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Seizure vulnerability and anxiety responses following chronic co-administration and acute withdrawal of caffeine and ethanol in a rat model

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Abstract

Background: Caffeine antagonizes the intoxicating effects of alcohol. Consequently, there has been a dramatic global increase in the consumption of caffeinated drinks together with alcohol, especially among young adults. We assessed the seizure vulnerability and anxiety responses following the chronic co-administration of, and withdrawal from, caffeine and ethanol in male rats.

Methods: The rats were randomly assigned to six groups consisting of 10 animals each: 10 mg/kg of caffeine, 20 mg/kg of caffeine, 4 g/kg of 20% ethanol, combined caffeine (20 mg/kg) and ethanol (4 g/kg of 20%), 4 mL/kg distilled water, and an untreated control group. The test substances were administered intragastrically twice daily for 29 days. On day 29, the rats were tested on the elevated plus maze to assess anxiety-related responses. On day 30, pentylenetetrazol (PTZ), a chemoconvulsant, was administered intraperitoneally at a dose of 40 mg/kg to the animals. Seizure responses and mortality up to 72 h were recorded.

Results: Compared with the control group, the rats that received chronic treatment with low-dose caffeine, ethanol alone, and combined caffeine and ethanol exhibited significant anxiogenic-like effects, unlike with high-dose caffeine. Both low- and high-dose caffeine significantly increased PTZ seizure latency. Ethanol alone and combined caffeine and ethanol both lowered PTZ seizure latency. No significant difference occurred between the controls and the untreated group for either anxiety or seizure expression. Combined caffeine and ethanol increased the seizure-induced mortality from withdrawal effects at 72 h.

Conclusions: These findings suggest that the chronic co-administration of caffeine and ethanol and the acute withdrawal from these drugs lead to anxiogenic effects and increased seizure vulnerability.

Keywords: acute withdrawal; anxiety; caffeine; chronic; elevated plus maze; ethanol; pentylenetetrazol; seizures.

Introduction

Increasing evidence suggests a dramatic global increase in the use of caffeine-containing energy drinks together with alcohol due to the rise in popularity of caffeinated energy drinks among the youth [1–3]. A national survey done in Uganda showed that 33% of the youth acknowledged having used alcohol and stimulant drugs, including marijuana and caffeinated energy drinks for pleasure or to help them through difficult situations [4]. Uganda is among the leading consumers of alcohol *per capita* in Africa [5] and there is an increasing use of caffeinated energy drinks in Uganda as well [6]. A significant number of adolescents now use caffeine-containing drinks and ethanol together to mitigate alcohol-induced sedation and intoxication [2].

The consumption of caffeine-containing drinks together with alcohol has been shown to increase self-reported brain stimulation and to reduce sedation compared with consuming alcohol alone [2, 3, 7]. The consumption of a caffeine-containing energy drink with alcohol, therefore, leads to an increased alcohol intake within an individual drinking episode, and may eventually lead to the development of ethanol dependence [2, 3, 7, 8]. The appeal of combining caffeine-containing drinks and alcohol is most notable among underage and adolescent drinkers [2], who are likely to be inexperienced drinkers seeking to avoid the sedation and intoxication that are considered minor problems in more established drinkers. Adolescent brains are still undergoing structural development and may be distinctively sensitive to the neurotoxic effects of binge-drinking as well as chronic heavy drinking resulting from the consumption of caffeine-containing drinks and alcohol [8, 9]. Significantly, the greater stimulant effect resulting from the combined

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caffeine and alcohol may also increase the likelihood of seizure activity and anxiety following chronic use via central nervous system (CNS) interactions between caffeine and alcohol [3].

Caffeine is a non-selective adenosine antagonist that is responsible for the direct blockade of the inhibitory effects of the adenosine and for the inhibition of the indirect presynaptic release of several neurotransmitters such as dopamine [10]. Adenosine inhibition thus causes increased wakefulness as a consequence of caffeine intake. Caffeine also leads to increased dopamine activity through the blockade of the adenosine activity. Caffeine is a methylxanthine derivative that, at high doses, induces seizure activity in rodents, similar to other methylxanthine derivatives (theophylline, pentoxifylline) [11].

Ethanol is a CNS depressant that disturbs the fine balance between the excitatory and inhibitory influences in the brain, thereby acutely producing disinhibition, ataxia, and sedation. Ethanol enhances the inhibitory neurotransmission of γ -aminobutyric acid (GABA) via the GABA_A receptor, and indirectly blocks the excitatory transmission through the glutamate and N-Methyl-D-Aspartate (NMDA) receptors, an action responsible for its anxiolytic and sedative effects [12]. Chronic ethanol administration in rats is known to create a neurochemical imbalance in the cortical neurons, thus resulting in excitatory neurotransmission upon withdrawal [13]. Tolerance to ethanol develops after chronic use, and physical dependence is demonstrated via alcohol withdrawal [14].

