].

## DISCUSSION

Sudden cooling of the spinal cord of amphibians generates a typical pattern of convulsive activity linked to release of EAAs [29]. We have recently showed that deep cooling produces nuclear pyknosis and severe neuronal cell damage characterized by swelling, vacuolated cytoplasm with distended neuronal bodies of spinal neurons [28]; here we present evidence that ethanol effectively protects spinal neurons against sudden cooling-evoked seizures.

After pretreatment with ethanol, the first observation was inhibition of the tonic phase of seizures accompanied by a depression of the clonic muscle contractions, but this phase was not prolonged in time. When recorded using sucrose gap technique, the clonic phase of seizure is coincident with the large, long lasting motoneuron depolarization and the tonic phase corresponded with the paroxysmal motoneuron repetitive firings which are superimposed on the declining phase of cold induced depolarization. These repetitive firings are eliminated by using DL-2-amino-7-phosphonoheptanoic acid (APH) and $Mg^{2+}$ [30]. In physiographically recorded myograms, the pattern of muscle contractions resembles those obtained after pretreatment with valproate, an antiepileptic drug [31] and ethyl carbamate or urethane, an anesthetic agent chemically derived from ethanol and urea [32].

The second observation was that ethanol was effective in increasing the latency of seizure onset, an effect that may be attributed to activation of $GABA_A$ receptors, because anticonvulsants such as diazepam, which acts by enhancing $GABA_A$ receptor activity, has a powerful effect prolonging the latency of seizure onset [31]. Based on the observation that the beginning of seizures is not altered by selective NMDA receptor antagonists such as DL-2-amino-7-phosphonoheptanoicacid (APH) or dizocilpine (MK-801) [29, 31], we concluded that an increase in latency of seizure onset may be associated with $GABA_A$ receptor activation. However, the effect of ethanol on $GABA_A$Rs seems to be much more complex than that of benzodiazepines and barbiturates [3] and it is difficult to demonstrated at anesthetic concentrations using urethane, an ethanol derivative, which is easily incorporated in physiologic solutions [33].

The third observation was the lack of effect of ethanol on the duration of the clonic phase. The clonic phase which probably occurs by activation of AMPARs and KARs within the spinal cord, is prolonged by pretreatment with selective NMDAR antagonists such as APH or MK-801 [29, 31]; however, ethanol, like urethane [33], inhibits both NMDARs and AMPARs [6] and elicited a clonic phase of seizures with a similar duration to that of control.

The dose of ethanol required to protect the spinal cord against cooling-induced seizures was similar to that used in others animal models of experimental seizures. Doses between 0.5-2.5 g/kg have been used in MES-, PTZ-, isoniazid-, nicotine-induced seizures [19, 20, 21, 23] and NMDA-evoked neuronal activity in rats [25]. The doses reported here are similar to those used in mammals; however, in amphibians, we have seen motor impairment only at 5 g/kg.

