Neuroscience Letters 670 (2018) 1–7

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Behavioral effects of the combined use of alcohol and energy drinks on alcohol hangover in an experimental mice model

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ARTICLE INFO

Keywords:
Alcohol
Energy drinks
Alcohol hangover
Locomotor activity
Neuromuscular coordination
Anxiety-like behavior
Mice

ABSTRACT

In last few years it has been a significant increase in the consumption of alcohol combined with energy drink. The aim of this work was to study the effect of this mixture in motor and affective behaviors during an alcohol hangover episode. Male Swiss mice received one of the following treatments: saline + sucrose; saline + energy drink; ethanol + sucrose; ethanol + energy drink. Ethanol dose was 3.8 g/kg BW (i.p.) and energy drink dose was 18 ml/kg BW (gavage) at ZT1 (8 am) (ZT: Zeitgeber time; ZT0: 7 am; lights on). The behavioral tests used were tight rope test to determine motor coordination; hanging wire test to study muscular strength; elevated plus maze and open field tests to evaluate anxiety like-behavior and locomotor activity. Tests were carried out at basal point that matched with lights onset and every 6 h up to 18 h after treatments. Hangover onset was established at ZT7 when blood alcohol concentration (BAC) was almost zero. Our results showed that the mixture of alcohol and energy drink altered significantly motor skills. Specifically, a significant decrease was observed in the performance of the animals in the tightrope and hanging wire tests in groups treated with the mixture of alcohol and energy drink. A significant impairment in the anxiety-like behavior was observed mainly at the beginning of alcohol hangover. These findings suggest that energy drink added to alcohol extends motor disabilities observed during an alcohol hangover episode in comparison with animals that received alcohol alone.

1. Introduction

Mixing alcohol with highly caffeinated energy drinks (AmED) has become increasingly popular among teenagers and young adults due to the prevailing view that the stimulant properties of energy drinks (ED) decrease the depressant effects of alcohol, leading individuals to believe they are less drunk and can drink more or for longer periods of time [1,2]. AmED may produce a false sense of confidence that induces to the drinker to carry out risk tasks [3–5]. Thus, the co-consumption of ED and alcohol has become a topic of concern and an increasingly important public health problem [6,7].

Alcohol hangover (AH) refers to the combination of cognitive and physical symptoms experienced the day after a single episode of heavy drinking, starting when blood alcohol concentration approaches zero [8,9]. Otherwise, the effects of alcohol hangover could overlap with withdrawal symptoms [9,10]. The hangover is an important issue in light of ED and alcohol co-use because cognitive, emotional and motor functions are negatively affected during AH with significant individual, social and economic consequences [11,12]. In this sense, perhaps the most important aspect is that adolescents believe that ED and alcohol co-use mitigates hangover symptoms which could play a role in motivation to consume this mixture, highlighting that this age group may be at particularly high risk for consequences arising from AmED consumption[13]. Interestingly, Costa et al. [14] have reported that more than a third of ED Australian adolescent consumers (12–18 years) exceed the daily limit of ED considered appropriate for adults (two standard ED/day). In addition, the amount of ED consumed was positively correlated with the presence of negative physiological symptoms and adolescents risk taking.

In humans, AH is characterized by headache, thirst, nausea, vomiting, tremors, diarrhea, sleepiness, fatigue, diminution in motor coordination and impaired cognitive functioning [15–17]. In addition, it has been suggested that other alterations such as dehydration, electrolyte imbalances, hypoglycemia, sleep and biological rhythm disturbances are produced by AH [15,18–20]. AH physiopathology is unknown and although several articles discuss a number of hypotheses it remains unclear. Acetaldehyde, the principal metabolite of ethanol, has been suggested as one of the causes of perturbations observed during AH [18]. Maxwell el al. [21] proposed that acetate is responsible of AH headache throughout inflammatory mechanism.

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https://doi.org/10.1016/j.neulet.2018.01.030
Received 5 October 2017; Received in revised form 7 January 2018; Accepted 16 January 2018
Available online 02 February 2018

In experimental animal models it has been demonstrated that during AH there is a decrease in neuromuscular coordination, motor strength and locomotion together with gait variability and slowness in exploratory activity [22]. Also, anxiety-like behavior together with fear related phenotype and depression signs have been shown [23]. Bustamante et al. [24] have related motor performance alterations with mitochondrial dysfunction during AH in mice. Greater locomotor activity, increased anxiety-like behavior, lost their righting reflexes sooner and poorer motor coordination, were observed in mice treated with AmED indicating that alcohol-induced deficits are aggravated by ED [25]. The combined administration of alcohol and ED may trigger rewarding effects in mice that were not stimulated by alcohol alone [26]. Preclinical studies in rodents indicated that adolescents may respond differently than adults to the combination of alcohol with ED. In these sense, adolescent ED consumption was not correlated with changes in adult alcohol intake or preference, suggesting that exposure to large amounts of caffeine does not alter future alcohol intake [27]. In addition, Fritz et al. [28] have reported that caffeine increased binge consumption of alcohol in adolescent and adult mice, but produced additive motor stimulation only in adolescent animals and also later alcohol intake and preference was not influenced by prior consumption history.