The adenosine neurotransmission has been implicated in the mechanistic link between caffeine and ethanol, and plays a pivotal role for potentially risky effects when the two substances are combined [10]. During chronic alcohol intake, the addition of caffeine enhances the A_{2A} receptor blockade and decreases the adenosine inhibition in the brain [15]. Neuroadaptation studies on the adenosine receptor signaling have demonstrated that chronic ethanol exposure induces adaptive changes that make the brain more vulnerable to various excitotoxic insults, such as ethanol withdrawal-related behavior and seizures [16, 17].

Therefore, the goal of this study was to investigate seizure vulnerability and anxiety responses as potential consequences associated with the chronic caffeine and ethanol co-consumption among juvenile males. We hypothesized that the chronic co-administration of caffeine and ethanol increases seizure vulnerability and anxiety responses in juvenile male Sprague-Dawley (SD) rats. As there have been no controlled studies that compared the differences in the behavioral responses to separate and interactive caffeine and ethanol following chronic

use, our objectives were to (i) assess anxiety responses following chronic caffeine administration alone, ethanol administration alone, and concurrent chronic caffeine and ethanol administration using an elevated plus maze; and (ii) compare the different seizure parameters following chronic caffeine administration alone, ethanol administration alone, and concurrent chronic caffeine and ethanol administration and acute withdrawal using a pentylenetetrazol (PTZ) seizure model. Understanding the behavioral manifestations resulting from the consumption of caffeinated drinks together with alcohol can contribute not only to policy development regarding the manufacture and sale of these products, especially to juveniles and young adults, but can also advance the clinical management of this vulnerable subpopulation, especially if targeted interventions have to be made.

Materials and methods

Animal treatment and experimental design

The subjects were juvenile male SD rats weighing between 100 and 200 g, and approximately 60 days old. The rats were group-housed in rat cages in the animal research facility of the Department of Pharmacology of the Mbarara University of Science and Technology. The cages were cleaned and bedding changed thrice per week. The rats were kept on a 12 h light/dark cycle and fed on standard rodent pellets with water provided *ad libitum*. Following a week of acclimatization, the animals were assigned into groups. The computer-generated random numbers were assigned to all groups at the beginning of the experimental period. The sample was divided into six groups; each group included 10 rats and the treatments were intragastrically administered as follows: Group 1: Untreated group; Group 2: received 4 mL/kg distilled water that served as a negative control; Group 3: received 10 mg/kg of caffeine; Group 4: received 20 mg/kg of caffeine; Group 5: received 4 g/kg of 20% ethanol; and Group 6: received combined caffeine (20 mg/kg) and 4 g/kg of 20% ethanol. The rats were weighed twice a week to ensure appropriate dosing based on weight changes. All rats, except for the untreated group, received single gavage of the test substances twice daily for 28 days, and on the day of the first behavioral test (elevated plus maze). There was no mortality associated with the gavage procedure. All treatments were administered between 8 and 10 a.m. and repeated between 3 and 5 p.m. daily. This dosing regimen was based on earlier studies that showed reliable induction of ethanol dependence and positive reinforcement [18].

Caffeine administration

Animals received caffeine (Sigma-Aldrich, St. Louis, MO, USA) by gavage for 28 days at a dose of 10 mg/kg or 20 mg/kg body weight, and on the day of the first behavioral test (elevated plus maze). The caffeine dosages were based on prior related studies, which showed that the metabolism of caffeine differs between rodents and humans

and that the half-life of the methylxanthine was much shorter in rats (0.7–1.2 h) than in humans (2.5–4.5 h) [19]. We assumed that 10 mg/kg in a rat represented about 250 mg of caffeine in a human weighing 70 kg (3.5 mg/kg), equivalent to about 2–3 cups of coffee [20]. The dosing regimen was designed to mimic the typical consumption in people who use both drugs concurrently.

Ethanol administration

The animals received 4 g/kg body weight of 20% ethanol (VWR International, Fontenay-sous-Bois, France) by gavage twice daily for 28 days, and on the day of the first behavioral test (elevated plus maze). This dose was based on a study, which showed that the oral administration of caffeinated energy drinks did not significantly alter the effects of moderate oral doses of ethanol (0.5, 1.0, 1.5, or 2 g/kg) but reduced the suppressant effect of a higher dose of ethanol (4 g/kg) [21]. All treatments ended by the beginning of the active period (starting at 6 p.m. for alternating 12 h light/dark periods), during which rats were more active [22].

Control animals

The control animals received distilled water (4 mL/kg) only by gavage for 28 days, and on the day of the first behavioral test (elevated plus maze). An untreated animal group was included, to ensure that measurements were valid by providing a point of contrast with the distilled water treated (control) group. This ensured that the experimental system, as outlined for the other treatments, did not in itself confound the outcome.