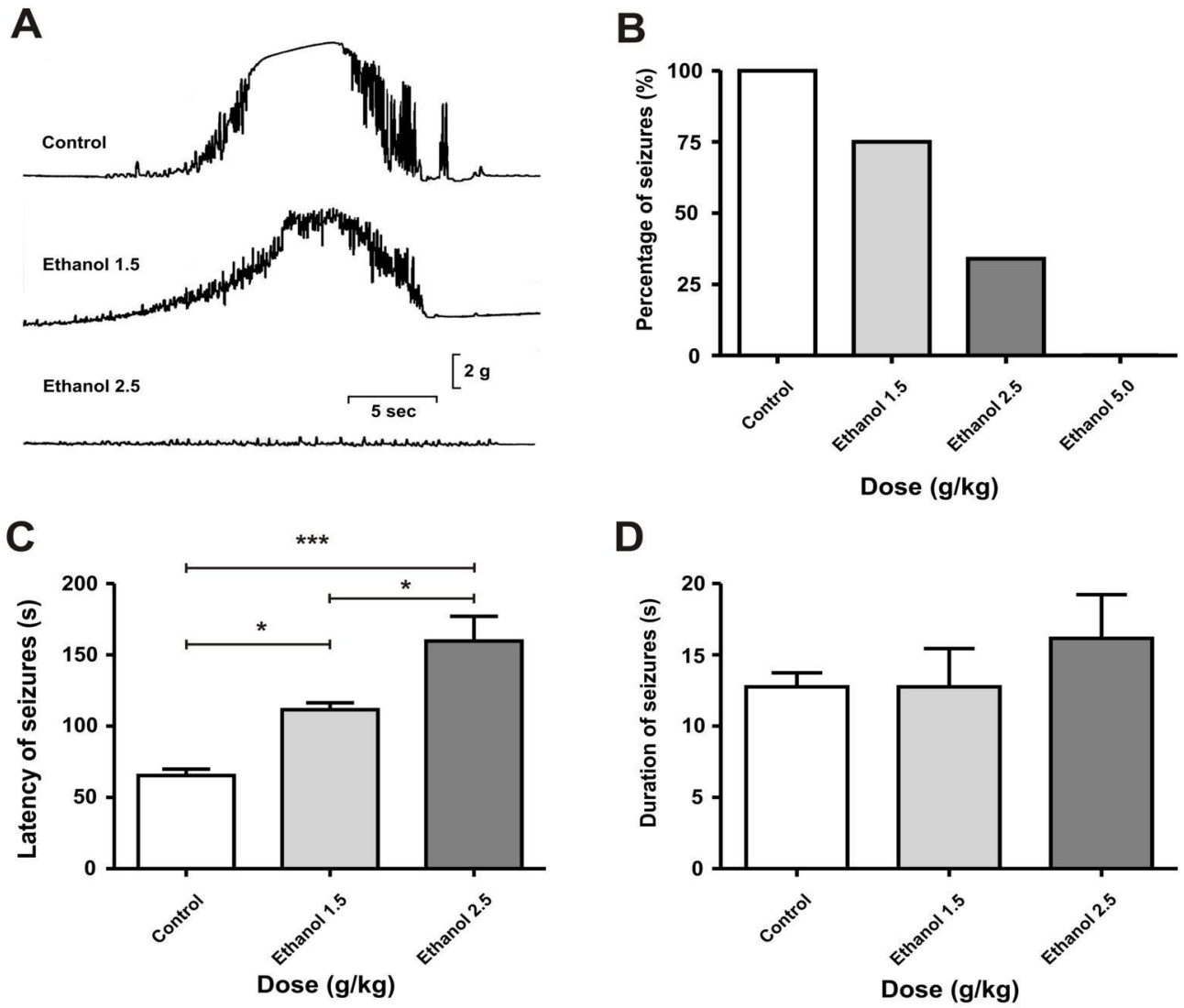


**Figure 1**. Ethanol prevents cooling-induced seizures in the isolated spinal cord. **A**) Pattern of muscle contraction during seizures. The horizontal bracket represents the paper speed (in seconds) and the vertical bracket the calibration of myograph (in grams); **B**) Percentage of seizure was inhibited in a dose-dependent manner by ethanol; **C**) Significant enhancement of latency of seizure onset. *p < 0.05, ***p < 0.0001; **D**) Duration of spinal seizures was not altered by ethanol.

The pattern of anti-seizure activity of ethanol is very similar to that reported for the anesthetic urethane (ethyl-carbamate) at doses of 0.15 and 0.2 g/kg [32]. Urethane has a pharmacological profile very similar to ethanol [34] and it is used at concentration of 22 mM to protect mature spinal cord preparations during dissection and tissue manipulation [35].

This work presents evidence that ethanol prevents seizures induced by sudden cooling, an experimental model of seizures linked to the release of EAAs. Ethanol effects on the tonic phase of spinal seizure is probably due to its blocking activity on NMDARs, while depression of the clonic phase may be due to its effect on AMPARs, but the increased latency of seizure onset may be attributed to its activity on $GABA_A$Rs.

**Acknowledgments**. This work was supported by the Research Council (CDCHT), Grant 002RVE2007, Universidad Centroccidental Lisandro Alvarado, Venezuela.

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