BASIC RESEARCH

Chronic consumption of distilled sugarcane spirit induces anxiolytic-like effects in mice

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OBJECTIVE: Chronic ethanol consumption is a major public health problem throughout the world. We investigated the anxiolytic-like effects and the possible ever injury induced by the chronic consumption of ethanol or sugarcane spirit in mice.

METHOD: Adult mice were exposed to a two-bottle free-choice paradigm for 6 weeks. The mice in Group A (n = 16) had access to sugarcane spirit + distilled water, the mice in Group B (n = 15) had access to ethanol + distilled water, and the mice in Group C (control, n = 14) had access to distilled water + distilled water. The ethanol content in the beverages offered to Groups A and B was 2% for the first week, 5% for the second week and 10% for the remaining four weeks. At the end of the experimental period, the mice were evaluated using the elevated-plus maze and the hole-board test to assess their anxiety-related behaviors. We also determined the serum aspartate aminotransferase and alanine aminotransferase levels.

RESULTS: In the elevated-plus maze, the time spent in the open arms was increased in the mice exposed to chronic ethanol (32 $\pm$ 8 vs. 7 $\pm$ 2 s, n = 9) or sugarcane spirit (36 $\pm$ 9 vs. 7 $\pm$ 2 s, n = 9) compared to the controls. In the hole-board test, the mice exposed to ethanol or sugarcane spirit displayed increases in their head-dipping frequency (16 $\pm$ 1 for the control group, 27 $\pm$ 2 for the ethanol group, and 31 $\pm$ 3 for the sugarcane-spirit group; n = 9 for each group). In addition, the mice exposed to sugarcane spirit displayed an increase in the aspartate aminotransferase / alanine aminotransferase ratio compared to the ethanol group (1.29 $\pm$ 0.17 for the control group and 2.67 $\pm$ 0.17 for the sugarcane spirit group; n = 8 for each group).

CONCLUSION: The chronic consumption of sugarcane-spirit produces liver injury and anxiolytic-like effects and the possible liver injury in mice.

KEYWORDS: Alcoholism; elevated plus maze; anxiety; liver; behavior.

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INTRODUCTION

Chronic ethanol consumption is a major public health problem throughout the world and one of the main causes of mortality in developing countries. Ethanol reinforcement and subsequent addiction are the prominent factors in the etiology of its abuse.[1,2]

Studies have shown that acute ethanol consumption exerts anxiolytic effects,[3,4] while its abrupt cessation following prolonged consumption leads to withdrawal anxiety.[5,6] Although several experimental models have investigated the consequences of the anxiety-like effects induced by ethanol withdrawal after chronic consumption, there is a lack of information on the anxiolytic effects of chronic ethanol consumption while ''under the influence''.

In Brazil, alcoholism has been an important public health problem, especially in the regions with significant poverty.[7] Interestingly, the most consumed alcoholic beverage in the Northeast region of Brazil is the distilled sugarcane spirit, known as cachaça, because it is relatively inexpensive compared to other alcoholic drinks, such as beer and wine.[8]

Distilled sugarcane spirit is a an authentic Brazilian beverage that is produced by distillation after the fermentation of sugarcane. Distilled sugarcane spirit is appreciated not only in Brazil but also throughout the world. The chemical composition of sugarcane spirit is rather complex; it typically contains organic molecules (ethanol, higher alcohols, acids, esters, aldehydes, and ketones) and inorganic species (Ca, Fe, Mg, K, and Na).[9] Although sugarcane-spirit consumption is common in Brazil, little is known about its chronic effects on anxiety.

In this study, we investigated whether the chronic consumption of ethanol and sugarcane spirit induces anxiolytic-like effects and liver injury in mice.

METHODS AND MATERIALS

Animals

We used forty-five male Swiss mice (3 to 3.5 months old), weighing 40-50 g. The mice were housed in collective cages in a temperature-controlled room (23 ± 1°C) that was maintained on a 12 h light-dark cycle (lights on at 06: 00, lights off at 18: 00). Food and water were provided *ad libitum*. All the experimental procedures were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Federal University of Paraiba Animal Care and Use Committee (CEPA # 0305/10).

Chronic sugarcane spirit / ethanol exposure

The two-bottle choice protocol was implemented as previously described.[10] In short, the two-bottle drinking protocol was employed over a six-week period. The mice were divided into three experimental groups. Group A (n = 16) had access to sugarcane spirit + distilled water, group B (n = 15) had access to ethanol + distilled water, and group C (n = 14) had access to distilled water + distilled water; group C served as the control group. The sugarcane spirit was provided by local certified producers in Paraiba State, Northeast Brazil. The level of ethanol in the sugarcane spirit was determined before the experiments. In the first week, the ethanol content of the beverages offered to groups A and B was adjusted to 2%; the ethanol content was increased to 5% in the second week and to 10% in the 4 remaining weeks.

