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Research report

# Alterations in affective behavior during the time course of alcohol hangover

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## HIGHLIGHTS

- Anxiety-like behavior and fear-related signs are evidenced during alcohol hangover.
- Signs of depression were found 14 h after hangover onset.
- Pain perception disabilities were detected at the beginning of hangover.
- Changes in affective behavior are evidenced for 14–16 h during hangover.

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## ABSTRACT

Alcohol hangover is a temporary state described as the unpleasant next-day effects after binge-like drinking. Hangover begins when ethanol is absent in plasma and is characterized by physical and psychological symptoms. Affective behavior is impaired during the acute phase of alcohol intoxication; however, no reports indicate if similar effects are observed during withdrawal. The aim of this work was to study the time-extension and possible fluctuations in affective behavior during a hangover episode. Male Swiss mice were injected i.p. either with saline (control group) or with ethanol (3.8 g/kg BW) (hangover group). Anxiety, fear-related behavior and despair phenotype were evaluated at a basal point (ZT0) and every 2 h up to 20 h after blood alcohol levels were close to zero (hangover onset). Also, anhedonia signs and pain perception disabilities were studied. Mice exhibited an increase in anxiety-like behavior during 4 h and 14 h after hangover onset when evaluated by the elevated-plus maze and open field test respectively ($p < 0.05$). Fear-related behavior was detected in hangover animals by the increase of freezing and decrease of line crossings and rearing frequency during 16 h after hangover onset ($p < 0.001$). Depression signs were found in hangover mice during 14 h ($p < 0.05$). Hangover mice showed a significant decrease in pain perception when tested by tail immersion test at the beginning of hangover ($p < 0.05$). Our findings demonstrate a time-extension between 14 and 16 h for hangover affective impairments. This study shows the long lasting effects of hangover over the phase of ethanol intoxication.



## 1. Introduction

Alcohol hangover (AH) is a temporary state described as the unpleasant next-day effects after a binge-like drinking [1]. In humans, AH begins when ethanol (EtOH) is absent in plasma and is characterized by a cluster of physical symptoms which include drowsiness, nausea, diarrhea, fatigue and tremors along with psychological signs that involve anxiety and guilt [2,3]. In addition, it was demonstrated that humans suffering from acute alcohol withdrawal (hangover) self-reported depression [4] or a general "decreased mood" [5]. Moreover, it is known that pain-related effects of ethanol such as headache and hyperalgesia are evidence at least at the onset of AH [6,7].

In the case of experimental animals, hypo-activity [8], fluctuations in body temperature, anxiety-like behavior [9] and reduced wheel running activity are observed during the hangover state [10,11]. In addition, we have previously demonstrated a reduction in motor performance at the beginning of AH in mice [12] establishing also an association between this motor impairments and changes in brain cortex energetic metabolism [13].

*Abbreviations:* %FEO, proportion of entrance into open arms; %TSO, proportion of time spent in open arms; BAC, blood alcohol concentration; AH, alcohol hangover; EMP, elevated-plus maze; i.p., intraperitoneally; TE, total number of entries; ZT, zeitgeber time.

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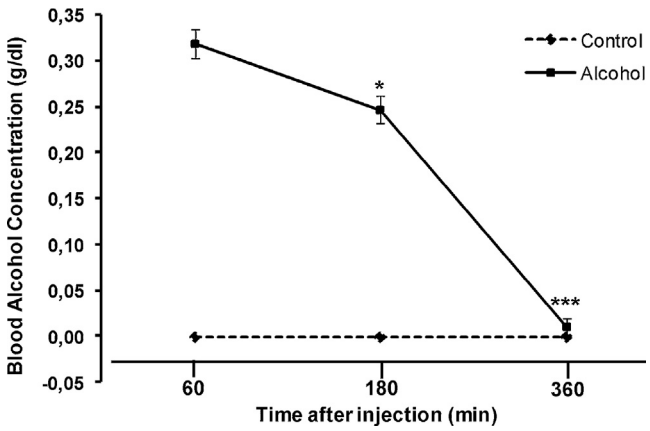


**Fig. 1.** Blood alcohol concentration after EtOH treatment.
Blood alcohol concentration (BAC) in controls (dotted line) and ethanol-treated (solid line) male Swiss mice was measured 60, 180 and 360 min after acute i.p. injections to determinate the onset of hangover. Values are expressed as mean ± SEM ($n = 15$ each group). $^{*}p < 0.05$ and $^{***}p < 0.001$, significantly different from BAC at 60 minutes. Independent samples *t*-test.

Particularly, it was shown that affective behavior is impaired during AH. In this sense, Gauvin et al. have shown that rats injected intraperitoneally (i.p.) with high doses of ethanol (3–4 g/kg) displayed a hangover-related anxiety behavior when tested 9 h after acute ethanol challenge [14,15]. Together with this, it has been recently demonstrated that, 18 h after acute EtOH administration (4 g/kg, i.p.), adult male rats present a reduced exploration into the elevated plus maze [16], and a significant social suppression [17] being both a response pattern that is consistent with an anxiogenic profile [18,19]. Similarly, Morse et al. reported a significant conditioned place aversion in the rat during hangover [20]. Likewise, Prediger et al. demonstrated a time-dependent development of anxiety-like behavior during AH in mice [21]. The evidence presented here together with the convergent findings from naturalistic methodology and the experimental investigations firmly suggest an increased anxiety-like behavior during alcohol hangover [22,23]. Nevertheless, the time-extension and possible fluctuations in affective behavior from the beginning to the end of a hangover episode were not explored.

In addition to hangover-related anxiety, it was demonstrated that rats exhibited brain reward deficits following acute exposure to ethanol [24]. This would indicate a negative affective component that, as mentioned above, is also observed in humans. Furthermore, Getachew et al. have verified that after chronic EtOH administration, rats exhibit an exaggerated immobility in the forced swim test, which reveal the "depressogenic" effects of alcohol [25]. Additionally, Walker et al. demonstrated an ethanol-induced depressive-like behavior mediated by alterations in the expression of brain neuropeptides [26]. Beyond anxiety and depression signs, it was established that hangover induced hyperalgesia and pain enhancement [11,27,28].

Although scientific evidences state that emotional behavior is affected during AH, it is unknown for how long these signs are presented and when individual physical and psychological symptoms are restored. Related to this, we have recently reported a time extension between 16 and 20 h for hangover motor and exploratory impairments in mice [29]; however, the different types and length of affective alterations together with the possible influence of light changes during AH throughout a day were not assessed. Taking all together into account, the aim of this work was to study the fluctuations in emotional behavior during AH in mice in order to achieve several goals: (1) to characterize the time course of anxiety and fear related-behavior; (2) to examine the possible manifestation of a despair phenotype; (3) to determinate the presence of anhedonia signs during a complete episode of alcohol induced hangover; (4) to evaluate possible pain perception disabilities at the beginning of the hangover and thus (5) to establish the time-extension of the possible affective behavior alterations.

## 2. Materials and methods

### 2.1. Animals

A total of 130 from five cohorts of male Swiss mice (*Mus musculus*) weighing 30–40 g were acquired from the School of Pharmacy and Biochemistry, Universidad de Buenos Aires, and housed in a soundproof room under conditions of controlled temperature (22 ± 2 °C) and humidity, with a 12-h light/dark cycle. Standard rat chow and tap water were provided ad libitum.