Elevated plus maze test

On the 29th day after the initiation of drug administration, the animals were randomly introduced to the elevated plus maze. The elevated plus maze was used on the basis of its documented ability to detect both anxiolytic and anxiogenic-like drug effects in rodents [23]. Briefly, the apparatus was made of medium-density fiber board with a matte-black acrylic surface and consisted of four arms (two open and two enclosed by 30 cm-high walls) 50 cm long and 10 cm wide. The open arms of the rat maze had 5 mm-high railings to increase open arm exploration. Each arm of the rat maze was attached to sturdy wooden legs 50 cm from the base. Plus-maze experiments were conducted in a sound-attenuated room under low-intensity light. The animals were placed on the central platform facing the closed arm and their behavior recorded for a 5-min test period. The criterion for arm visit was considered only when the animal decisively moved all its four limbs into an arm. The maze was cleaned with 70% ethanol after each trial. The percentage of time spent in the arms was calculated as time in open arms or closed arm/total time \times 100. The number of entries into the arms were calculated using number of entries into open or closed arms/total number of entries \times 100 [24]. To ensure that animals did not experience withdrawal from caffeine or ethanol administration, after completion of the elevated plus maze experiments, the test substances were administered twice at the same doses as those given during the chronic administration, 1 h after elevated plus maze (EPM) testing, and between 6 and 8 p.m. on the same day.

Pentylentetrazol seizure model

We used the PTZ seizure model. This model produces reliable and reproducible seizure behavior seen during ethanol dependence and withdrawal that is generalizable to co-dependence [13, 25, 26].

Determination of seizure latency, duration and severity

On the 30th day, the test animals were randomly administered a bolus dose of PTZ (a chemoconvulsant) intraperitoneally, at a dose of 40 mg/kg body weight to determine three seizure responses, namely, seizure latency, seizure duration, and seizure severity. The dose of PTZ used (Sigma-Aldrich, St. Louis, MO, USA) was based on prior studies, which showed reliable inductions of seizures without a maximal effect on seizure severity (death) in most of the animals [18]. Seizure latency was measured as the time (in seconds) following the injection of PTZ to the first sign of seizure. Seizure duration was measured from the first sign of the seizure to seizure cessation. The severity of the seizure was recorded following PTZ administration for each animal and was graded using a modified scoring system [27] as follows: Grade 0: No signs of motor seizure activity during the 30 min observation period, Grade 1: Staring, mouth or facial movements, Grade 2: Head nodding or isolated twitches, Grade 3: Unilateral/bilateral forelimb clonus, Grade 4: Rearing, Grade 5: Loss of posture, jumping, Grade 6: Clonic/tonic seizures, Grade 7: Full tonic seizures, Grade 8: Death. Mortality following testing with PTZ was recorded for up to 72 h post-administration.

Ethical considerations

Ethical approval was obtained from the Faculty of Medicine Research Committee (FRC) and the Institutional Ethics Committee of MUST (approval number 09/04-15). Animal care followed the guidelines for the care and use of laboratory animals [28]. At the end of the experimental period, the animals that did not die during the experiment were euthanized by injecting each with 0.5 mL of 70% ethanol intraperitoneally; at this dose, rats demonstrated gross loss of muscle control, coma, and death within 2–4 min [29]. Animal carcasses were incinerated. All procedures were conducted in accordance with animal guidelines [28, 30].

Data analysis

The numerical data obtained from the experiments were subjected to the D'Agostino and Pearson Omnibus Normality test in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA; www.graphpad.com), to determine whether data were parametric or non-parametric. The outcome measures were seizure latency, seizure duration, seizure severity, and anxiety responses (open arm entries, closed arm entries, and proportion of time in arms). The results were presented as mean \pm standard error of the mean. One-way analysis of variance (ANOVA) was used for parametric data (anxiety responses, seizure latency, and duration) to determine a statistical difference between groups, whereas Kruskal-Wallis test was used for non-parametric data (seizure severity). Bonferroni's correction for multiple comparisons was used as the *post hoc* test for one-way ANOVA, whereas Dunn's test was used as the post-test for Kruskal-Wallis. Here,

$p < 0.05$ was considered significant. Graphing and statistical analysis were done using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism® version 6 (GraphPad Software Inc., La Jolla, CA, USA), respectively.

Results

The animals in the different treatment groups achieved normal progress in their body weights through the 28-day administration of treatments (Figure 1). By the end of these treatments, there was no statistical difference in body weights between the treatment groups.