Determination of body weight, food intake and water intake

Weekly body weight, food intake and water intake were determined for each mouse.

Detection of liver marker enzymes levels

Following the completion of the behavioral experiments, blood was obtained by cardiac puncture and separated by centrifugation (3,000 rpm for 10 min). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., China). The enzyme activities were expressed by the AST/ALT ratio. An elevated serum AST in relation to serum ALT has been proposed as an indicator that ethanol has induced liver damage, and an AST/ALT ratio > 1.5 has been found to be highly suggestive of alcohol-induced liver injury.[11,12]

Anxiety-like behavioral measurements

Elevated-Plus Maze Test. On the morning of the 42nd day, while the animals were under the influence of the ethanol/sugarcane spirit, anxiety-like responses were measured in the elevated-plus maze (EPM) test. The procedure was similar to the method described by Lister.[13] The experimental apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) was shaped like a 'plus' sign and consisted of two open arms (30 × 5 cm) and two equal-sized closed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). The maze was made of opaque grey PVC and was kept elevated at a height of 50 cm above the floor. The test consisted of placing a mouse on the central platform facing an enclosed arm and allowing it to freely explore the maze for 5 min. Entry into an arm was defined as the animal placing all four paws past the line dividing the central square from the arm. The test arena was wiped with a damp cloth after each trial. The time spent in the open arms was measured by an observer blind to the drug treatment. Anxiolytic-like activity was inferred from an increase in the time spent in the open arms.

Hole-Board Test. The hole-board test for anxiety in mice was implemented as previously described by Clark et al.[14] The apparatus was 60 × 30 cm, with 16 evenly spaced holes. The number of head dips into the holes was counted for each animal during a 3 min period. An increase in the head-dipping response was taken to indicate an anxiolytic-like effect, as described by File and Pellow.[15] In addition, motor activity in the hole-board test was evaluated by counting the number of quadrants transversed during the 3 min spent on the board.

Statistical analysis

The data are expressed as the mean ± SEM. The results were analyzed by the Student's *T* test or by a one-way analysis of variance (ANOVA) model followed by Tukey's post-hoc test when appropriate. In all the statistical analyses, the level of significance was set at $p < 0.05$.

RESULTS

Chronic sugarcane spirit consumption increases water intake

The body weights of the mice did not change significantly over the six-week experimental period in any of the groups (distilled water, week 0 = 46.0 ± 1.2 g and week 6 = 47.5 ± 1.1 g; ethanol, week 0 = 45.7 ± 0.9 g and week 6 = 47.1 ± 0.7 g; sugarcane spirit, week 0 = 46.6 ± 0.9 g and week 6 = 47.7 ± 0.7 g; n = 15 for each group, and $p > 0.05$). In addition, the weekly food intake per mouse was not significantly different among the groups (distilled water, week 0 = 42.7 ± 0.5 g and week 6 = 43.0 ± 1.0 g; ethanol, week 0 = 43.0 ± 0.8 g and week 6 = 42.1 ± 0.9 g; sugarcane spirit, week 0 = 42.6 ± 1.1 g and week 6 = 43.1 ± 0.7 g; n = 15 for each group, and $p > 0.05$). Moreover, the ethanol intake did not differ significantly from the sugarcane-spirit intake during the six-week experimental period, as illustrated in (Figure 1A). Interestingly, the water intake was significantly greater in the sugarcane spirit + distilled water group starting at the second week (53.7 ± 2.1 mL for the sugarcane spirit + distilled water group vs. 44.3 ± 1.8 mL for the ethanol + distilled water group; n = 15 for each group, and $p < 0.05$). The water intake remained elevated until the end of the sixth week (62.2 ± 2.5 mL for the sugarcane spirit + distilled water group vs. 47.0 ± 1.5 mL for the ethanol + distilled water group; n = 15 for each group, and $p < 0.05$), as illustrated in Figure 1B.

Liver marker enzyme levels are increased during chronic sugarcane spirits

Although the percentage of ethanol was adjusted to be identical for all the groups that received alcoholic beverages, the mice from the sugarcane spirit + distilled water group displayed a significantly greater than both control and ethanol groups increase in the AST/ALT ratio (1.29 ± 0.17