Animal handling, treatment 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 Universidad de Buenos Aires where the protocol was performed. All efforts were made to minimize suffering and reduce the number of animals used.

### 2.2. Experimental procedure

Animals received intraperitoneal (i.p.) injections of 15% EtOH at a dose of 3.8 g/kg. Ethanol dose was previously applied in alcohol-induced hangover animal models [8,11]. Control mice received saline i.p. injections. In order to determine the animals' response to ethanol and the onset of hangover, fifteen mice from each group

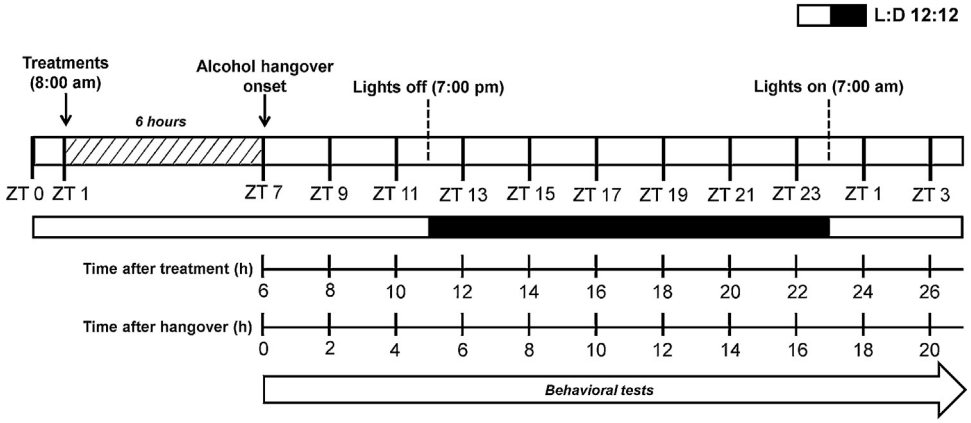


**Fig. 2.** Timeline and experiments.
Male mice received intraperitoneal treatment with saline or ethanol at a dose of 3.8 mg/kg or an equivalent of normal saline at 8:00 a.m. Behavioral tests were performed before and six hours after treatment when alcohol hangover began. ZT: Zeitgeber time; ZT12: 7:00 p.m.

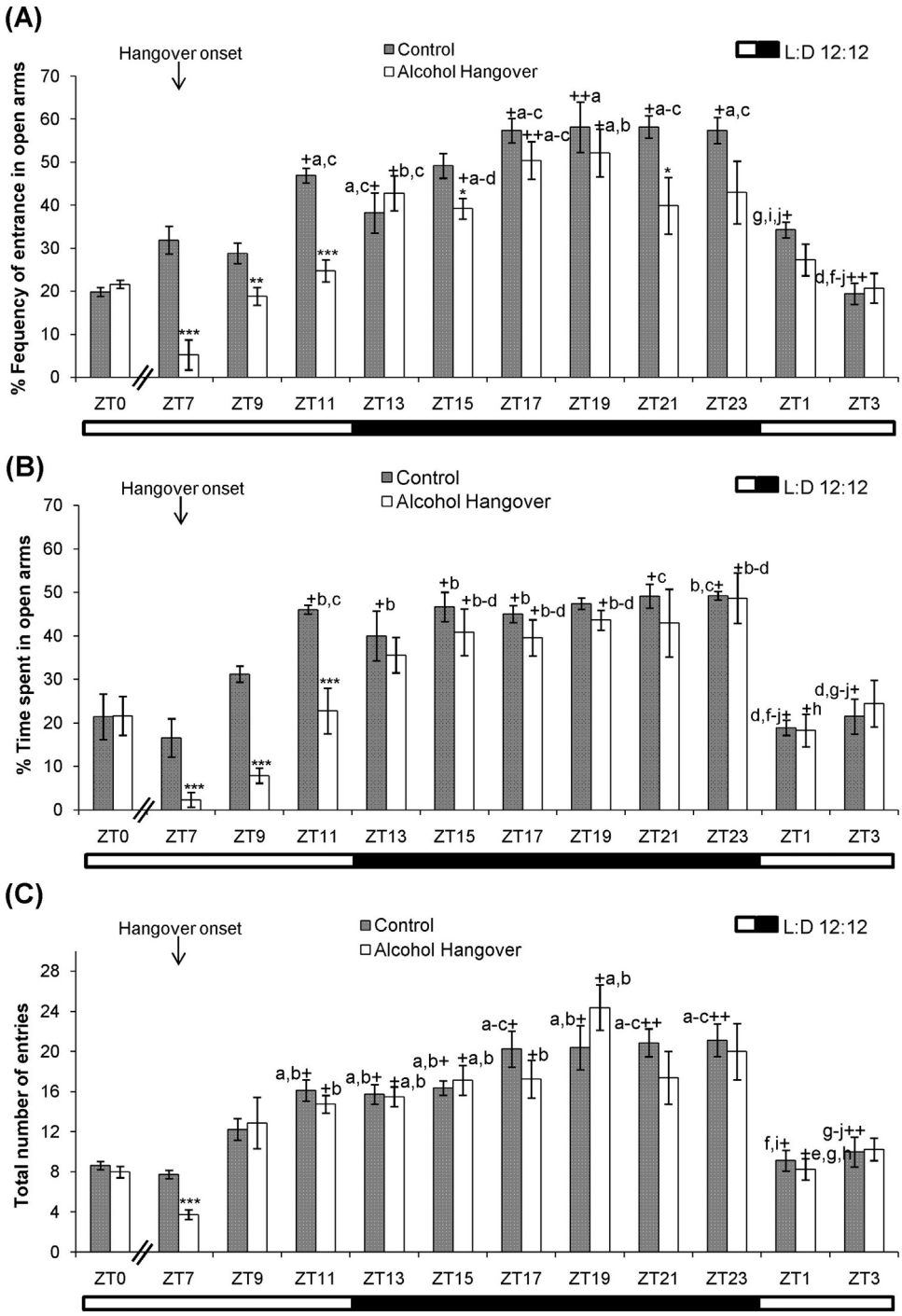


**Fig. 3.** Anxiety-like behavior on the elevated-plus maze during alcohol hangover.
Values are expressed as mean ± SEM ($n = 10$ each group). ZT: Zeitgeber time; L:D; light:dark. Unpaired independent samples Student's $t$ test was used ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) for intergroup differences. Repeated-measures two-factor ANOVA was used ($^{+}p < 0.05$, $^{++}p < 0.01$) for in-group difference. (A): proportion (%) of entrance into open arms, (B): proportion (%) of time spent in open arms and (C): total number of entries. Letters indicate the time point of the comparison as follows: a:ZT0, b:ZT7, c:ZT9, d:ZT11, e:ZT13, f:ZT15, g:ZT17, h:ZT19, i:ZT21, j:ZT23 and k:ZT1. Bars shading indicate group: gray, control; white, alcohol hangover.

were decapitated 60, 180 or 360 min after the injection ($n = 5$ each time point). Blood was collected from the trunk and blood alcohol concentration (BAC) was measured by gas chromatography (Hospital Británico, Buenos Aires, Argentina). Experiments were conducted in the morning (9:00 a.m.). The criteria used to establish the onset of alcohol hangover was when BAC was less than or equal to 10% of the maximum value reached (Fig. 1).