On this basis, the aim of the present study was to describe the motor and anxiety-like behavior effects of the AmED when BAC is closed to zero (onset of alcohol hangover). Specifically, we studied the effects of alcohol alone or mixed with ED at high dose on the spontaneous locomotor activity and anxiety-like behavior in mice. For this propose, tightrope, hanging wire, open field and elevated-plus maze (EPM) tests were used to evaluate the interaction produced by the AmED still when the amount of ED consumed exceeds the daily limit considered appropriate for adults.

2. Materials and methods

2.1. Animals

Male Swiss mice (Mus musculus) weighing 30–40 g were acquired from the School of Biochemistry, University of Buenos Aires, and housed in a soundproof room under conditions of controlled temperature (22 ± 2 °C) and humidity, with a 12:12 h light/dark cycle. Standard mice chow and tap water were provided *ad libitum*.

Animal handling, treatments and experimental procedures were reviewed in accordance with the guidelines of the National Institutes of Health (USA) and with Regulation 6344/96 of Argentina's National Drug, Food and Medical Technology Administration (ANMAT). Moreover, the present study had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling of the School of Medicine from University of Buenos Aires (CICUAL) where the protocol was performed. All efforts were made to minimize suffering and reduce the number of animals used.

2.2. Drugs and experimental procedure

We used Red Bull®, a widely consumed and advertised ED, which composition, according to the manufacturer is:100 ml of Red Bull has 11.3 g of sucrose and glucose, 400 mg of taurine, 32 mg of caffeine, 240 mg of gluconolactone, 20 mg inositol, 7.2 mg of niacin, 2.4 mg of pantenol, 0.4–0.8 mg of vitamins B2/B6/B12, citric acid, caramel coloring, artificial flavoring and sparkling water. ED was administrated by gavage in a dose of 18 ml/kg BW. ED dose was chosen to exceed the daily limit considered appropriate for adults and was calculated considering the injected dose of alcohol and the amount of ED necessary to achieve a relationship between ED and alcohol equivalent to that used by Ferreira et al. (2.4 g/kg EtOH: 10.71 ml/ kg ED) [26]. Ethanol (EtOH) was used at a concentration of 15% (3.8 g/kg BW, i.p.). EtOH dose was previously applied in alcohol-induced hangover animal models [22–24]. The sucrose solution was given by gavage at a dose of 18 ml/kg BW (8.03 kilocalories/kg, isocaloric respect to ED). Animals were randomly divided in four groups: SAL (saline) + SS (Sucrose solution); EtOH + SS; SAL + ED; EtOH + ED. It is important to note that although human beings consume the mixture orally, under our experimental conditions it was not possible. Previously, we had conducted a pilot test and observed that the volume of the mixture required achieving the desired dose of alcohol and energy drink exceeded mice stomach capacity [29] and most of animals showed a backflow of the mixture. This very important physical limitation prevented us to administer the ED by gavage.

2.3. Determination of the onset of hangover

In order to determine the animal's response to ethanol and the onset of hangover, blood alcohol concentrations (BAC) were evaluated for each group of animals. They were decapitated 60, 180 or 360 min after the injection (n = 5 each time point). Blood was collected from the trunk and was measured by gas chromatography. Experiments were conducted in the morning (9:00 am). The criteria used to establish the onset of AH was when BAC was less than or equal to 10% of the maximum value reached.

2.4. Behavioral assessments

Treatments were administrated one hour after lights on: ZT1 (8 am) (ZT: Zeitgeber time). Behavioral tests were carried out at a basal point that matched with lights onset (ZT0: 7 am, 1 h before treatment) and every 6 h after treatment: ZT7 (2.00 pm, when AH began), ZT13 (8 pm, 12 h after treatment) and ZT19 (2 am, 18 h after treatment). (See Fig. 1). Each subject was tested every 6 h in only one behavioral test avoiding multiple tasks for animal groups. Motor performance, motor strength, locomotion and anxiety like-behavior were evaluated at specific times described above, using a battery of different behavioral tests. During experimental procedures, test boxes or the apparatus used for behavioral studies were cleaned with 10% EtOH solution after every individual test session to prevent the next mouse from being influenced by the odors deposited in the urine and feces of the previous mouse.