The chronic caffeine and ethanol administration altered anxiety responses following exposure to the EPM (Figure 2). There was a significant effect of chronic drug administration on anxiety-time responses [$F_{(5, 54)} = 26.04$, $p < 0.0001$] with regard to time spent in the open and enclosed arms [Figure 2 (B) and (W), respectively]. Bonferroni's *post hoc* tests for multiple comparisons showed that, in comparison with controls, the administration of caffeine at 10 mg/kg, ethanol at 4 g/kg of 20%, and combined caffeine (20 mg/kg) and 4 g/kg of 20% ethanol, significantly reduced time spent in the open arms and increased enclosed arm duration ($p < 0.01$). In addition, male SD rats treated with combined caffeine 20 mg/kg and 4 g/kg of 20% ethanol spent more time in the enclosed

arm than in the open arm compared with the untreated group ($p < 0.0001$). There were no differences between the treatment groups given high-dose caffeine (20 mg/kg) and 4 g/kg of 20% ethanol in time spent in either arm, when compared with the combined caffeine and ethanol group ($p > 0.05$).

The chronic caffeine and ethanol administration showed significant effects on anxiety responses towards entry exploration [$F_{(5, 54)} = 9.44$, $p < 0.0001$], with regard to open and enclosed arms [Figure 3 (B) and (W), respectively]. The administration of caffeine 10 mg/kg, 4 g/kg of 20% ethanol, and combined caffeine 20 mg/kg and 4 g/kg of 20% ethanol significantly reduced the exploration of entries in the open arms and increased enclosed arm entries ($p < 0.05$) compared with the controls. Furthermore, the juvenile male SD rats treated with combined caffeine 20 mg/kg and 4 g/kg of 20% ethanol made more entry explorations in the enclosed arms than the open arms compared with the untreated group ($p < 0.0003$). There were no differences between the treatment groups of high-dose caffeine 20 mg/kg and 4 g/kg of 20% ethanol alone in the entry explorations in either arm when compared with the combined caffeine and ethanol group ($p > 0.05$).

The chronic caffeine and ethanol administration showed significant effects [$F_{(5, 54)} = 9.93$, $p < 0.0001$] on seizure latency following intraperitoneal PTZ administration (Figure 4). *Post hoc* tests showed no statistical significance in the seizure latency of the juvenile male SD rats administered 4 g/kg of 20% ethanol, combined caffeine (20 mg/kg) and 4 g/kg of 20% ethanol, and the untreated group compared with the controls ($p > 0.05$). However, compared with the high-dose caffeine (20 mg/kg) group, the control animals (distilled water at 4 mL/kg), 4 g/kg of 20% ethanol, untreated group, and combined caffeine and ethanol group had significantly lowered seizure latency ($p < 0.01$). There was no significant difference in the seizure latency between animals treated with low-dose caffeine at 10 mg/kg and combined caffeine and 4 g/kg of 20% ethanol ($p > 0.05$).

The chronic treatment had a significant effect [$F_{(5, 54)} = 60.11$, $p < 0.0001$] on the seizure duration following PTZ exposure to the animals (Figure 5). The *post hoc* tests showed a significant decrease in seizure duration for caffeine at 10 mg/kg, caffeine at 20 mg/kg, and combined caffeine (20 mg/kg) and 4 g/kg of 20% ethanol, compared with the control group ($p < 0.001$). Additionally, the rats treated with 4 g/kg of 20% ethanol had a prolonged seizure duration compared with all groups ($p < 0.001$). However, the animals treated with combined caffeine (20 mg/kg) and 4 g/kg of 20% ethanol showed a significant reduction in seizure duration compared with the untreated group,

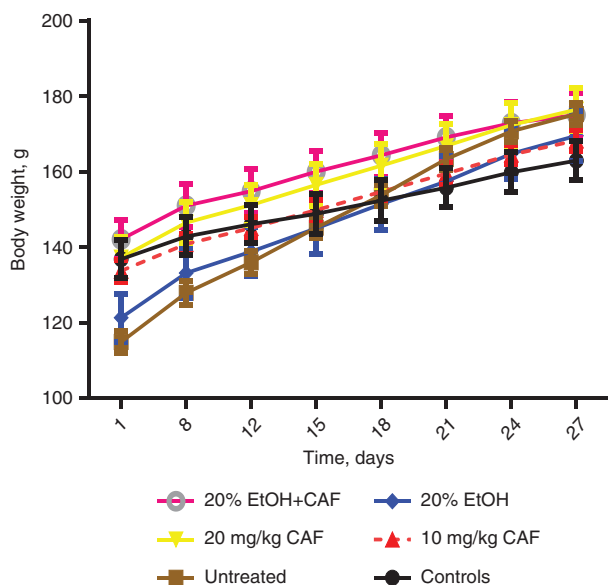


Figure 1: The changes in body weight with time (days) of controls, untreated, caffeine, ethanol, and combined caffeine and ethanol were considered.