### 2.3. Behavioral assessments

Behavioral tests were carried out at a basal point that matched with lights onset (ZT0) and every 2 h up to 20 h after alcohol hangover onset (ZT3 of the following day) (see Fig. 2) [29]. Animals were randomly assigned to saline or ethanol treatment before baseline tests ($n = 10$ per treatment and for each behavioral task). Each subject was tested every two hours in only one behavioral test avoiding multiple tasks for animals groups. Control groups (saline treatment) let observe in- and between-group differences due to time-course (photoperiod), acute treatments and carry-over effects. Anxiety and fear-related behavior together with depression signs, anhedonia and antinociceptive responses were evaluated using a battery of different behavioral tests. During experimental procedures, test boxes or the apparatus used for behavioral studies were cleaned with 10% EtOH 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. Testing was conducted during the mouse's normal lights-on sleeping time.

*2.3.1. 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 closed arms, and were allowed to explore it for 5 min as previously described [30]. The animal's behavior was analyzed by video tracking system &%Annotation-xml.content; Maze (Stoelting Co., Wood Dale, Illinois). The proportion of entrance into open arms (%FEO) and the proportion of time spent in open arms (%TSO) together with the total number of 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. The elevated-plus maze 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 built-in control measure for general hyperactivity or sedation.

*2.3.2. Open field test*

Anxiety-like tendency and fear-related behavior were evaluated by the open field test [31]. The test box consisted of a 60 cm × 60 cm square arena surrounded by a 50 cm high wall divided in two zones: center (30% 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 and its movement throughout the duration of the session was recorded and analyzed by the video tracking system &%Annotation-xml.content; Maze (Stoelting Co., Wood Dale, Illinois). The latency to the first exit from central area (s) and the time in the central zone (s) was scored during the open field test session. In this sense, less time spent in the central area or low exit latencies indicate a possible anxiety-like behavior. Also, the number of line crossings, rearing frequency, freezing episodes and fecal boli deposition were quantified. In this case, low line crossings and rearing frequency together with high freezing episodes and defecation represent a potential fear-related behavior.

*2.3.3. Tail suspension test*

The total duration of immobility induced by tail suspension was based on a method described by [32]. Briefly, mice were isolated and suspended upside down by taping their tails to a flat metal bar with adhesive tape. The mice were positioned so that they could not reach the top or sides of the chamber. The mice were left in this position for 6 min. During this period mice generally stop struggling and hang immobile. The latency to the first immobility episode and the duration of immobility were observed and measured during the final 4 min of the test. A depressive-like behavior is exhibited by a decrease in the latency to the first episode of immobility and an increase in immobility time.

*2.3.4. Forced swim test*

Depression-related phenotype was evaluated by the modified Porsolt forced swim test [33]. For this purpose, a cylinder of 20 cm diameter was filled with warm water (30 °C) to a depth of at least 10 cm, which exceeds the distance to which the tail can extend, so the mouse cannot balance on its tail at the bottom of the cylinder. The top of the cylinder was 15 cm above the upper surface of the water, such that the mouse cannot climb out of the cylinder. All mice were forced to swim for 6 min, and the duration of immobility and the latency to the first immobility episode were observed and measured during the final 4 min of the test. The immobility time was regarded as the time the mouse spent floating in the water without struggling and making only those movements necessary to keep its head above water. Despair behavior is manifested by a decrease in the latency to the first episode of immobility and an increase in immobility time.

*2.3.5. Two-bottle sucrose preference test*

Consumption of a 2% sucrose solution between ZT7 (2:00 p.m.) and ZT3 (10:00 a.m.) was recorded in all mice to measure sucrose anhedonia [34]. Mice were simultaneously given a free choice between two bottles, one with 2% sucrose solution and another with tap water, for 20 h. The beginning of the test started with the onset of alcohol hangover (ZT7). No previous food or water deprivation was applied before the test. Special attention was dedicated to sugar storage to avoid its contact with flavors and plastic, and to washing of bottles, where minimal amounts of detergent were used. Bottles were filled in advance (during the preceding evening) and were kept upside down for at least 12 h prior to testing. In order to balance the air temperature between the room and the drinking bottles, they were kept in the same room where the testing took place. To prevent the possible effects of a side-preference in drinking behavior, the position of the bottles in the cage was switched every 5 h during the test. The bottles were weighed before and after the 20-h sample time. Preferences were calculated in percent from total volume of liquids consumed. A decrease in sucrose intake and preference over water is generally taken as a putative sign of anhedonia in rodents [35–37].

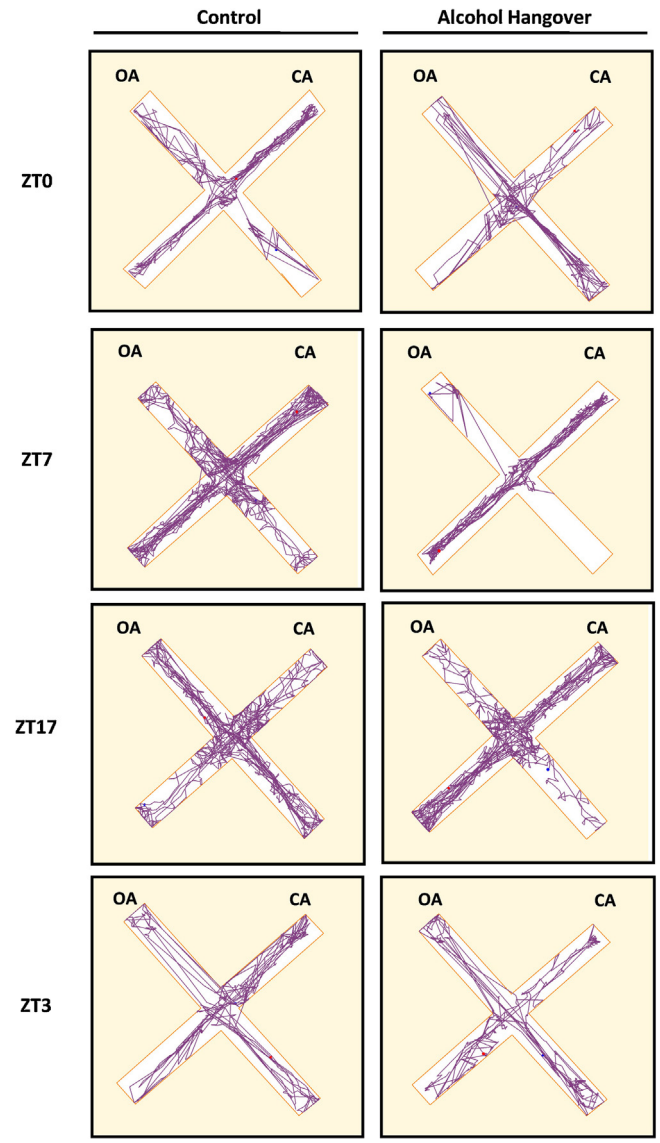


**Fig. 4.** Elevated-plus maze during alcohol hangover.
Representative traces in the elevated-plus maze from control and hangover groups at ZT0, ZT7, ZT17 and ZT3. OA: open arms, CA: close arms.

*2.3.6. Antinociceptive responses*

Two different nociceptive models, the tail-immersion and the hot-plate test were used to evaluate the antinociceptive responses elicited at the onset of alcohol hangover.

*2.3.6.1. Tail-immersion test.* The spinal reflex of tail-flick was assessed by the tail immersion test as previously described [38–40]. The test measures the latency for the mouse to withdraw the tail when immersed in a beaker of water maintained at 52.5 °C. The trial was terminated once the animal flicked its tail. In the absence of tail-flick, a 10 s cut-off was used to prevent tissue damage.