2.5. Tightrope test

Motor coordination was evaluated with a modified tightrope test [30]. Briefly, the procedure consisted in placing the animal on the middle of a 60 cm long horizontal rope suspended 30 cm above the floor and time was recorded until the animal either reached the end of the rope or fell down during a period of 60 s. A score was assigned accordingly: animals reaching the end of the rope in ≤6 s were given 1 point and an additional point was given for every additional 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end obtained 11 points. When mice fell down, while test was running, 11 points were assigned and 1 extra point was added for every 6 s before the test ending time (60 s). The test evaluates the motor performance of the animal as a mean of its intrinsic neuromuscular

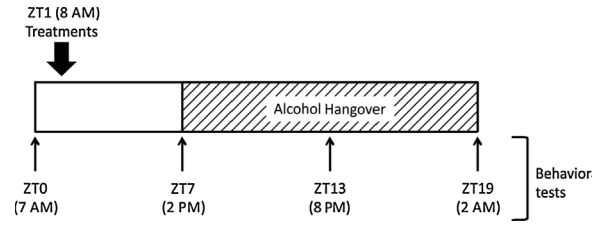

Fig. 1. Timeline and experiments Male mice received different treatments at 8:00 AM. Behavioral tests were performed before (ZT0) and after treatment: ZT7 (when alcohol hangover began), ZT13 and ZT19. Real time figure between brackets. ZT: Zeitgeber time; ZT12: 7:00 PM lights off; photoperiod 12:12 l:D.

coordination. For this work, results were shown as a percentage of the motor performance which was calculated considering the maximum score for the test (20 points) and the score reached for each animal.

2.6. Hanging wire test

Neuromuscular abnormalities were detected by the evaluation of balance and grip strength in a hanging wire [31]. A standard wire cage lid was used. The perimeter was masked by duct tape to prevent the mice from walking off the edge. The hanging wire test was performed by placing the mouse on the top of a wire cage lid. The lid was lightly shaken three times to cause the mouse to grip the wires, and then the lid was turned upside down. The upside-down lid was held at a height approximately 20 cm above the cage litter. The latency to fall off the wire lid was quantified. Normal mice can hang upside down for at least one minute. A cut-off time of 60 s was used for each test session.

2.7. Elevated-plus maze

Anxiety-like behavior was evaluated by the elevated-plus maze (EPM). The apparatus (made of Plexiglas) consisted of two open arms (10 cm × 50 cm) alternating at right angles with two closed arms (10 cm × 50 cm × 10 cm), delimiting a central area. The whole maze was elevated 50 cm above the floor. Mice were placed in the central area of the maze, facing one of the open arms, and were allowed to explore it for 5 min as previously described [23]. The percentages of entries in the open arms (%FEO) and the time spent in the open arms (%TSO) were calculated as entries or time in the open arms over the total entries or time, respectively. These parameters and total entries (TE) were measured following a four-paw criterion; entry into the arm of the EPM was defined as the animal placing all four paws in that particular part of the maze. The maze's arms were equally illuminated, so that the animals did not perceive lighting differences. EPM rests on the conflict between the tendency of mice to explore a novel environment and the aversive properties of a brightly lit, open area. It is considered that anxiety-like behavior is characterized by a decreased in% FEO and%TSO. Also, the parameter of TE provides a locomotor activity measure.

2.8. Open field test

Anxiety-like tendency and locomotor activity were evaluated by the open field test. The test box consisted of a 60 cm × 60 cm square arena surrounded by a 50 cm high wall divided in two zones: center (11% of the entire area) and periphery. The apparatus (made of Plexiglas) was elevated 80 cm off the floor level. Mice were individually tested in the open field during a 5 min session. At the onset of the session, mice were placed at the center of the apparatus. The time in the central zone (s) was scored during the open field test session. In this sense, less time in the central area indicates a possible anxiety-like behavior. Also, the number of line crossings was considered as a locomotor activity measure.

2.9. Statistical analysis

Results are presented as means ± SEM. Before each analysis, test variables were checked for normality, so all data were evaluated by the Kolmogorov–Smirnov test to follow a posterior parametric or non-parametric statistical analysis. In elevated plus maze and open field cases, data obtained were analyzed using univariate ANOVA (post-hoc test Bonferroni) to test the significance of differences between the groups. In group along time comparisons, differences were examined by repeated-measures ANOVA; post-hoc test Bonferroni. In hanging wire and tight rope tests, data were analyzed using Kruskal-Wallis test; post-hoc Mann Whitney (Bonferroni correction) to compare unpaired groups. In the case of related samples, Friedman and Wilcoxon were used. In all the cases, the statistical software used was SPSS (version 22.0) and a difference was considered statistically significant when $p < 0.05$.