This figure shows that all animals in their respective groups attained normal weight progression throughout the treatment period and no statistical divergence was observed; $n = 10$ animals per group.

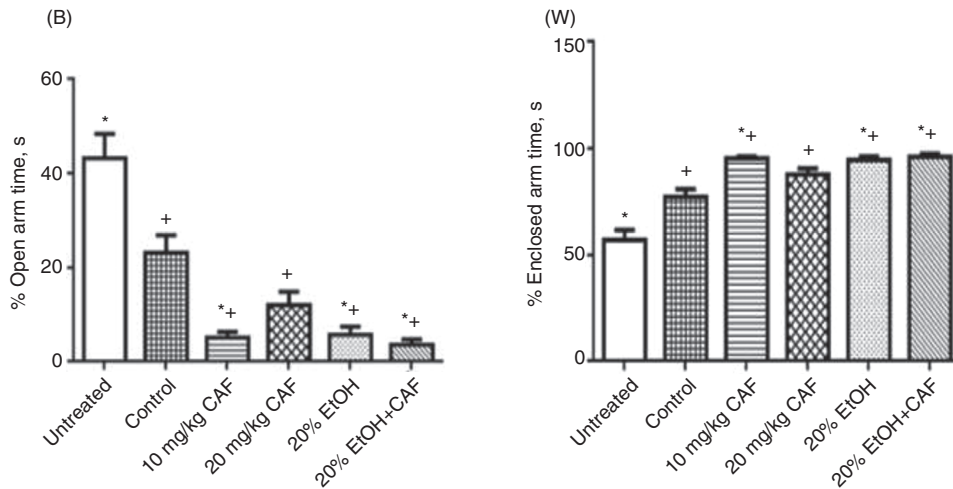


Figure 2: The effects of chronic administration of caffeine, ethanol, and combined caffeine and ethanol on anxiety-time responses in the EPM test.

Figures (B) and (W) represent the effects of 28 days treatment on percentage time spent in the open arms and enclosed arms, respectively. Data are expressed as the mean \pm SEM: * $p < 0.05$ vs. the control group; $n = 10$; + $p < 0.05$ vs. the untreated animals.

the negative control group, and the 4 g/kg of 20% ethanol group ($p < 0.0001$).

There was a significant difference ($H = 32.39$; 4 df, critical χ^2 , $\alpha [0.05 = 15]$, $p < 0.0001$) among the treatment groups in terms of seizure severity grade, following exposure to a bolus dose of 40 mg/kg of PTZ (Figure 6). The *post hoc* tests showed that male SD rats administered low-dose caffeine at 10 mg/kg showed a diminished severity score of PTZ-induced seizures compared with the control group ($p < 0.0125$). The animals treated with 4 g/kg of 20% ethanol, combined caffeine (20 mg/kg) and 4 g/kg of

20% ethanol, distilled water (4 mL/kg), and the untreated group, showed significantly higher seizure severity compared with the low-dose caffeine at 10 mg/kg ($p < 0.01$) but not at the high-dose caffeine ($p > 0.05$) groups. No significant seizure severity difference was noted between the combined caffeine and ethanol group and the 4 g/kg of 20% ethanol-only group ($p > 0.05$).

Finally, the assessment of mortality at 24, 48 and 72 h following PTZ administration showed no deaths among the animals treated with both low- and high-dose caffeine at all three time points (Table 1). The mortality of the ethanol-only

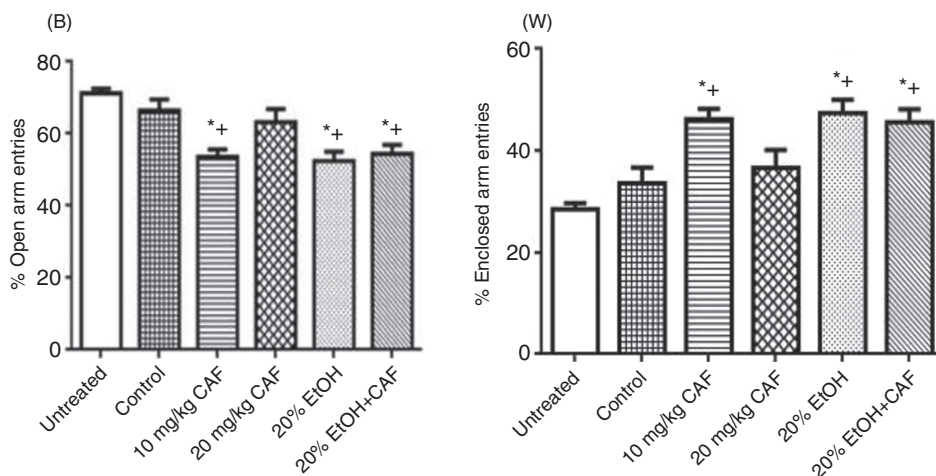


Figure 3: The effects of the chronic administration of caffeine, ethanol, and combined caffeine and ethanol on anxiety-entry responses in the EPM test.