*2.3.6.2. Hot-plate test.* The hot-plate test measures a reflex that requires circuitry in the brain as well as in the spinal cord [41–43]. The mouse is placed on a horizontal surface that is heated to 52–55 °C. The hot-plate temperature is calibrated by the experimenter to produce a response within about 10 s in control mice. A tall plastic cylinder or square is placed on the surface, around the mouse, to prevent the animal from walking off the surface or jumping out of the test apparatus. Pain perception was evaluated by the licking of forepaws or a jumping response. In absence of paw-licking or jump, a 15 s cut-off was used to prevent tissue damage.

*2.4. 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

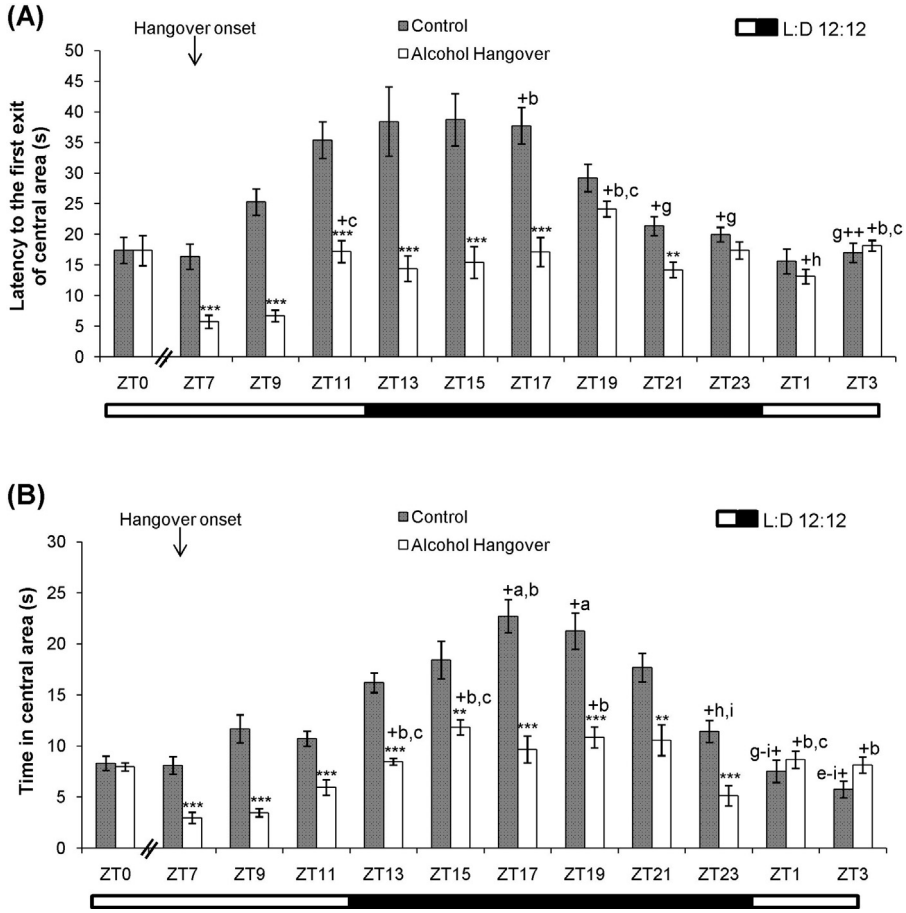


**Fig. 5.** Exploring behavior and anxiety-like tendencies on the open field test during alcohol hangover.
Values expressed as mean ± SEM ($n$ = 10 each group) ZT: Zeitgeber time; L:D; light:dark. Student's t test was used for intergroup differences ($^{**}p < 0.01$, $^{***}p < 0.001$). Repeated-measures two-factor ANOVA was used for in-group difference ($^{+}p < 0.05$; $^{++}p < 0.01$). (A): Latency to the first exit of central area (s) and (B): Time in central area (s). Letters indicate the time point of the comparison as follows: a:ZT0; b:ZT7; c:ZT9; d:ZT11; e:ZT13; f:ZT15; g:ZT17; h:ZT19; i:ZT21; j:ZT23 and k:ZT1. Bars shading indicate group: gray, control; white, alcohol hangover.

to follow a posterior parametric or nonparametric statistical analysis. Data obtain from behavioral test were analyzed using the unpaired independent Student's t test to analyze the significance of differences between hangover and control groups. In-group differences were examined by repeated-measures two-factor ANOVA. SPSS (version 13.0) statistical software was used and a difference was considered statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Anxiety-like phenotype by the elevated-plus maze (Figs. 3 and 4)

When animals were tested in the EPM test, a significant reduction in the frequency of entrance in open arms (%FEO; Fig. 3A) was observed in hangover mice from ZT7 to ZT11 ($p < 0.001$, compared with controls). During the dark period, hangover mice displayed a gradually and significant increase in %FEO ($p < 0.05$, compared with same group from ZT0 to ZT11) that was sustained up to ZT23 when %FEO levels dropped being similar to basal levels at ZT1 and ZT3. Together with this, control mice showed a significant increase in the frequency of visits into open arms during the dark period ($p < 0.05$, compared with same group from ZT0 to ZT9) which returned to baseline at ZT1. No significant differences were observed between control and hangover mice at ZT1 and ZT3 or between same groups with its basal levels (ZT0). A similar behavioral pattern was observed in the proportion of time spent in open arms (%TSO; Fig. 3B). A significant reduction in %TSO was observed in hangover mice from ZT7 to ZT11 ($p < 0.001$, compared with controls). During the dark period, hangover mice displayed a significant increase in %TSO ($p < 0.05$, compared with same group at ZT7 and ZT9). During same phase, control mice exhibited a significant increase in %TSO ($p < 0.05$, compared with same group at ZT7 and ZT9) which was not different from the level reached by hangover mice. Control and hangover groups returned to baseline at ZT1 when no significant differences were found between both of them. In addition, total entries (TE; Fig. 3C) as an indicator of general activity were measure in the EPM. A significant decreased in TE was observed at the onset of hangover ($p < 0.001$, compared with controls at ZT 7). Although control and hangover mice displayed a significant increase in TE during the dark period ($p < 0.05$, compared with same groups from ZT0 to ZT9), there were no significant differences between them. Both groups recovered TE baseline at ZT1.

Representative traces in the EPM are shown in Fig. 4. Control and hangover mice displayed a similar behavior at ZT0. Both groups exhibited more traces into the open arms compared with close arms showing no qualitative preference for a particular arm quarter. At ZT7, controls showed a markedly increment in the exploring behavior into closed arms and a slight increase in the exploration into open arms compared with was observed at ZT0. Mice undergoing the beginning of the hangover (ZT7) avoided touring into the open arms and kept walking into the close arms. In the middle of the dark period (ZT17) controls increased the exploration

into open and close arms compared with was observed at ZT0. Hangover mice showed an increment in walking into the open arms and a qualitative strong increase in the exploration into the close arms; however, this group still avoided touring the open arms compared with controls. At the end of the experiment (ZT23), hangover mice exhibited a similar behavioral pattern in the EPM to controls.