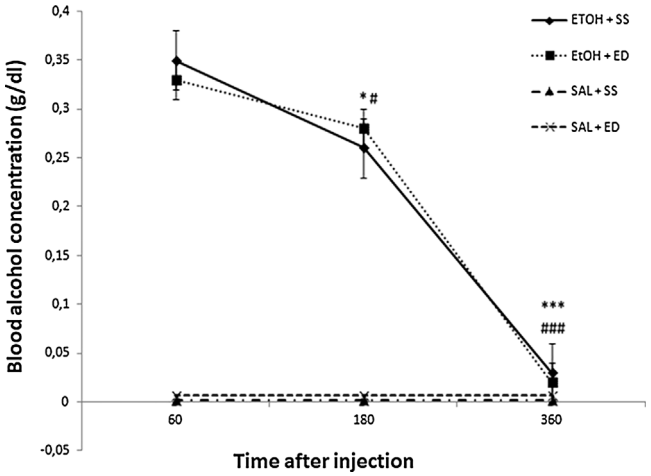

Fig. 2. Blood alcohol concentration (BAC) after acute i.p. injection of ethanol and/or energy drink administration by gavage. BAC was measured 60, 180 and 360 min after treatments to determinate the onset of hangover. SAL: saline; SS: sucrose; ED: energy drink; EtOH: ethanol. Values are expressed as mean ± SEM (n = 15 each group). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs 60 min in EtOH + SS group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs 60 min in EtOH + ED group. Independent samples *t*-test.

3. Results

3.1. Determination of the onset of hangover (Fig. 2)

After 180 and 360 min post- injection BAC decreased significantly ($p < 0.05$ and 0.001, respectively) in EtOH + SS and EtOH + ED groups. At 360 min, the BAC was 95% decreasing from its starting value at 60 min in these groups. On this basis, it was considered that AH started six hours (360 min) after EtOH treatment. Also, there were no significant differences in the BAC between EtOH + SS and EtOH + ED groups at 60 and 360 min post injection ().

3.2. Tight rope test (Fig. 3)

The results were expressed as percentage of motor performance (calculated as it was indicated in Materials and methods section). When compared different treatments at the same ZT, significant differences were found ($K(3) = 25.948$, $p < 0.001$). At the onset of AH (ZT7), groups treated with alcohol, showed significant differences in motor coordination compared with the respective control group: EtOH + SS vs Sal + SS ($U = 2.500$, $p < 0.001$); EtOH + ED vs Sal + ED ($U = 5.000$, $p < 0.001$). At ZT13 a significant decrease in percentage of motor performance was observed in EtOH + ED vs SAL + ED ($U = 13.500$, $p = 0.004$) and EtOH + ED vs EtOH + SS ($U = 17.000$, $p = 0.007$). When compared each group of treatment along time, alcohol treated-animals showed a significant decrease in this parameter ($X^2(3) = 14.912$, $p = 0.002$; EtOH + ED: $X^2(3) = 15.918$, $p = 0.001$). In these groups, significant differences were found in motor coordination at the beginning of AH (ZT7) with respect to the basal time (ZT0): EtOH + SS ($Z = -2.936$, $p = 0.003$); EtOH + ED ($Z = -2.683$, $p = 0.007$) and also in EtOH + ED at ZT13 compared to ZT0 ($Z = -2.492$, $p = 0.013$). No significant changes were detected in% of latency to fall between the animals treated with SAL +SS and SAL + ED as well as in each of these groups at different times after treatment ().

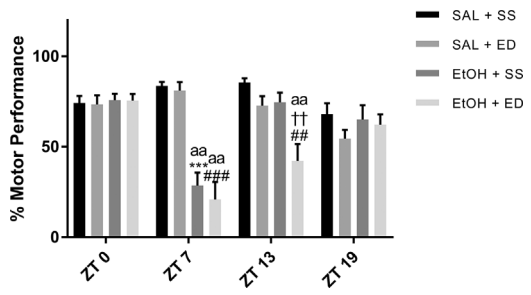

Fig. 3. Effect of alcohol mixed with energy drink in motor performance on the tight rope test during alcohol hangover. SAL: saline; SS: sucrose; ED: energy drink; EtOH: ethanol; ZT: Zeitgeber time. The colors of the bars indicate different treatments. Behavioral tests were performed 1 h before (ZT0) and 6 h after treatment when alcohol hangover began: ZT7 (2 pm), ZT13 (8 pm) and ZT19 (2 am). Values are expressed as mean ± SEM (n = 12 each group). Statistical comparisons were performed using Friedman test for intragroup differences in related samples, followed by Wilcoxon test when appropriate (^{aa} $p < 0.01$ vs ZT0). For intergroup differences was used Kruskal-Wallis test followed by Mann Whithney test ($^{***}p < 0.001$ vs Sa l + SS; $^{\#\#}p < 0.01$ vs Sa l+ ED·$^{\#\#\#}p < 0.001$ vs Sal + ED;$^{\dagger\dagger}p < 0.01$ vs EtOH + SS). In all cases, the significant level was fixed at $p < 0.05$.