Figures (B) and (W) represent the effects of the 28-day treatment on percentage entry exploration in the open arms and enclosed arms, respectively. Data are expressed as the mean \pm SEM: * $p < 0.05$ vs. the control group; $n = 10$; + $p < 0.05$ vs. the untreated animals.

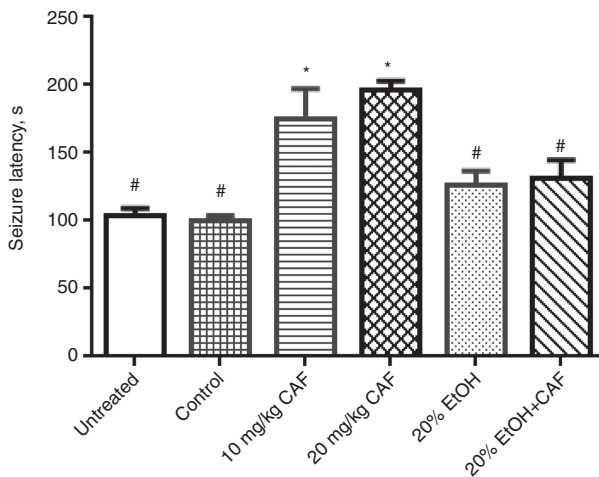


Figure 4: The effects of the chronic administration of caffeine, ethanol, and combined caffeine and ethanol on seizure latency using a PTZ model.

The figure represents the time of seizure onset of juvenile male SD rats following a bolus dose of 40 mg/kg of PTZ after the 28-day treatment with caffeine, ethanol, and combined caffeine and ethanol. Data are expressed as the mean \pm SEM: * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the 20 mg/kg caffeine group; $n = 10$; one-way ANOVA and Bonferroni post-test.

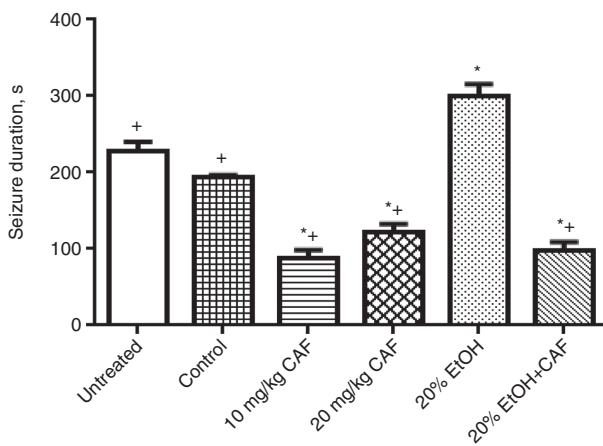


Figure 5: The effects of the chronic administration of caffeine, ethanol, and combined caffeine and ethanol on seizure duration using a PTZ model.

The figure represents the duration of seizure occurrence in juvenile male SD rats following a bolus dose of 40 mg/kg of PTZ after the 28-day treatment with caffeine, ethanol, and combined caffeine and ethanol. Data are expressed as the mean \pm SEM: * $p < 0.05$ vs. the control group; + $p < 0.05$ vs. the 20% ethanol group; $n = 10$; one-way ANOVA and Bonferroni post-test.

treated animals was comparable to that of the untreated animals and the controls at 24 h through 72 h. Whereas the combined caffeine and ethanol-treated animals showed no deaths at 24 and 48 h, this group showed 70% mortality by 72 h, indicating an increased trend towards seizure-induced

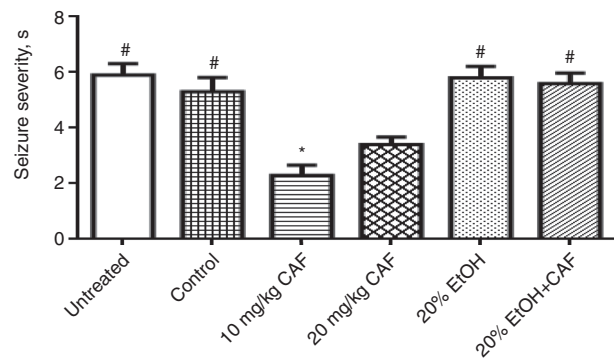


Figure 6: The effects of the chronic administration of caffeine, ethanol, and combined caffeine and ethanol on seizure severity using a PTZ model.

The figure depicts the severity of the seizures of juvenile male SD rats following a bolus dose of 40 mg/kg of PTZ after the 28-day treatment with caffeine, ethanol, and combined caffeine and ethanol using a graded scoring system. Data are expressed as the mean \pm SEM: * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the 10 mg/kg caffeine group; $n = 10$; Kruskal-Wallis and Dunn post-tests.

mortality from the withdrawal effects due to the combined use ($\chi^2 = 12.54$, $p = 0.05$).