### 3.2. Anxiety-like behavior by the open field test (Figs. 5 and 6)

Mice exhibited a significant decrease in the latency to the first exit of central area at the onset of alcohol hangover compared with controls ($p < 0.001$, Fig. 5A). This parameter remained lower than controls up to ZT21. At the middle of the dark period (ZT17), controls showed a significant increase in the latency to the first exit of central area ($p < 0.05$). No significant differences were observed between control and hangover mice from ZT23 to ZT3. Hangover mice displayed a decrease in the total time spent in the central area from the beginning of alcohol hangover (ZT7) to 22 h after ethanol exposure (ZT23) ($p < 0.001$, compared with controls; Fig. 5B). Control mice exhibited a significant increase in the time spent in central area at ZT17 and ZT19 compared with same group at ZT0 ($p < 0.05$). Time spent in the central area starts to significantly decrease in controls at ZT23 and ZT1 ($p < 0.05$, compared with same at ZT21) being not different from basal levels. Hangover mice showed no differences in the time spent in central area at ZT1 compared either with its basal point (ZT0) or controls at ZT1.

Representative records of the open field test are shown in Fig. 6. Control and hangover mice exhibited a similar behavior at the beginning of the experiment (ZT0). Both experimental groups toured across the open field homogeneously showing a basal preference for the peripheral area as seen in Fig. 5B. At ZT7, controls walked across the whole field, showing a similar tracking pattern as ZT0. At the onset of the hangover, mice avoided the central zone and explored the periphery in a non homogeneous way. In the middle of the dark period (ZT17) controls exhibited more activity manifested by a high number of line crossings and an increase in central zone exploration compared with same group at ZT0 or ZT7. Hangover mice displayed an increment in open field exploration at ZT17; however, this group avoided the central area showing a higher exploration around the periphery compared with same group at ZT7. At the end of the experiment (ZT23), hangover mice recovered the behavioral pattern in the open field being similar to controls.

### 3.3. Fear-related behavior in the open field (Table 1)

Four different variables were measured in the open field test in order to evaluate a potential fear-related behavior during the alcohol hangover. Control mice exhibited an increase in line crossings and rearing frequency during the dark period ($p < 0.05$); however, no significant changes were observed in freezing episodes throughout the experiment. On the other hand, hangover mice displayed a significant different behavioral patter than controls. In this sense, treated mice showed a significant decrease in line crossings and rearing frequency at the onset of alcohol hangover ($p < 0.001$) which remained lower than controls during the dark period (up to ZT19 for line crossings and ZT23 for rearing frequency). Both groups returned to baseline at ZT1 (18 h after treatment). Furthermore, freezing episodes increased very significantly at the onset of alcohol hangover ($p < 0.001$, compared with same group at ZT0). Hangover mice showed a decrease in freezing events during the dark; however, the episodes remained to be higher than controls at the same stage ($p < 0.001$). No significant difference where observed between control and hangover mice in freezing episodes from ZT1 to ZT3. In the case of fecal boli deposition, both groups exhibited a slightly increased in defecation during the dark; nevertheless, no markedly significant difference were observed between control and hangover groups.

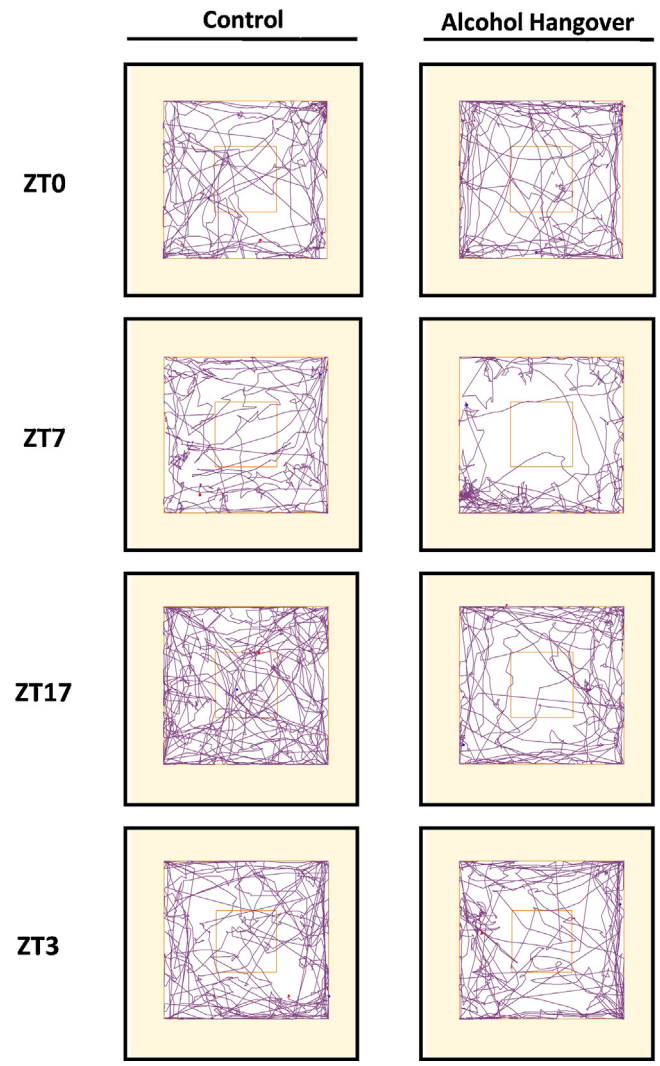


**Fig. 6.** Open field test during alcohol hangover.
Representative traces of the exploring behavior on the open field test from control and hangover groups at ZT0, ZT7, ZT17 and ZT3. Is illustrated the test box divided in two zones: center (30% of the entire area) and periphery.

### 3.4. Depression signs by the tail suspension test (Fig. 7)

At the onset of alcohol hangover, mice showed a significant decrease in the latency to the first immobile episode ($p < 0.01$, compared with controls; Fig. 7A). During the first experimental period, hangover mice ceased moving at around the first 7 s of the test. In the middle of the night period, a significant increase in the latency to the first immobile episode was observed both in hangover and control mice ($p < 0.05$ and $p < 0.001$ respectively, compared with same group at ZT17 and ZT19; Fig. 7A). Control and hangover mice reached the first immobile episode at similar time points from ZT23 to ZT0. The total immobile time was also recorded by the tail suspension test. Hangover mice showed a 60% increase in the total immobility time from ZT7 to ZT13 compared either with controls or same group at ZT0 ($p < 0.001$ and $p < 0.05$ respectively; Fig. 7B). Treated mice recovered basal immobility time in the tail suspension test at ZT23.

