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EFFECT OF ALCOHOL-WITHDRAWAL ON PENTYLENETETRAZOL-INDUCED SEIZURES IN RATS: A PROTECTIVE FUNCTION OF VITAMIN C

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ABSTRACT:

Oxidative injury in the brain has been suggested as a common pathway of several neurologic diseases including epilepsy. Pentylenetetrazol (PTZ)-induced seizure is a proposed animal model of epilepsy. Further, animals have been shown to exhibit a PTZ-like stimulus after cessation of chronic alcohol treatment. The purpose of the present study is to further investigate the effect of alcohol withdrawal on acute PTZ-induced seizures, and the possible protective function of vitamin C.

Seventy male rats were randomly allocated to control (saline-injected) and alcohol-treated groups. Animals were divided into seven groups, 10 animals each. Group 1, animals were injected with normal saline (0.9%), i.p., daily for two weeks. Other groups (Groups 2-7) were treated with ethyl alcohol (20% w/v) at a dose of 2 gm/kg body weight, i.p., daily for two weeks. Group 3, animals were pretreated with Vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection. Group 4, animals were pretreated with Diazepam at a dose of 1 mg/kg body weight one hour before PTZ injection. Group 5, animals were pretreated with Vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection; in addition, animals were pretreated once with Diazepam at a dose of 1 mg/kg body weight, i.p., one hour before PTZ injection. Group 6, animals were pretreated with Diazepam at a dose of 0.5 mg/kg body weight one hour before PTZ injection. Group 7, animals were pretreated with Vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection; in addition, animals were pretreated once with Diazepam at a dose of 0.5 mg/kg body weight, i.p., one hour before PTZ injection. All groups were treated acutely with PTZ at a dose of 30 mg/kg body weight, i.p., one day after the last injection of saline or alcohol. Seizures were evaluated using the modified Racine's scale.

The undetected effects of the subconvulsive dose of PTZ (30 mg/kg) was clearly observed in alcohol-withdrawing animals. Pretreatment with Vitamin C alone could not prevent PTZ-induced seizures. In contrast, Diazepam (1 mg/kg) could decrease seizures score. This protective effect of diazepam has dissipated upon dose reduction (DZP, 0.5 mg/kg). However, the combined effects of Diazepam (0.5 mg/kg) and Vitamin C (400 mg/kg) proved to be effective in suppressing the convulsant effect of PTZ in alcohol-withdrawing animals. It is concluded that alcohol withdrawal enhanced the sensitivity of animals to the acute convulsant effects of PTZ. Vitamin C could not block seizure activity, however, it enhanced the anticonvulsant effects of diazepam.

INTRODUCTION

Epilepsy is one of the most common neurological disorders worldwide. Several models of epilepsy have been proposed including repeated electrical or chemical stimulation of the amygdala and hippocampus or repeated administration of subconvulsive doses of convulsants such as pentylenetetrazol (PTZ). PTZ interacts competitively with picrotoxin binding sites of the γ-aminobutyric acid ($GABA_A$) receptor, thereby decreasing Cl^- flux across the membrane[1] and inducing generalized clonic seizures. This property is used as an experimental animal model of epilepsy and epileptogenesis[2].

Ballenger and Post[3] suggested that episodes of ethanol intoxication and withdrawal could serve as a kindling stimulus. Subsequently, this concept has been supported using several models of repeated alcohol dependence and withdrawal[4]. Furthermore, Prior exposure to kindling regimens, including PTZ, has been shown to increase the severity of symptoms associated with chronic ethanol withdrawal[5]. Withdrawal from chronic ethanol exposure could produce changes in sensitivity to convulsant drugs[6,7]. Seizure susceptibility to PTZ was increased in ethanol-withdrawing mice[8]. Both chronic ethanol and PTZ-kindling have been shown to reduce GABA-stimulated Cl^- uptake[9,10]. There are other parallels between PTZ-kindling and ethanol dependence and withdrawal; these include the action of MK801, which prevents ethanol withdrawal behavior and development of PTZ-kindling[11,12].