Discussion

The goal of our study was to investigate how the chronic co-consumption of caffeine and ethanol and the acute withdrawal from these substances affect seizure vulnerability and anxiety responses in male SD rats. We used a PTZ seizure model to determine the behavioral characteristics of the seizures following the chronic combined use of ethanol and caffeine and extending into the acute withdrawal phase (72 h). The elevated plus-maze was used to assess the anxiety-like behaviors. Our main findings indicated that chronic co-administration of caffeine and ethanol and a brief period of withdrawal increased anxiety, whereas seizure vulnerability increased during an acute phase of withdrawal in male SD rats.

Previous studies suggest that chronic low-dose caffeine given to juvenile rats triggers anxiogenic effects, whereas high-dose caffeine elicited anxiolytic effects [31]. Additionally, an experimental study conducted on male and female PVG/c rats administered caffeine (25 or 50 mg/kg), and exposed to an open field (OF) or EPM showed decreased anxiety indicated by increased OF rearing and decreased grooming, immobility, and corner occupancy in the presence of bright light after either higher or both doses of caffeine [32]. Furthermore, in the same study, male rats had increased caffeine-related entries and observations in

Table 1: The effects of chronic caffeine, ethanol, and combined caffeine and ethanol on PTZ-induced mortality at 24, 48 and 72 h (number of deaths in a treatment group/total number of rats in the group).

| Dose group | Mortality at 24 h | Mortality at 48 h | Mortality at 72 h | Total |
|---------------|-------------------|-------------------|-------------------|------------|
| Untreated | 1/10 (10%) | 2/10 (20%) | 2/10 (20%) | 5/10 (50%) |
| Control | 2/10 (20%) | 0/10 (0%) | 2/10 (20%) | 4/10 (40%) |
| 10 mg/kg CAF | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) |
| 20 mg/kg CAF | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) |
| 20% Ethanol | 1/10 (10%) | 3/10 (30%) | 2/10 (20%) | 6/10 (60%) |
| Ethanol + CAF | 0/10 (0%) | 0/10 (0%) | 7/10 (70%) | 7/10 (70%) |

The PTZ treatment for animals chronically administered with low-dose caffeine at 10 mg/kg and high-dose caffeine at 20 mg/kg showed no mortality at 72 h. Conversely, the chronic combined caffeine (20 mg/kg) and 20% ethanol (4 g/kg) as well as the 20% ethanol alone, showed marked mortality following the PTZ treatment, thus indicating increased vulnerability to the seizure-induced lethality due to the combined use ($\chi^2 = 12.54$; $p = 0.05$).

the EPM open arms in the presence of bright light, consistent with the anxiolytic effect demonstrated by the high-dose caffeine group. The pronounced trend for the observed behaviors indicative of anxiety as well as seizure susceptibility in our animal model might be attributable to adenosine neurotransmission in the CNS [33].

The anxiolytic effect of 4 g/kg ethanol is normally implicated in the development of alcohol dependence [34]. Acute ethanol ingestion has well-known anxiolytic effects. However, a recent animal study showed that chronic intermittent alcohol administration might produce persistent anxiety in both juvenile and adult rats [35], depending on the scheme of administration (intervals between administration) and dose. The chronic ethanol-treatment of juvenile male SD rats in our study demonstrated the anxiogenic effects in the EPM test. There was some degree of withdrawal in the rats, considering that the EPM testing was done the day after stopping the administration of the test substances, a situation that likely contributed to the rebound anxiogenic effect observed. A previous study that explored alcohol-induced adaptations and patterns of c-Fos activation in the locus coeruleus of male and female SD rats found that chronic ethanol exposure produces an anxiogenic-like response in both sexes but may render males less vulnerable to subsequent stress exposure [12]. Additionally, emerging evidence suggests that juvenile chronic ethanol dependence stimulates stress hormones that have neuromodulatory effects on the dopaminergic system and other associated neurobehavioral functions [36], possibly contributing to the anxiogenic behavior identified in the ethanol-only treated rats in the present study.

Seizure expression in male juvenile SD rats that were administered combined caffeine and ethanol showed shortened seizure latency and increased severity compared with those that received caffeine-only treatment, but significantly shortened seizure duration compared

with the ethanol-only treatment or control, following a bolus dose of PTZ. PTZ, a GABA_A receptor antagonist, specifically targets the Cl⁻ ionophore and not the GABA binding of the receptor complex [37, 38]. PTZ causes long-term alterations in the synaptic anatomy and physiology of the hippocampus, and these alterations are responsible for the depressant effect of adenosine and decrease in GABAergic inhibition [39].