3.3. Hanging wire test (Fig. 4)

The results were expressed as percentage of latency to fall. When compared different treatments at the same ZT, significant differences were found in latency to fall ($K(3) = 27.617$, $p < 0.001$). At ZT7 significant decreases in latency to fall were observed between groups: EtOH + SS vs SAL + SS ($U = 8.000$, $p < 0.001$); EtOH + ED vs SAL + ED ($U = 2.000$, $p < 0.001$) and at ZT13 in EtOH + ED vs SAL + ED ($U = 17.500$, $p = 0.004$) and EtOH + ED vs EtOH + SS ($U = 15.000$, $p = 0.007$). When compared each treatment along time, alcohol treated-animals showed significant differences in latency to fall (EtOH + SS: $X^2(3) = 15.822$, $p = 0.001$; EtOH + ED: $X^2(3) = 14.226$, $p = 0.003$). In these groups, significant differences were found at the beginning of AH (ZT7) with respect to the respective basal time (ZT0): EtOH + SS ($Z = -2.701$, $p = 0.007$); EtOH + ED: ($Z = -2.677$, $p = 0.007$) and also in EtOH + ED at ZT13 compared to ZT0 ($Z = -2.371$, $p = 0.018$). No significant changes were detected in% of latency to fall between the animals treated with SAL +SS and SAL + ED as well as in each of these groups at different times after treatment ().

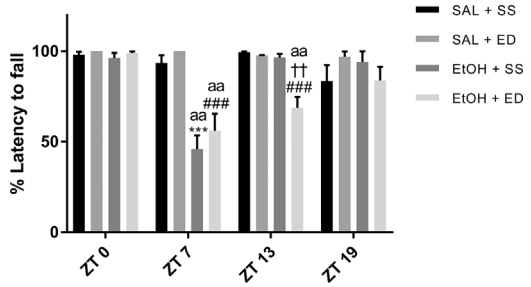

Fig. 4. Effect of alcohol mixed with energy drinks in motor strength during alcohol hangover evaluated trough Hanging Wire test. SAL: saline; SS: sucrose; ED: energy drink; EtOH: ethanol; ZT: Zeitgeber time. The colors of the bars indicate different treatments Behavioral tests were performed 1 h before (ZT0) and 6 h after treatment when alcohol hangover began: ZT7 (2 pm), ZT13 (8 pm) and ZT19 (2 am). Values are expressed as mean ± SEM (n = 12 each group). Statistical comparisons were performed using Friedman test for intragroup differences in related samples, followed by Wilcoxon test when appropriate (^{aa} $p < 0.01$ vs ZT0). For intergroup differences was used Kruskal-Wallis test followed by Mann Whithney test ($^{***}p < 0.001$ vs Sal + SS; $^{\#\#}p < 0.01$ vs Sal + ED·$^{\#\#\#}p < 0.001$ vs Sal + ED;$^{\dagger\dagger}p < 0.01$ vs EtOH + SS). In all cases, the significant level was fixed at $p < 0.05$.

3.4. Elevated plus maze (Fig. 5)

The results are expressed as% TSO, % FEO and TE (calculated as it was indicated in Materials and Methods section). No significant interaction of treatments x ZT was found in% TSO ($F(9199) = 1.085$, $p = 0.376$) and% FEO ($F(9209) = 1.336$, $p = 0.220$). However, the interaction of treatments x ZT ($F(9233) = 3.086$, $p = 0002$) influenced the number of total entries in the open and closed arms. In addition, there were no direct treatment differences in% TSO ($F(3199) = 1.439$, $p = 0.233$). By the contrary, treatment significantly affected% FEO ($F(3209) = 4.019$, $p = 0.008$) and TE ($F(3.233) = 8313$, $p = 0.001$). ZT differences between groups were observed in% TSO ($F(3.199) = 13.058$, $p = 0.001$), % FEO ($F(3.209) = 7.364$, $p = 0.001$) and TE ($F(3.233) = 87.297$, $p = 0.001$). Compared with their respective same ZT control group: at ZT7 there were significant differences in% TSO between EtOH + SS vs SAL+ SS ($p < 0.001$) and EtOH + ED vs SAL+ ED ($p < 005$); % FEO: EtOH + SS vs SAL + SS ($p < 0.01$) and EtOH + ED vs SAL + ED ($p < 0.05$); TE: EtOH + SS vs SAL + SS ($p < 0.001$) and EtOH + ED vs SAL + ED ($p < 0.001$). In addition, there were significant differences in TE at ZT13 between EtOH + ED vs SAL + ED ($p < 0.001$) and EtOH + ED vs EtOH+ SS ($p < 0.05$). In group along time comparisons, significant differences were observed in% TSO and% FEO between EtOH + SS at ZT7 vs the same group at ZT0 (%TSO: $F(3.55) = 7.835$, $p < 0.01$; % FEO: $F(3.53) = 4.035$, $p < 0.01$). Also, a significant difference was observed in% TSO and% FEO when compared ETOH + ED at ZT7 vs ZT0 (% TSO: $F(3.51) = 7.068$, $p < 0.001$; % FEO: $F(3.53) = 4.643$, $p < 0.01$) ().