It has been suggested that reactive oxygen species (ROS) could be involved in the neural damage induced by PTZ[13]. In addition, ethanol has been shown to induce DNA strand breaks through the generation of reactive oxidants[14]. In general, ROS has been implicated in a variety of neurologic conditions[15,16], including convulsions[17,18]. Thus, the antioxidants may have a potential role in preventing excitotoxicity-induced seizure genesis.

The antioxidant system comprises various compounds with different functions. These include enzymes (e.g., catalase, glutathione peroxidase, glutathione transferase, and superoxide dismutase) and the sequestration of metals (e.g., iron, copper and hence by chelating agents), all of that suppress the generation of free radicals. In addition, hydrophilic and lipophilic compounds (e.g., vitamin C and vitamin E, respectively), which can act by scavenging or suppressing the generation of free radicals, are also important for control of intracellular levels. Vitamin C is a water-soluble antioxidant, which protects cells from oxidative stress by scavenging free radicals. It has a reduction potential and can therefore, inactivate

highly damaging radicals, including OH and lipid peroxyl radicals.

The aim of the present study was to investigate (i) the enhancing effect of alcohol-withdrawal on PTZ-induced seizures in rats (ii) the possible protective effect of vitamin C against PTZ-induced seizures in alcohol-withdrawing rats (iii) the modulatory role of vitamin C on the anticonvulsant effect of diazepam.

MATERIALS AND METHODS

Animals

The experiments were performed on 70 male albino rats, obtained from the National Institute of Drug Control and Research, weighing about 100±20 g. Animals were kept under controlled laboratory conditions (normal light-dark cycle, temperature 25-30°C, and relative humidity 50-60 %. The animals were housed as triads of rats per cage. All animals had free access to food and water. All experiments were performed between 12:00 a.m. and 3:00 p.m. to minimize circadian influences on seizure susceptibility.

Drugs and chemicals

Pentylenetetrazol (PTZ, Sigma): It was dissolved in saline. Rats were injected with subconvulsive dose of PTZ (30 mg/kg body weight, i.p.). Ethyl Alcohol (Alcohol, 95%, ADWIC, El-Nasr Pharmaceutical Chemicals): Ethyl Alcohol was used at a concentration of 20% (w/v). Animals were treated with alcohol at a dose of 2 gm/kg, i.p., daily for a two-week period. Diazepam (DZP, Valpam® ampoule, Amoun Co., Egypt): Rats were pretreated with Diazepam at a dose of 1 mg/kg or 0.5 mg/kg body weight, i.p., one hour before PTZ injection. Vitamin C (Vit. C, Cevarol® ampoule, Memphis Co., Egypt): Rats were pretreated with Vitamin C at a dose of 400 mg/kg, i.p., one hour before each alcohol injection. Selection of doses and injection-dose intervals was made according to the pharmacokinetic data from the literature and our preliminary studies. Vitamin C is sensitive to light, so all glassware used for mixing and injections were opaque and covered with aluminum foil.

Evaluation of Seizures:

Rats were injected with a subconvulsive dose of PTZ (30 mg/kg, i.p.). After the injection of PTZ, the convulsive behavior was observed for 20 minutes and scored using a modified rating scale according to Racine scale[19]. The scale ranges from 0-5; zero score indicates no convulsions, whereas 5 indicate generalized clonic seizures. Seizure duration was the duration of limbic seizures (stage 1-2) and motor seizures (stage 3-5). Table 1 describes Racine scoring system of seizures.

Table 1: Racine rating scale for evaluation of seizures.