**Table 1**
Fear-related behavior on the open field test during alcohol hangover.

| | Line crossings | | Rearing frequency | | Freezing episodes | | Fecal Boli | |
|---|---|---|---|---|---|---|---|---|
| | Control | Alcohol Hangover | Control | Alcohol Hangover | Control | Alcohol Hangover | Control | Alcohol Hangover |
| ZT0 | 18.67 | 20.00 | 11.50 | 10.83 | 2.17 | 2.20 | 3.50 | 4.17 |
| ZT7 | 21.67 | 11.33 | 11.00 | 1.67 | 1.67 | 18.17 | 2.83 | 3.17 |
| ZT9 | 21.00 | 11.17 | 10.67 | 2.00 | 2.33 | 13.67 | 3.00 | 3.50 |
| ZT11 | 20.33 | 10.33 | 14.67 | 2.83 | 1.67 | 12.00 | 2.67 | 2.50 |
| ZT13 | 23.00 | 14.33 | 20.33 | 5.50 | 2.00 | 10.33 | 3.50 | 4.67 |
| ZT15 | 29.00 | 21.67 | 21.00 | 6.17 | 1.33 | 8.50 | 5.00 | 5.17 |
| ZT17 | 32.50 | 23.67 | 20.50 | 10.00 | 1.17 | 7.50 | 6.83 | 7.33 |
| ZT19 | 27.83 | 22.83 | 19.50 | 10.00 | 1.00 | 7.17 | 6.33 | 6.67 |
| ZT21 | 24.50 | 23.33 | 13.83 | 9.83 | 1.67 | 5.00 | 4.83 | 5.00 |
| ZT23 | 22.33 | 21.50 | 11.67 | 10.33 | 2.00 | 3.00 | 4.33 | 4.50 |
| ZT1 | 19.00 | 17.83 | 11.00 | 10.33 | 2.00 | 2.17 | 3.83 | 3.50 |
| ZT3 | 18.50 | 18.33 | 11.33 | 11.17 | 1.50 | 1.83 | 4.00 | 3.50 |

### 3.5. *Despair behavior by the forced swim test (Fig. 8)*

The latency to the first immobile episode and the proportion (%) of time immobile were analyzed by the forced swim test. At ZT7 (hangover onset), treated mice showed a significant decrease in the latency to the first immobile episode ($p < 0.001$, compared with controls; Fig. 8A). During night period, control mice took longer to reach immobility compared with same group at the early stage of

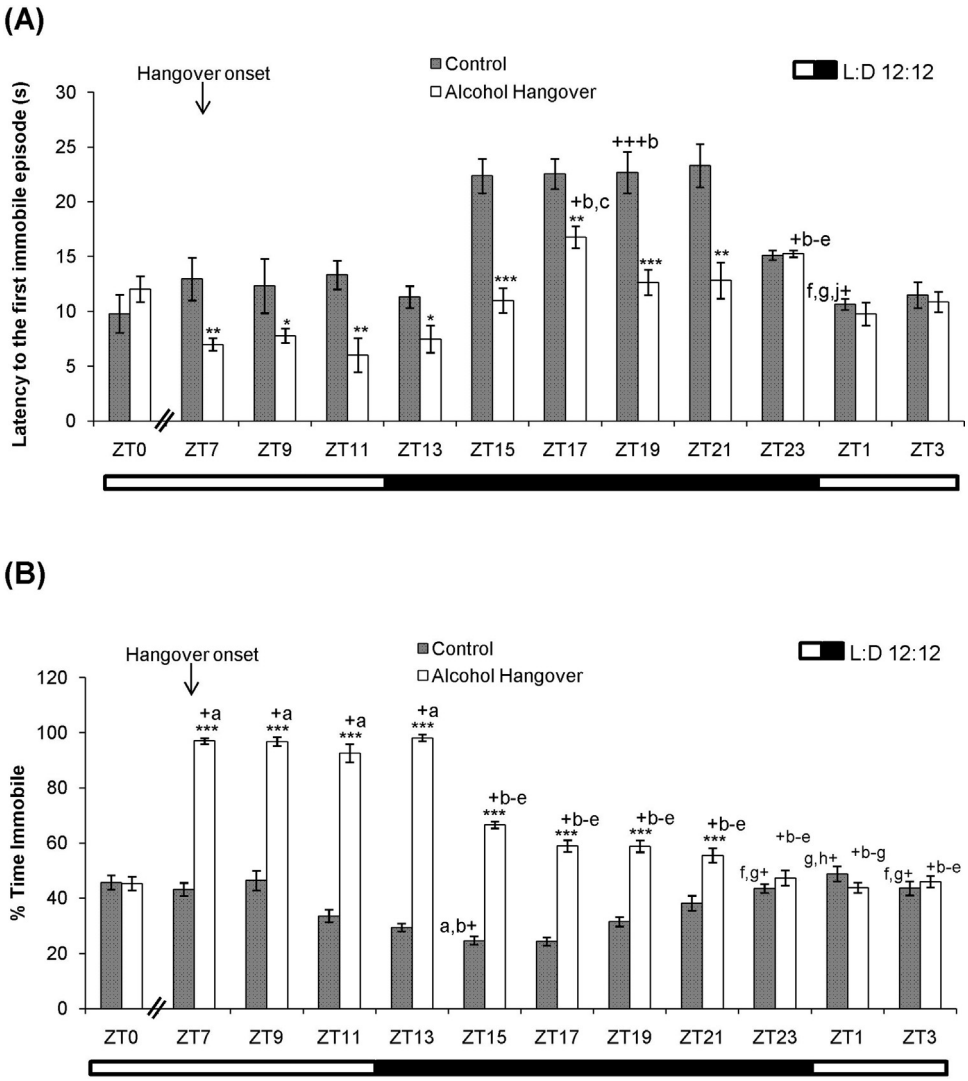


**Fig. 7.** Depressive-like behavior by the tail suspension test during alcohol hangover.
Values are expressed as mean ± SEM ($n = 10$ each group). ZT: Zeitgeber time; L:D; light:dark. Student's $t$ test was used for intergroup differences ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Repeated-measures two-factor ANOVA was used for in-group difference ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). (A): Latency to the first immobility episode (s) and (B): the proportion (%) of time immobile. Letters indicate the time point of the comparison as follows: a:ZT0; b:ZT7; c:ZT9; d:ZT11; e:ZT13; f:ZT15; g:ZT17; h:ZT19; i:ZT21; j:ZT23 and k:ZT1. Bars shading indicate group: gray, control; white, alcohol hangover.