3.5. Open field test (Fig. 6)

Anxiety and locomotor activity was measured using time spent in central area and number of line crossings respectively at open field test. No significant interaction of treatments x ZT was found in time spent in the central area ($F(9153) = 1.473$, $p = 0.162$). However, a significant interaction was observed in line crossings x ZT ($F(9.156) = 2.142$, $p = 0.029$). In addition, there were direct ZT differences in: time spent in central area ($F(3.153) = 15.094$, $p < 0.01$) and number of line crossings ($F(3156) = 21.729$, $p < 0.001$). Also, there were direct treatment significant differences in number of line crossings ($F(3156) = 11.201$, $p < 0.01$; but treatment did not influence time spent in central area ($F(3153) = 1.685$, $p = 0.173$). Compared with their respective same ZT control group, at ZT7 there were significant differences in time spent in central area between groups EtOH + SS vs SAL + SS ($p < 0.05$) and EtOH + ED vs SAL + ED ($p < 0.01$); number of line crossings: EtOH + SS vs SAL+ SS ($p < 0.05$) and EtOH+ ED vs SAL + ED ($p < 0.01$). At ZT13, there were also significant differences between groups EtOH +ED and EtOH + SS ($p < 0.05$) and EtOH + ED vs SAL +ED ($p < 0.01$). In group along time comparisons, it there were significant differences in time in central area between ZT7 and ZT0 in groups EtOH + SS ($F(3.41) = 6.199$, $p = 0.001$) and EtOH +ED ($F(3.43) = 6.522$, $p = 0.001$). In addition, when compared number of line crossings with respect to basal time, there were significant differences in EtOH + ED at ZT7 and ZT13 ($F(3.48) = 9.047$, $p < 0.001$) and in group EtOH + SS at ZT7 ($F(3.44) = 11.786$, $p < 0.001$) ().

4. Discussion

Our results showed that the mixture of alcohol and energy drink modified motor and anxiety-like behavior in mice during the time course of AH. Specifically, locomotor disturbances were observed in the performance of AmED treated-animals at ZT7 and ZT13, meanwhile perturbances in anxiety-like behavior were observed mainly only at the beginning of the AH (ZT7).

It was observed that the AmED group took longer than alcohol alone group to return to control values in motor tests. In fact, a significant