Symptoms	Score
No seizure response.	0
Immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus.	1
Head nodding associated with more severe facial clonus.	2
Clonus of one forelimb.	3
Bilateral forelimb clonus without rearing.	3.5
Bilateral forelimb clonus with rearing.	4
Falling on a side (without rearing), loss of righting reflex accompanied by generalized clonic seizures.	4.5
Rearing and falling on back accompanied by generalized clonic seizures.	5

Study Design:

Rats were randomly allocated to control (saline-injected) and alcohol-treated groups. Animals were divided into seven groups, 10 animals each. Group 1, animals were injected with normal saline (0.9%), i.p., daily for two weeks. Other groups (Groups 2-7) were treated with ethyl alcohol at a dose of 2 gm/kg body weight, i.p., daily for two weeks. Group 3, animals were pretreated with vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection. Group 4, animals were pretreated with diazepam at a dose of 1 mg/kg body weight one hour before PTZ injection. Group 5, animals were pretreated with vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection; in addition, animals were pretreated once with diazepam at a dose of 1 mg/kg body weight, i.p., one hour before PTZ injection. Group 6, animals were pretreated with diazepam at a dose of 0.5 mg/kg body weight one hour before PTZ injection. Group 7, animals were pretreated with vitamin C at a dose of 400 mg/kg body weight one hour before each alcohol injection; in addition, animals were pretreated once with diazepam at a dose of 0.5 mg/kg body weight, i.p., one hour before PTZ injection. All groups were treated acutely with PTZ at a dose of 30 mg/kg body weight, i.p., one day after the last injection of saline or alcohol. A summary of the experimental design is shown in Table 2.

Table 2: Summary of the study design.

Group 1 n = 10	Group 2 n = 10	Group 3 n = 10	Group 4 n = 10	Group 5 n = 10	Group 6 n = 10	Group 7 n = 10
Saline + PTZ	Alc. + PTZ	Alc. + Vit.C + PTZ	Alc. + 1mg/kg DZP + PTZ	Alc. + Vit.C + 1mg/kg DZP + PTZ	Alc. + 0.5mg/kg DZP + PTZ	Alc. + Vit.C + 0.5mg/kg DZP + PTZ

Statistical analysis

Values are expressed as mean ± SEM for statistical analysis, one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests were applied for statistical analysis. For all comparisons, differences were considered significant at $p \leq 0.05$.

RESULTS

In the present study, it was evident that alcohol withdrawal has increased the sensitivity of animals to the convulsant effects of PTZ. Injection of a subconvulsive dose of PTZ (30 mg/kg) in saline-treated animals produced minimal changes in the rats' convulsive behavior on Racine's scale of seizures (Saline, 0.6±0.17). However, the same dose of PTZ, when administered acutely to alcohol-withdrawing animals, produced a significant increase in the convulsive behavior of these animals (Alc, 3.813±0.16, $p \leq 0.001$, Fig. 1).

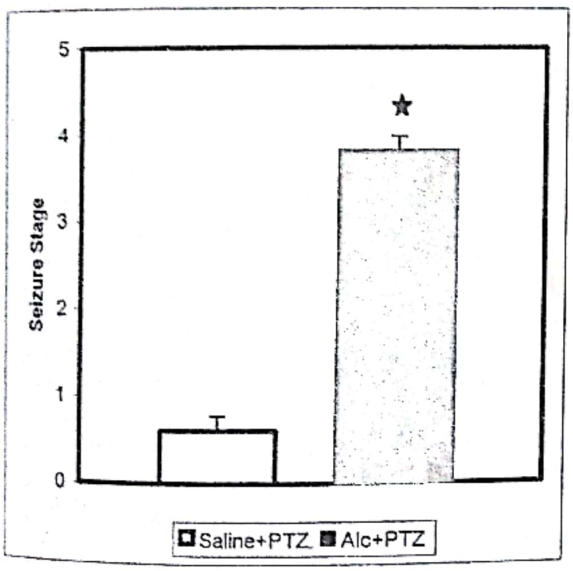

Figure 1: Effect of cessation of chronic alcohol administration (2 gm/kg body weight, i.p., daily, for a 2-week period) on the acute convulsant effect of PTZ (30 mg/kg body weight) in rats.
* Significantly different at $p<0.001$:

Although ANOVA analysis showed a significant change in the seizure scores of animals among all treated groups ($F=45.5$, $p \leq 0.001$), pretreatment of alcohol-treated rats with vitamin C (400 mg/kg) failed to protect the animals against the convulsant effects of PTZ. No significant difference was observed between the mean score of group 2 and 3 (Alc, 3.813±0.16 vs. Alc + Vit. C, 3.375±0.07, Fig. 2).