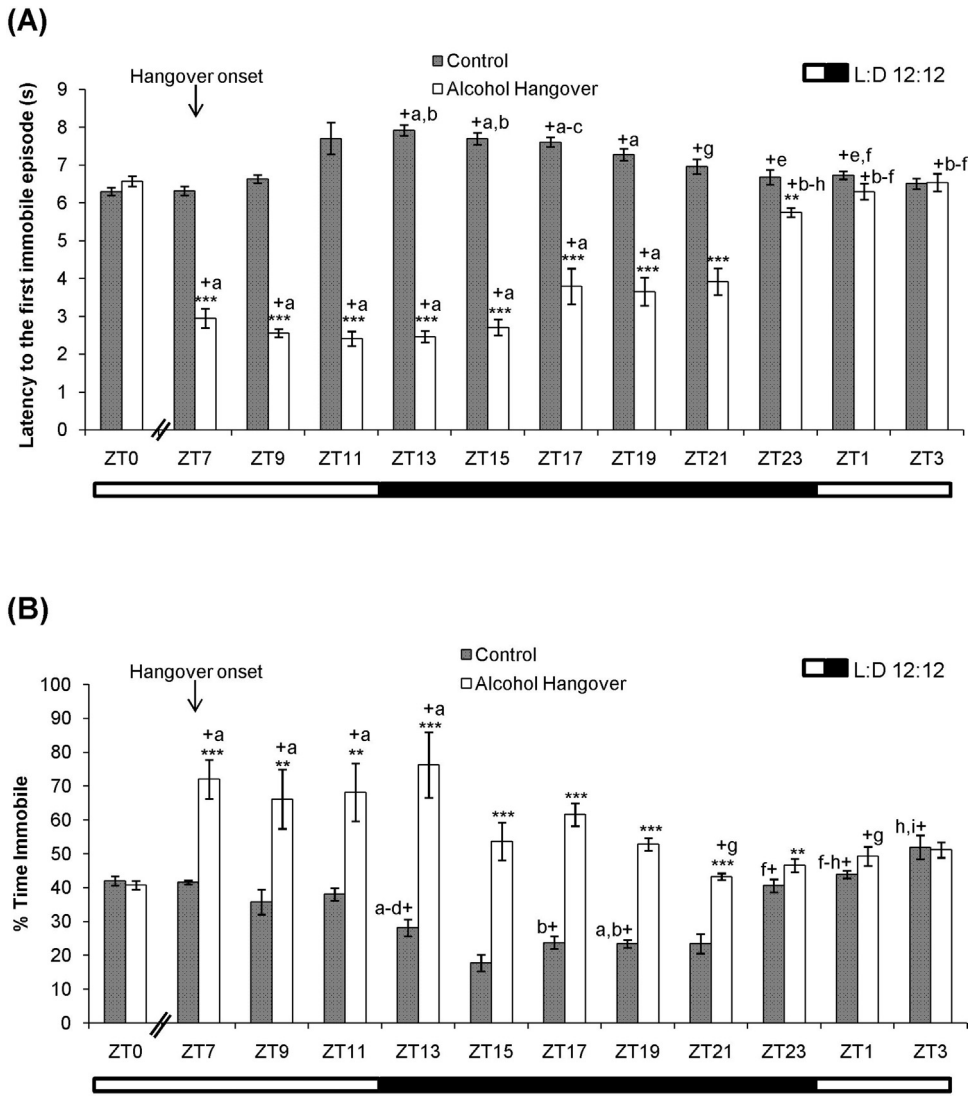


**Fig. 8.** Despair behavior by the forced swim test during alcohol hangover.
Values are expressed as mean ± SEM ($n = 10$ each group). ZT: Zeitgeber time; L:D; light:dark. Student's $t$ test was used for intergroup differences ($^{**}p < 0.01$, $^{***}p < 0.001$). Repeated-measures two-factor ANOVA was used for in-group difference ($^{+}p < 0.05$). (A): Latency to the first immobility episode (s) and (B): the proportion (%) of time immobile. Letters indicate the time point of the comparison as follows: a:ZT0; b:ZT7; c:ZT9; d:ZT11; e:ZT13; f:ZT15; g:ZT17; h:ZT19; i:ZT21; j:ZT23 and k:ZT1. Bars shading indicate group: gray, control; white, alcohol hangover.

the experiment ($p < 0.05$). Same group recovered basal immobility levels at ZT21. Similarly, hangover mice displayed the recovery in the latency to the first immobile episode at ZT1 (18 h after hangover onset). Mice showed a significant increase in the % of time immobile at the hangover onset (ZT7) compared with controls ($p < 0.001$, Fig. 8B). During the dark period, hangover mice showed a decrement in the % time immobile; however, this remained to be significant higher than controls ($p < 0.001$). On the other hand, controls exhibited a decrease of % of time immobile at the start of the dark period (ZT13) compared with same group at ZT0 and ZT11 ($p < 0.05$) and an increase at ZT23 ($p < 0.05$, compared with same group at ZT15). Both groups reached baseline levels 18 h after the start of the alcohol hangover.

### 3.6. Anhedonia by the two bottle sucrose preference test (Fig. 9)

Sucrose preference was tested from ZT7 to 20-h after hangover onset. There was no significant difference between hangover and control mice [$t(18) = -0.69$; $p = 0.498$]. Results showed an average sucrose preference of around 70% being for controls 68.31 ± 2.87% and for hangover mice 71.26 ± 3.15%.

### 3.7. Pain perception at the onset of alcohol hangover (Fig. 10)

At the onset of alcohol hangover (ZT7), mice displayed a jumping response at 1.38 ± 0.18 s on the hot plate test (Fig. 10A). Meanwhile, control mice exhibited a jumping response at 1.63 ± 0.38 s. However, there was no significantly difference between both groups [$t(14) = 0.60$; $p = 0.559$]. On the other hand, tail-flick response in the tail immersion test was measured at ZT7 in both groups (Fig. 10B). Hangover mice showed a significant decrease in the tail-flick response as compared with controls (2.00 ± 0.33 s vs. 3.38 ± 0.56 s, respectively); [$t(14) = 2.11$; $p = 0.050$].

## 4. Discussion

An increasing amount of evidence suggests that alcohol hangover (AH) is a pathophysiological state that compromises the

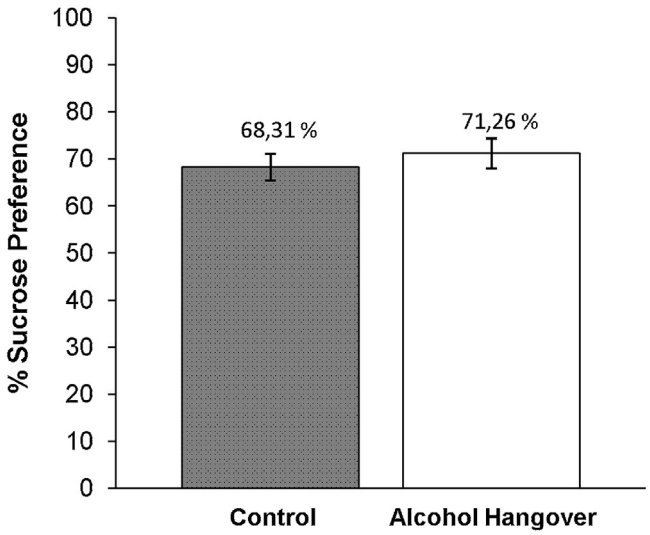


**Fig. 9.** Signs of anhedonia by two-bottle sucrose preference test during alcohol hangover.
The proportion of consumption of a 2% sucrose solution of between ZT7 (2:00 p.m.) and ZT3 (10:00 a.m.) was measured in control and hangover mice to quantify sucrose anhedonia. Values are expressed as mean ± SEM ($n$ = 10 each group). Results were analyzed by Student's $t$ test, no significant differences were found between control and hangover mice. Bars shading indicate group: gray, control; white, alcohol hangover.

physical condition along with cognitive functions and subjective capacities being one of the most important causes of inefficiency, reduced productivity and even absenteeism in the workplace, driving impairments and poor academic achievement. Although psychophysical impairments were previously established both in humans and in experimental animal models, no previous studies were carried out to evaluate the affective behavior during acute ethanol withdrawal.