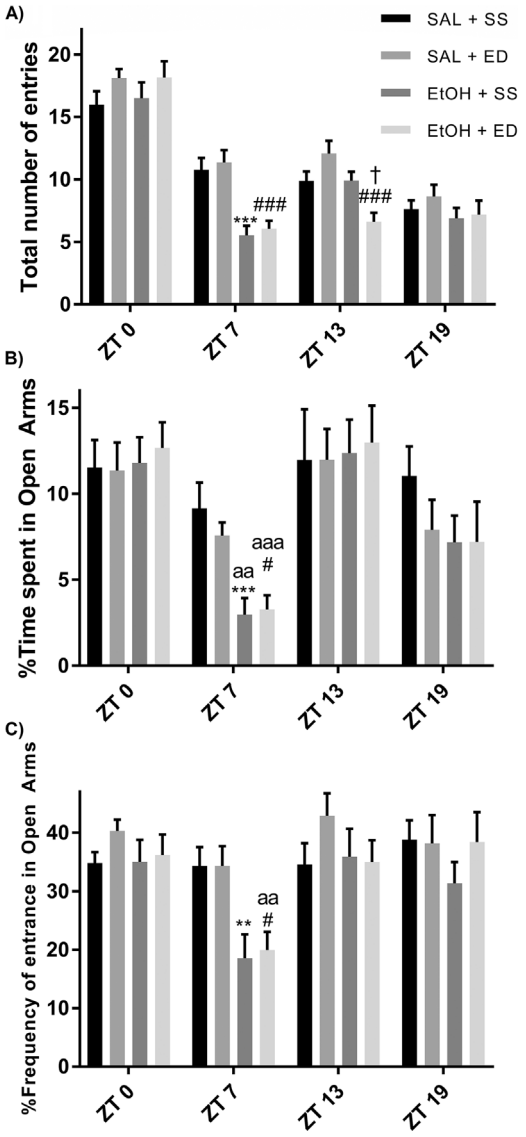

Fig. 5. Effect of alcohol mixed with energy drinks in locomotion and anxiety-like behavior on the Elevated plus maze test during alcohol hangover. A) Total number of entries (TE); B): proportion (%) of time spent in open arms (%TSO); C) proportion of entrance into open arms (%FEO). SAL: saline; SS: sucrose; ED: energy drink; EtOH: ethanol; ZT: Zeitgeber time. The colors of the bars indicate different treatments. Behavioral tests were performed 1 h before (ZT0) and 6 h after treatment when alcohol hangover began: ZT7 (2 pm), ZT13 (8 pm) and ZT19 (2 am). ZT: Zeitgeber time. Values expressed as mean ± SEM (TE: n = 12; %FEO: n = 12; %TSO: n = 12). Statistical comparisons were made using ANOVA (post hoc Bonferroni) to test the significance of differences between the groups (**p < 0.01 vs Sal + SS; ***p < 0.001 vs Sal + SS; #p < 0.05 vs Sal + ED; ###p < 0.001 vs Sal + ED; †p < 0.05 vs EtOH + SS). In group along time comparisons, differences were examined by repeated-measures ANOVA; post hoc test Bonferroni (aap < 0.01 vs ZT0; aaap < 0.001 vs ZT0. In all cases, the significant level was fixed at p < 0.05.

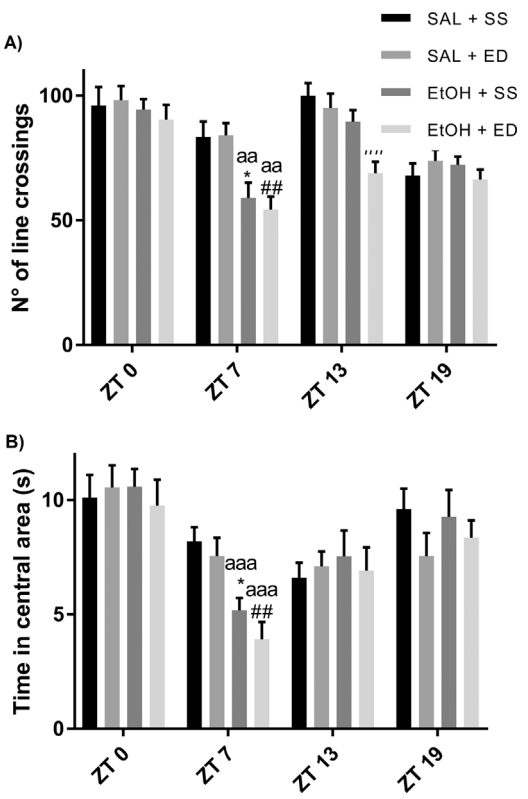

Fig. 6. Open field activity during alcohol hangover after the co-administration of energy drinks and alcohol A) Number of line crossings and B) Time spent in central area.). SAL: saline; SS: sucrose; ED: energy drink; EtOH: ethanol; ZT: Zeitgeber time. The colors of the bars indicate different treatments. Behavioral tests were performed 1 h before (ZT0) and 6 h after treatment when alcohol hangover began: ZT7 (2 pm), ZT13 (8 pm) and ZT 19 (2 am). ZT: Zeitgeber time. Values expressed as mean ± SEM (n = 12 each group). Statistical comparisons were made using ANOVA (post hoc Bonferroni) to test the significance of differences between the groups (*p < 0.05 vs Sal + SS; ##p < 0.01 vs Sal + ED; †p < 0.05 vs EtOH + SS). In group along time comparisons, differences were examined by repeated-measures ANOVA; post hoc test Bonferroni (ap < 0.05 vs ZT0; aap < 0.01 vs ZT0; aaap < 0.001 vs ZT0). In all cases, the significant level was fixed at p < 0.05.

decrease in percentages of motor performance and latency to fall was observed at ZT13 in mice treated with AmED with respect to alcohol alone, supporting the fact that mixture prolongs AH. In addition, at the beginning of AH the decrease in motor performance showed by the animals treated with alcohol alone (about 40% respect to the respective control) was similar to the reported in a previous study in animals injected with the same dose (3.8 mg/kg) of alcohol alone [24]. On the other hand, our results showed that BAC was similar in all groups at the beginning of AH, indicating that neither the addition of sucrose nor ED could alter alcohol metabolism. This finding is in agreement with Wang et al. [32] who demonstrated that the mixture of alcohol and Red Bull®

do not modify alcohol and acetaldehyde blood levels, the activity of the metabolizing enzymes nor the absorption of alcohol. On this basis, here we reported that the mixture prolongs the alterations in motor performance during hangover in mice.