In contrast, pretreatment of alcohol-withdrawing animals with diazepam (1 mg/kg) could produce a significant reduction in seizure scores (Alc, 3.813±0.16 vs. Alc + DZP, 1.857±0.33, $p \leq 0.001$, Fig. 2). This protective effect of diazepam (1 mg/kg) was augmented even more in animals pretreated with vitamin C (400 mg/kg) during ethanol exposure (Alc + Vit. C + DZP, 0.6±0.17, Fig. 2).

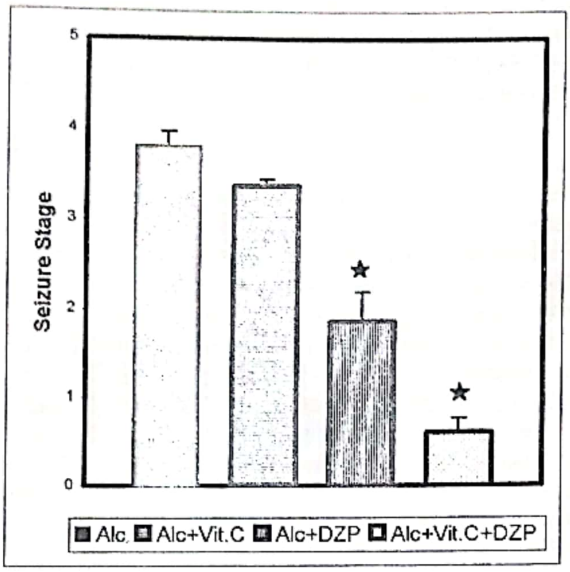

Figure 2: The anticonvulsant effects of diazepam (1mg/kg body weight) and/or vitamin C (400 mg/kg body weight, i.p., daily, for a 2-week period) on the acute convulsant effect of PTZ (30mg/kg body weight) in alcohol-withdrawing rats.
* Significantly different at $p<0.001$

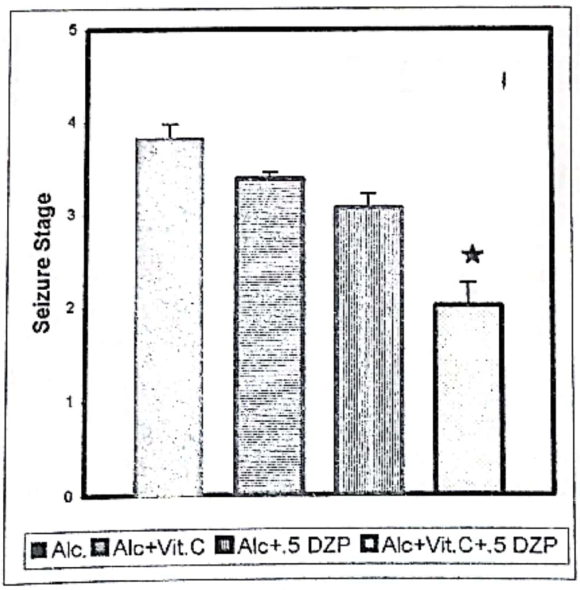

Figure 3: The anticonvulsant effects of diazepam (0.5mg/kg body weight) and/or vitamin C (400 mg/kg body weight, i.p., daily, for a 2-week period) on the acute convulsant effect of PTZ (30 mg/kg body weight) in alcohol-withdrawing rats.
* Significantly different at $p<0.001$

In contrast to the protective effects of diazepam (1 mg/kg), the lower dose of diazepam (0.5 mg/kg) failed to protect the animals against the convulsant effects of PTZ in alcohol-withdrawing rats (Alc, 3.813±0.16 vs. Alc + 0.5 DZP, 3.06±0.15, Fig. 3). However, ANOVA

analysis showed a significant change in the mean score of various treatment groups ($F=33.45$, $p\leq0.001$). Bonferroni's multiple comparisons demonstrated that pretreatment with vitamin C could disclose a suppressing effect of the subtherapeutic dose of diazepam (0.5 mg/kg) on the convulsive behavior of animals (Alc + Vit. C + 0.5 DZP, 2.0±0.25, Fig. 3).