We tested anxiety-like behavior on the elevated-plus maze (EPM) and open field test during alcohol hangover. We found that animals showed anxiety-like phenotype by decreasing the frequency of entrance and permanence in open arms in the EPM and a reduced exploration in the central zone of the open field. Signs of anxiety were markedly evidenced at the beginning of AH and remained decreased up to ZT13 when evaluated on EPM and up to ZT21 when tested on the open field. Particularly, control and hangover mice showed in-group increments in the frequency of entrance and time spent in open arms during the dark period which matched with the active phase of animals. In this sense, it was observed that even when hangover mice increased open field exploration during the dark, it remained being lower than controls and finally recovered at ZT23. It is important to note that the time spent in central zone can be affected by many factors, including locomotor and sensory deficits [44]. Indeed, we have recently reported that hangover mice traveled less time and displayed a lower average speed while exploring the open field [29]. Because of these reasons, it could be thought that the decrease in open field exploration could be understood as hypo-activity or an anxiety-like phenotype due to AH. In addition, signs of sedation were observed at the onset of AH taking into account the total entries on EPM; however, no other differences respected to control behavior were observed. Our representative figures either for EPM or open field test were in line with the above results. In this sense, it was demonstrated that rats fed with a liquid diet containing 4.5% EtOH for 12 h exhibited anxiogenic-like symptoms during the acute withdrawal in the elevated plus-maze [45]. Here, we found that mice recovered their basal behavior 6 h on EPM and 14 h in the open field test after hangover onset. In accordance with this, Lal et al. have been

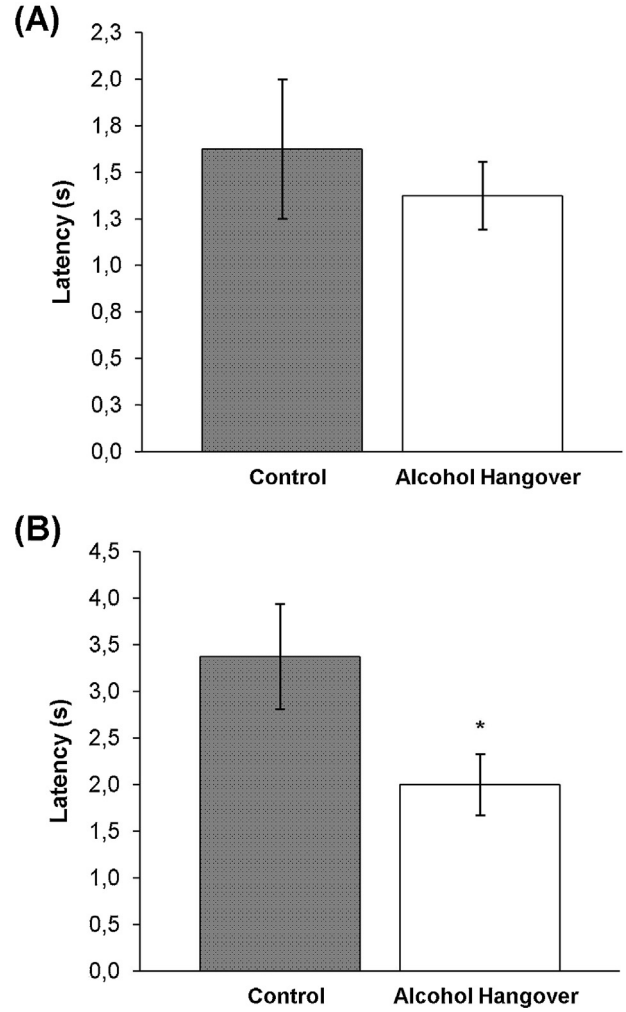


**Fig. 10.** Antinociceptive responses at the onset of alcohol hangover.
Hot-plate and tail-immersion tests were used to evaluate the antinociceptive responses elicited at the onset of alcohol hangover. Values are expressed as mean ± SEM ($n$ = 10 each group). Results were analyzed by Student's $t$ test (*$p$ < 0.05). (A): Latency to the jumping response on the hot plate test (s) and (B): latency to mice tail-flick in the tail-immersion test (s). Bars shading indicate group: gray, control; white, alcohol hangover.

evidenced a 36 h time course for withdrawal intensity on the EPM [46]. We also reported signs of fear-related behavior in both groups. As expected, hangover mice exhibited a strongly fear-related phenotype by a high rate of freezing episodes and low line crossings and rearing frequency which returned to baseline 18 h after treatment.

In the present work we found signs of anxiety which were previously stated by Goldstein including these symptoms as part of the opponent process model for ethanol's effects [47]. This one explained ethanol's initial effects, characterized as anxiolytic, anticonvulsant, and muscle relaxing followed by delayed compensatory opposite effects (anxiogenic, proconvulsant, and muscle rigidity). Although the negative hangover effects were announced, the time-extension of them was not assessed. Other research works proposed that anxiogenic responses due to AH may be related to a compensatory alteration in GABA neurotransmission [48] or due to other systems including the brain stress hormone CRF and the neurotransmitter serotonin [49,50].

One of the most important symptoms of AH in humans is the lack of interest on doing activities or unwillingness which is comparable with a mild depression pattern. In this sense, despair signs were analyzed by tail suspension and forced swim tests respectively based on the assumption that animals usually try to escape

from an "aversive situation" like being held by the tail or swimming to keep afloat as in the present work. Here, we deduced the loss of fight for survival as a sign of depression. In this study we found that hangover mice exhibited a markedly decreased in the latency to the first immobile episode and an increase in the time immobile (without making any escape movement) for both behavioral tests which indicate a clear sign of despair from the beginning to at least 14 h after AH onset. Related to this, it is known that alcohol induces a depressogenic state in rats being associated with a reduction in cortical norepinephrine [51]. Together with this, Walker et al. (2010) demonstrated that the depressive-like behavior induced by ethanol involves alterations in CRF and NPY systems in the brain [26]. However; there were no previous studies reporting the manifestation of despair behavior during acute alcohol withdrawal. Our results allow us to verify that symptoms of depression are also evidence during AH. Regarding the evaluation of depression signs by detecting mice' immobility, it might be thought that animals stopped moving because they experienced a transient fatigue like getting tired because of swimming or trying to escape from the suspended rope. However, this factor does not seem to explain this behavior, since the lightest disturbance reactivated the activity. Besides the determination of despair behavior, a two-bottle sucrose preference test was added in order to evaluate if hangover animals suffered from anhedonia. Results indicated that hangover mice did not experience anhedonia which is conceptualized as the loss in responsiveness to reward in rodents [52]. In this sense, it is well known that acute alcohol exposure has effects on blood sugar concentration [4]. Taking this into account, it might be expected a compensatory effect by an increase in sucrose consumption during AH which could make the sucrose relative intake similar than controls.

Previous studied demonstrated that the pathways related to affective behavior and autonomic arousal have direct effects on pain systems [27]. We tested pain perception by tail-immersion and hot-plate tests which evoke responses by spinal reflex and supraspinal integration respectively [53,54]. We found a significant AH effect over the spinal reflex which indicates an impairment in immediate automatic and involuntary response to a given stimulus. It was indicated that ethanol can modify neuropeptide release and nociception [55]. Moreover, it was established that the tolerance to the antinociceptive effects of ethanol continues through at least the acute phase of withdrawal in rats chronically exposed to alcohol [27]. However, there were no previous studies which verified this during AH. Here, we observed that alcohol impairs pain perception not only during alcoholism but also during hangover.

It was hypothesize that the exposure to acute binge doses of ethanol may result in activation of inflammatory processes in brain that could be responsible for withdrawal-associated changes in behavior, particularly to a sickness-related phenotype [56]. Here we observed a transient affective disorder during 14 h after the onset of AH. This adds to our previous evidence that motor, exploratory and locomotion impairments lasted over at least 16 h. Altogether, we demonstrate that alcohol hangover compromises both motor and affective state which could impair directly diary activities, job performances and cognitive and visuospatial skills as has been recently stated [57]. Summing up, the results obtained in the present work could contribute to build up an experimental model of alcohol hangover pathophysiological state which is the key for the development of a hangover cure.

## 5. Conclusion

During alcohol hangover there is an increment of anxiety-like behavior together with fear-related phenotype and depression signs. Moreover, our findings demonstrate a time-extension between 14 and 16 h for hangover affective impairments. As a whole, this study shows the long lasting effects of alcohol hangover over the acute phase of ethanol intoxication.

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