On the other hand, some human studies have shown that alcohol alone or in combination with ED was able to induce motor impairments. Ferreira et al. have reported that subjective perceptions of some symptoms could suggest that alcohol intoxication was less intense after the combined ingestion of the alcohol plus ED. However, these effects were not detected in objective measures of motor coordination [33]. Other authors have found that the addition of ED or caffeine to alcohol was not able to attenuate alcohol-induced decrements in psychomotor performance [34,35]. In this sense, Woolsey et al., [36] have reported that combined use of alcohol and ED may place drinkers at greater risk when compared with those who consume only alcohol. College students who combined alcohol and ED were more likely to participate in high-risk driving [37,38].

In our study, we tested anxiety-like behavior on the EPM and open field tests during AH. We found that animals of alcohol alone and AmED groups showed anxiety-like phenotype by decreasing% FEO and %TSO in the EPM and also reduced the time of exploration in the central zone of the open field. These signs of anxiety were markedly evidenced in both groups with respect to their controls at the beginning of AH (ZT7), but they were not detectable at ZT13. Respect to the total entries on EPM test, a significant decrease was observed in alcohol alone and

AmED groups at ZT7 and also extended to ZT13 in animals treated with the mixture. One possible explanation for this phenomenon could be due to sedation induced by alcohol at the beginning of the AH and the decrease in motor skills caused by the mixture at ZT13, which in turn could prolong motor disabilities during the hangover.

One important component of the ED is caffeine. It is a well-known stimulant that affects numerous neurotransmitter and endocrine signaling pathways [41]. Caffeine antagonizes signaling through adenosine receptors, and increases release of catecholamines. The interactive pharmacological effects of caffeine and alcohol have been studied in animal models and humans. Hilbert et al. found that combinations of ethanol and caffeine interacted to increase locomotor activity more than either drug given alone in a context of conditioned reinforcement [42]. Also, using a mouse model of binge in the paradigm Drinking- in the- Dark (DID), caffeine and alcohol co-consumption produced a stimulated, less a of ataxic and anxious, as well as cognitively altered state [43]. May et al. have reported that repeated exposure to caffeine and alcohol induced sensitization and tolerance in mice influencing the response of some alcohol-related behaviors, notably locomotion and ataxia, but appeared not to influence the expression of conditioned behaviors [44].

In humans, Marczinski et al. have reported that the combination of alcohol and ED did enhanced the desire to drink, which could be due to the interaction between the caffeine present in ED and alcohol [37,45].

Regarding the possible neurobiological mechanisms driving the interactions between alcohol and ED, it is known that caffeine and alcohol have a common biological substrate; both act on neurochemical processes related to the neuromodulator adenosine. Caffeine acts as a nonselective adenosine A_1 and A_{2A} receptor antagonist, while ethanol has been demonstrated to increase the basal adenosinergic tone via multiple mechanisms [46]. In view of the relationship between adenosine and sleep on the one hand and the effect of caffeine on adenosine for the other one, Rohsenow [47] comparing the acute effects of caffeinated beer vs. non caffeinated beer, demonstrated that the first one improved perceived sleep quality, effect sizes were greater for morning alertness than for quality while sleeping, with no effect on sleep latency or total sleep time. No effects were seen on hangover incidence or severity. Although it is believed that caffeine is mainly responsible for the effect produced by the use of alcohol and ED, the possible effect of taurine should also be taken into account given that it is another quantitatively important component of ED [48].

Taurine is a weak GABA agonist at the $GABA_A$ benzodiazepine receptor complex as same as alcohol [49]. Exogenous taurine addition has exhibited an interaction with $GABA_A$ receptors [50]. The microdialysis technique has shown that the acute administration of ethanol (1–2 g/kg i.p.) increased extracellular levels of taurine in the nucleus accumbens, amygdala, hippocampus and frontal cortex [51].

In summary, our results indicate that AmED impairs motor performance and increased anxiety-like behavior, extending hangover symptoms. It will be necessary to deeply understand the action(s) of caffeine and/or taurine as components of ED and theirs interactions with alcohol at central nervous system level to explain these behavioral effects observed herein.

5. Conclusion

The co-administration of alcohol and energy drinks prolongs the symptoms of alcohol hangover in motor skills when compared with only alcohol treated animals, at least in this experimental murine model.

Acknowledgments

This research was supported by grants from Universidad de Buenos Aires (UBA) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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