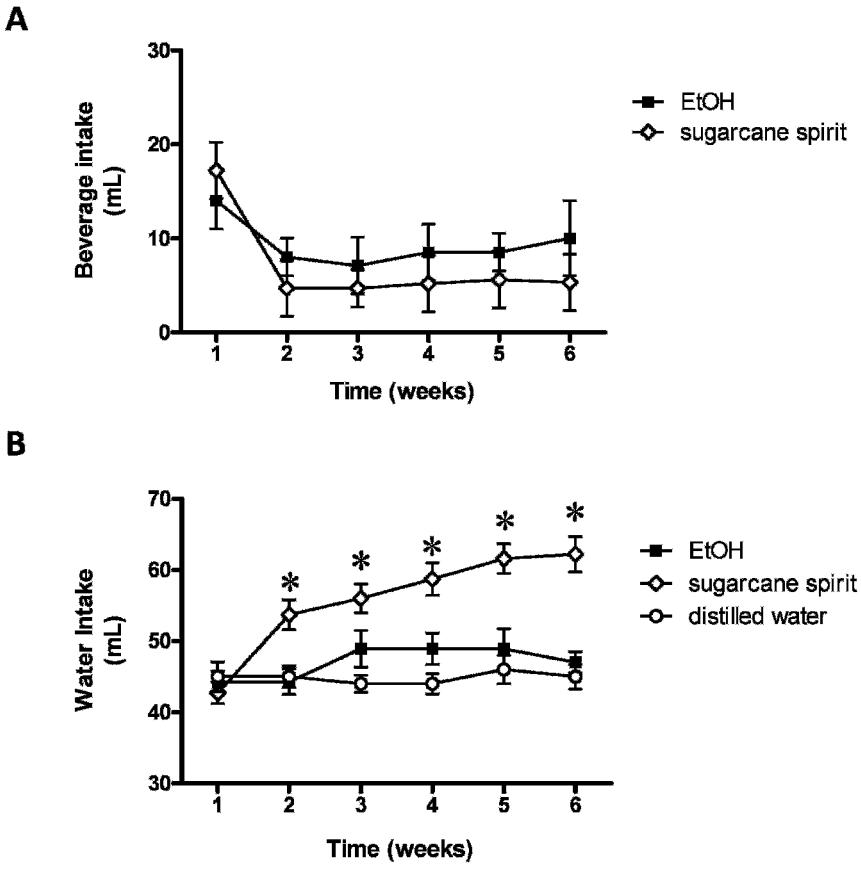

Figure 1 - A. The ethanol and sugarcane spirit consumption during the six-week experimental period. **B**. Water intake during the six-week experimental period in the three groups. The data are presented as the mean ± SEM over the six weeks of sugarcane-spirit or ethanol consumption. * $p<0.05$ compared to the ethanol or distilled-water group.

for the control group, 1.82 ± 0.18 for the ethanol + distilled water group, and 2.67 ± 0.17* for the sugarcane spirit + distilled water group; n = 8 for each group, and $p < 0.05$ compared to control), as is illustrated in Figure 2.

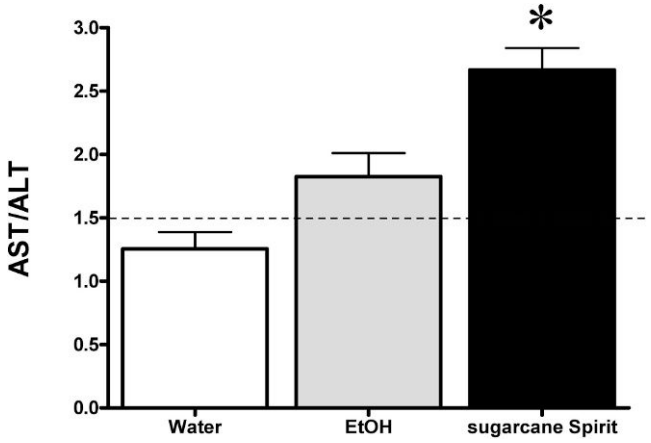

Figure 2 - *Detection of liver marker enzymes.* The data are presented as the mean ± SEM of the serum aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio. The dashed line represents the cutoff for suggesting liver injury (AST/ALT >1.5). * $p < 0.05$ compared to the controls (distilled water).

The chronic consumption of sugarcane spirit induces an anxiolytic-like effect in mice, as evidenced by the elevated plus maze and hole board test scores

The time spent in the open arms was increased in the mice exposed to chronic ethanol (32 ± 8 vs. 7 ± 2 s, n = 9, $p < 0.05$) or sugarcane spirit (36 ± 9 vs. 7 ± 2 s, n = 9, $p < 0.05$) compared to the control group. The time spent in the closed arms was significantly decreased in the animals consuming ethanol (183 ± 6 vs. 214 ± 4 s, n = 9, $p < 0.05$) or sugarcane spirit (150 ± 8 vs. 214 ± 4 s, n = 9, $p < 0.05$) compared to control group. In addition, the ratio of the time spent in the open arms to the total time spent in both the closed and open arms was significantly increased compared to the control (ethanol 34 ± 8 vs. 6 ± 2 s; sugarcane spirit 43 ± 12 vs. 6 ± 2 s, n = 9, $p < 0.05$). These data are presented in Figure 3.