Therefore, the undetected effects of the subconvulsive dose of PTZ (30 mg/kg) was clearly observed in alcohol-withdrawing animals. Pretreatment with vitamin C alone could not prevent PTZ-induced seizures. In contrast, diazepam (1 mg/kg) could significantly decrease seizure scores. This protective effect of diazepam has dissipated upon dose reduction (DZP, 0.5 mg/kg). However, the combined effects of diazepam (0.5 mg/kg) and vitamin C (400 mg/kg) proved to be effective in suppressing the convulsant effect of PTZ in alcohol-withdrawing animals.

DISCUSSION

In the current study, PTZ was used acutely to induce seizures in rats. It is well documented that repeated systemic administration of subconvulsive doses of PTZ results in a progressive increase in seizure intensity that is long lasting and irreversible[20]. The development of PTZ kindling has been suggested to be related to functional alterations in various neurotransmitter systems, including gradual reduction in $GABA_A$ receptor function and an enhancement of glutamatergic transmission that together result in hyperexcitability and seizure activity[21]. PTZ, at subconvulsive doses, has been extensively used as an animal model of ethanol-induced anxiety[22].

The $GABA_A$ receptors are the major inhibitory neurotransmitter system in mammalian central nervous system. Decreased GABA-mediated inhibitory synaptic transmission is considered a major factor contributing to the pathophysiology of alcoholism and epilepsy[23]; thus GABA receptor agonists, such as diazepam, are used to enhance GABAergic transmission during ethanol withdrawal. Therefore, diazepam was used in the current study to prevent PTZ-induced seizures in alcohol withdrawing rats.

The N-methyl-D-Aspartic Acid (NMDA) receptor complex was also suggested to be involved in the development of PTZ kindling. The ability of NMDA antagonists in blocking kindling development has led to the suggestion of a potential clinical utility in the prophylaxis of epilepsy[24]. Unfortunately, NMDA antagonists induce severe neurotoxicity, which prevents its clinical use[25]. The NMDA type of glutamate receptor is a prime target for ethanol action[26]. Thus the adaptive changes associated with chronic alcohol exposure may affect the sensitivity of the glutamatergic transmitter system to the effect of PTZ. In fact, McCown and Breese[27] showed that prior withdrawals from chronic ethanol treatment resulted in a decreased number of stimuli required for generation of seizures.

Exposure to chronic ethanol produces well-documented ion channel adaptation[28]. Briefly, GABA inhibitory neurotransmission is reduced, whereas glutamatergic excitatory transmission is increased following chronic ethanol administration[29]. In addition, an increase in the number of voltage-sensetive calcium channels after chronic ethanol administration also increases neuronal excitability[30]. Overall, the resultant changes in ion channel sensitivity or number lead to decreased inhibitory and increased excitatory receptor function during withdrawal from chronic ethanol exposure.

Idemudia et al.[31] showed that antagonism of the GABA system by bicuculline and picrotoxin increased the alcohol withdrawal stimulus in an additive manner. Further, after chronic exposure to ethanol-containing liquid diet, increased sensitivity to bicuculline was demonstrated in ethanol withdrawing rats[32]. In line with these observations, rats exhibited a PTZ-like stimulus as soon as the blood ethanol and ethanol intoxication declined after cessation of chronic ethanol treatment[31]. Withdrawal from prolonged ingestion of ethanol results in a number of signs and symptoms consistent with enhanced neuronal excitability, including increased sensitivity to convulsants[8]. These findings suggest some common mechanism in PTZ-kindled and ethanol-dependent rats. In the present study, PTZ was used acutely, at a subconvulsive dose, to further investigate the seizure susceptibility during ethanol withdrawal. In fact, a significant increase in the convulsive behavioral response to PTZ in alcohol withdrawing rats was recorded in the present results.