These findings suggest that habitual caffeine and ethanol co-consumption followed by acute withdrawal provoke alterations in seizure vulnerabilities by lowering seizure latency following PTZ exposure in male juvenile SD rats [40, 41]. Conversely, in the present study, chronic caffeine treatment alone significantly protected against seizure expression in male juvenile SD rats, a finding consistent with results obtained from a study, which showed the neuroprotective effect of low-dose, long-term caffeine exposure on epileptic neuronal damage [42]. Furthermore, other experimental findings showed that epileptiform bursting is reduced after caffeine treatment, implying a potential role in the modulation of epilepsy development after a severe head injury [43].

The ethanol-only treated rats exhibited significantly prolonged seizure duration, which can be excitotoxic to neurons, a finding related to a study, which showed that chronic ethanol and PTZ exposure induced neuronal death in the rat cortical and hippocampal neurons [44]. On the contrary, the chronic co-administration of caffeine and ethanol to male juvenile rats showed shortened seizure latency and duration, coupled with heightened seizure severity grade. The protective outcome of caffeine following seizure expression and vulnerability may be mediated by the adenosinergic system, which may modulate seizure expression [45].

Finally, the chronic combined caffeine and ethanol administration to the juvenile male rats was associated with increased PTZ-induced death observed after 48 h,

with 7/10 (70%) mortality recorded within the last 24 h of the 72-h period. Comparable to the caffeine-only treated groups, none of the animals in the combined caffeine and ethanol group died by the 24-h and 48-h time points, suggesting that caffeine may have initially contributed to the neuroprotective effect. The addition of ethanol to caffeine, unlike in the ethanol-only treated group, markedly increased the mortality at 72 h, whereas no mortality occurred in the presence of ethanol combined with caffeine by the end of 48 h. The mortality in the post-seizure period was attributed to the withdrawal phenomena subsequent to chronic combined caffeine and ethanol administration in the rats, since none of the rats in the caffeine-only or ethanol-only treatments died. This finding suggests that combined withdrawal effects from caffeine and ethanol may result in severe deleterious consequences that should be borne in mind, especially in the acute period.

One of the limitations of our study was the timing in the administration of test substances and the timing in the conduct of the two behavioral tests (anxiety response battery and seizure vulnerability). To prevent the development of severe withdrawal effects from either caffeine or ethanol prior to seizure susceptibility testing, we administered the test substances immediately after completing the EPM testing but considering the relatively large number of animals, it is possible that some withdrawal phenomena occurred. However, our data on mortality following PTZ injection did not support the premise that the withdrawal phenomena within 24 h were severe. None of the animals in the caffeine group died at the 24-, 48-, or 72-h time points of withdrawal following PTZ administration. Furthermore, no animal in the combined caffeine and ethanol group died by 24 or 48 h of withdrawal. Likewise, only one animal in the ethanol-only group died by 24 h of withdrawal, a number comparable to the untreated group. This suggests that while a brief period of withdrawal during the EPM testing (within 12 h) may confound interpretation, the nature of the withdrawal phenomena may not be severe enough compared with that seen at 48 or 72 h. Another limitation is that we did not measure blood ethanol concentrations (BEC) prior to the administration of the battery of behavioral tests. However, considering that chronic administration of caffeine and ethanol leads to the development of tolerance and dependence, the BEC measurement may not have provided an accurate assessment of withdrawal, given that the time during which animals were first tested was short (<12 h). Regardless, the third day of withdrawal, particularly from ethanol, appears to be the time at which manifestations of withdrawal are most pronounced, thus leading to extreme consequences including death [13, 26].

Our work contributes to the evidence showing that the combined intake of caffeinated drinks with ethanol may be deleterious to the brain if taken chronically. Together with human data reporting the dangers of the chronic adolescent consumption of caffeine-mixed alcohol [2, 10], our results should draw attention to the potential safety risks of using highly caffeinated drinks with alcohol by adolescents and young adults. A significant implication of these findings is that the treatment of adolescents and young adults who chronically use caffeinated drinks with alcohol should consider the likelihood of exaggerated adverse behavioral effects during use and in acute withdrawal. Targeted interventions for such individuals, may thus benefit from developing protocols that take into account a history of significant consumption of both caffeinated energy drinks and ethanol.

In conclusion, our findings demonstrated that the administration of caffeine alone protected against PTZ-induced seizures, whereas ethanol alone and combined caffeine and ethanol in juvenile male SD rats lead to anxiogenic responses and increased seizure vulnerability, particularly during acute withdrawal. The combined caffeine and ethanol treatment also produced high mortality in the acute withdrawal phase following PTZ administration. Further studies are recommended to understand the neurochemistry responsible for these PTZ-induced behavioral outcomes in the adolescent brain exposed to combined ethanol and caffeine as well as the consequences during the immediate withdrawal period.

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