The enhanced convulsive behavior of alcohol withdrawing rats after treatment with PTZ was not suppressed by the lower dose of diazepam (0.5 mg/kg). It required doubling the dose of diazepam (1.0 mg/kg) to inhibit seizures. In agreement with these results, it has been reported that chronic ethanol exposure produced reduced sensitivity to the anticonvulsant effect of diazepam in rats during ethanol withdrawal[33].

Oxidative injury in the brain is increasingly recognized as a common pathway of cellular injury in many acute neurologic insults, and in more chronic disease states such as Parkinson's or Alzheimer's disease[34,35]. ROS production has been considered to be a part of mechanisms involved in the glutamatergic excitotoxicity "in vitro"[15] and "in vivo"[36]. It has been proposed that activation of excitatory amino acid receptor can trigger the formation of ROS. These increased ROS, in turn, further release glutamate, thus forming a loop. This "vicious" cycle not only causes long-lasting seizure formation, but if not arrested may lead to neuronal death[37].

Ethanol may induce DNA strand breaks through the generation of reactive oxidants[14]. Several groups have proposed that ROS may be generated by the ethanol-inducible protein cytochrome P450 2E1[38]. The elevated levels of reactive oxyradicals caused by ethanol may also arise as a consequence of an increase in the $NADH/NAD^+$ redox ratio or as a result of the release of iron[39].

There is evidence that oxidative stress might occur during seizures and participate in the pathophysiology of epilepsy. Such evidence includes the high incidence of seizures during head trauma and hypoxia[40], conditions associated with a sharp increase of free radical (FR) production[41], as well as observations from a variety of experimental epilepsy models employing FR-generating systems[42]. The ability of antioxidative therapy to decrease lipid peroxidation and significantly alleviate seizure in these pathological conditions[43] is in agreement with this hypothesis. These suggest that ROS could be involved in the neural damage induced by PTZ. In various experimental studies, it has been demonstrated that antioxidants can prevent the excitotoxicity induced by agents like glutamate[44]. Therefore, the antioxidants may play a potential role in preventing excitotoxicity-induced seizure genesis.

In normal conditions, there is a steady state balance between the production of ROS and their destruction by cellular antioxidant system. However, this balance can be broken either by increasing the ROS production or by decreasing the defense system. When cells are exposed to an oxidative stress various defense mechanisms can be induced, including catalase and glutathione peroxidase[45]. Antioxidant defense mechanisms include removal of O_2, scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation, and up-regulation of endogenous antioxidant defense. Indeed, there is a relatively high concentration of the water soluble antioxidant vitamin C in the brain, which plays a variety of roles, one of which is the regeneration of the lipid soluble antioxidant vitamin E[46].

However, since our endogenous antioxidant defense mechanisms are not always completely effective, and since exposure to damaging environmental factors is increasing, it seems reasonable to propose that exogenous antioxidants could be effective in diminishing the cumulative effects of oxidative damage. Agus et al.[47] found that vitamin C can cross the blood-brain barrier (BBB). It readily enters the brain and retained in the brain tissue in the form of ascorbic acid. This transport is probably implemented via the glucose transporter in the BBB. The author concluded, therefore, that increasing blood concentrations of dehydroascorbic acid could increase vitamin C concentrations in the brain.

In the current study, it was demonstrated that alcohol withdrawal has increased the susceptibility to the convulsant effect of PTZ. This effect was abolished in animals pretreated with vitamin C and diazepam. Although neither diazepam (0.5 mg/kg) nor vitamin C alone could significantly decrease seizure scores, their combination proved effective in preventing seizures. This may indicate that free radical generation may, at least in part, mediate seizure genesis in the present model.

In line with the present results, Goodwin et al.[48] noted a correlation between memory function and vitamin C in blood of healthy volunteers aged 60 or over. Further, a number of investigations have revealed that vitamin C level is decreased by exposure to ethanol. For example, a decrease in the level of vitamin C in rat testis was observed after ethanol administration[49]. Furthermore, in healthy volunteers, pretreatment with vitamin C (1 g daily for 3 days) decreased alcohol toxicity (mediated by circulating acetaldehyde) after drinking 84 g of ethanol[50]. In addition, pretreatment with vitamin C prevented the ethanol-induced hepatic DNA strand breaks. The protective effect of vitamin C was correlated with the inhibitory effects of vitamin C on the generation of 1-hydroxyethyl radicals after ethanol exposure[14]. Therefore, vitamin C is proven to be effective against ROS that might be involved in seizure genesis.

However, in order to provide neuroprotection by antioxidants, potential measures should be taken into consideration. These include (i) the appropriate use of specific antioxidant(s) for a given disease or, disease stage of progression, (ii) the use of optimal doses of antioxidant(s), (iii) sufficient knowledge of blood-brain barrier penetration of different antioxidants when used systemically, (iv) methods of targeting drugs to specific sites within the brain are necessary to produce efficient drugs with minimal side effects, and (v) determination of whether antioxidants can be used as prophylactics, in order to slow down the progression of neurodegenerative diseases such as epilepsy.

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تأثير سحب الكحول على التشنجات الحادة المحثة بالبنتيلين تترازول في الفئران: التأثير الواقي لفيتامين ج .

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تهـدف الدراســة الحالية إلى بحث تأثير سحب الكحول في التشنجات الحادة المحثة بمادة البنتيلين تترازول ، وكذلك بحث إمكانية الوقاية بفيتامين ج .

اسـتخدم لهـذه الدراســة سبعون جرذا ، قسمت إلى سبع مجموعات بواقع عشر حيوانات في كل مجموعة. عولجت جميع المجموعات بالكحول الأثيلي (٢٠% وزن/حجم) بجرعة قدرها ٢ جم/كجم من وزن الجسم بالحقن فـي تجويف البطن يومياً ولمدة أسبوعين ، ما عدا مجموعة واحدة أعطيت محلول ملح بدلاً من الكحول (مجموعة ضـابطة). عولجـت أربع مجموعات بفيتامين ج (٤٠٠مجم / كجم – يومياً ولمدة أسبوعين) إما منفردا أو مجتمعا مع ديازيـبـام (١مجم/كجم أو ٠,٥ مجم/كجم) قبل ساعة من التعرض للكحول أو البنتيلين تترازول على التوالي. عولجـت الحيوانات بجرعة واحدة من البنتيلين تترازول (٣٠ مجم/كجم) بعد يوم واحد من انتهاء العلاج بالكحول أو محلول الملح . تم تقييم التشنجات بمقياس راسين.

بينـت النتائج أن الجرعة تحت التشنجية المستعملة من البنتيلين تترازول (٣٠ مجم/كجم) لم تحدث تشنجات واضحة في المجموعة الضابطة في حين أحدثت تشنجات بدرجة عالية في المجموعة المسحوبة من الكحول . في حيـن لم يستطيع العلاج بفيتامين ج حماية الحيوانات من التشنجات المحثة بالبنتيلين تترازول ، أظهر الديازيبام (١ مجـم/كجـم) حماية واضحة. إلا أن هذا التأثير الواقي للديازيبام قد أختفى بتخفيض الجرعة إلى ٠,٥ مجم/ كجم. وجديـر بالإهتمام أن التأثير الواقي – المختفي للديازيبام عند جرعة (٠,٥ مجم/كجم) عاد ليظهر من جديد عندما أعطي مجتمعاً مع فيتامين ج.

تخلـص النـتائج إلـى أن سحب الكحول المزمن يؤدي إلى زيادة حساسية الحيوانات لتأثير البنتيلين تترازول الحـاد في التشنجات ، وأن فيتامين ج يمكن أن يضيف إلى التأثير الواقى للديازيبام ضد التشنجات المحثة بالبنتيلين تترازول.