In the hole-board test, the mice treated with ethanol or sugarcane spirit showed an increase in the number of head-dipping behaviors compared to the control condition (16 ± 1 for the control group, 27 ± 2 for the ethanol group, and 31 ± 3 for the sugarcane-spirit group; n = 9 for each group, and $p < 0.05$) (Figure 4A). In addition, the motor activity in the hole-board test was also increased in both the ethanol and sugarcane-spirit groups compared to the control group (21 ± 2 for the control group, 34 ± 2 for the ethanol group, and 40 ± 4 for the sugarcane-spirit group, n = 9 for each group, and $p < 0.05$) (Figure 4B).

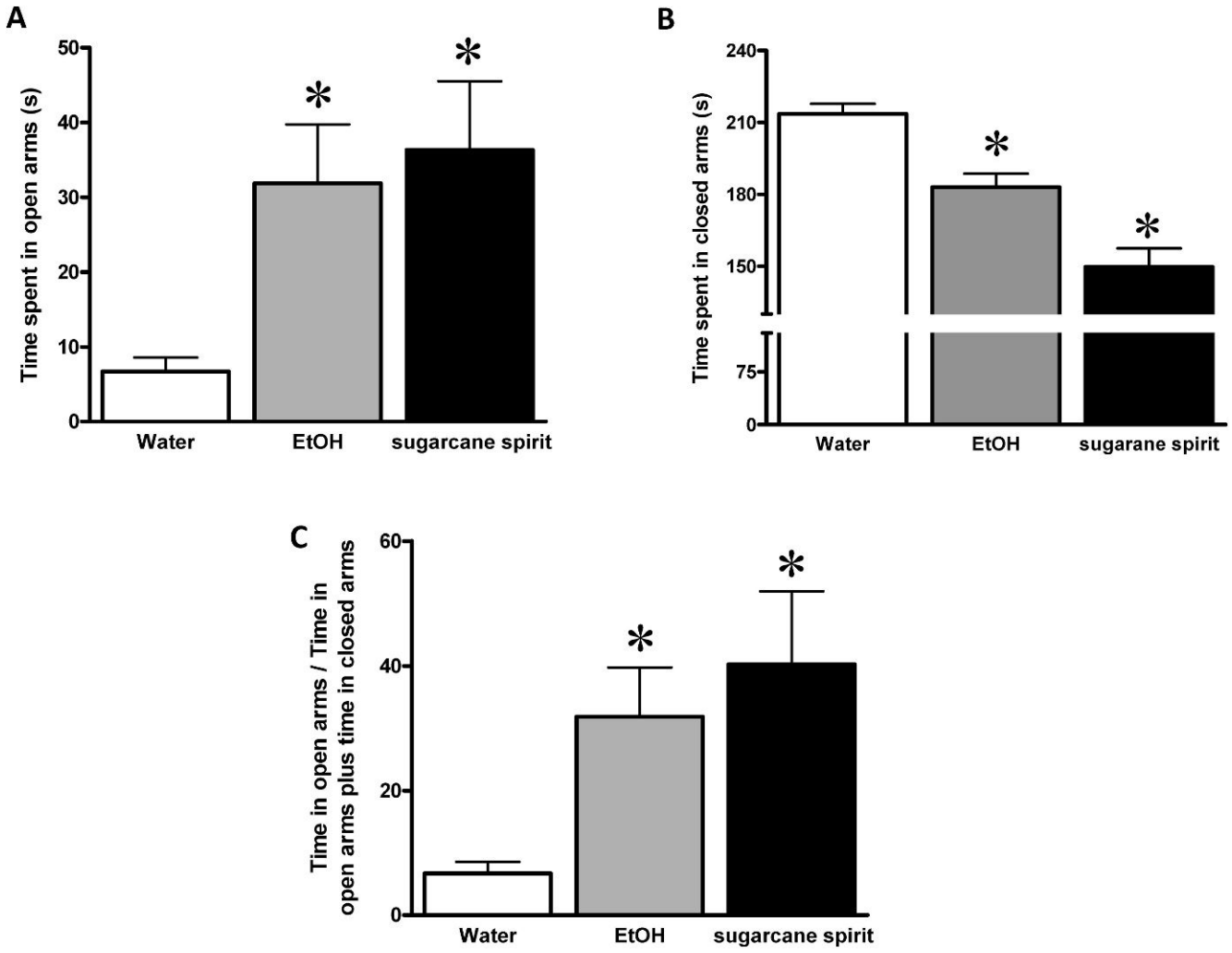

Figure 3 - The elevated-Plus Maze Test. A. The time spent (in seconds) in the open arms during the 5-minute testing period. **B**. The time spent (in seconds) in the closed arms during the 5-minute testing period. **C**. The ratio of the time spent in the open arms to the total time spent in both arms during the 5-minute testing period. The data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the controls (water).

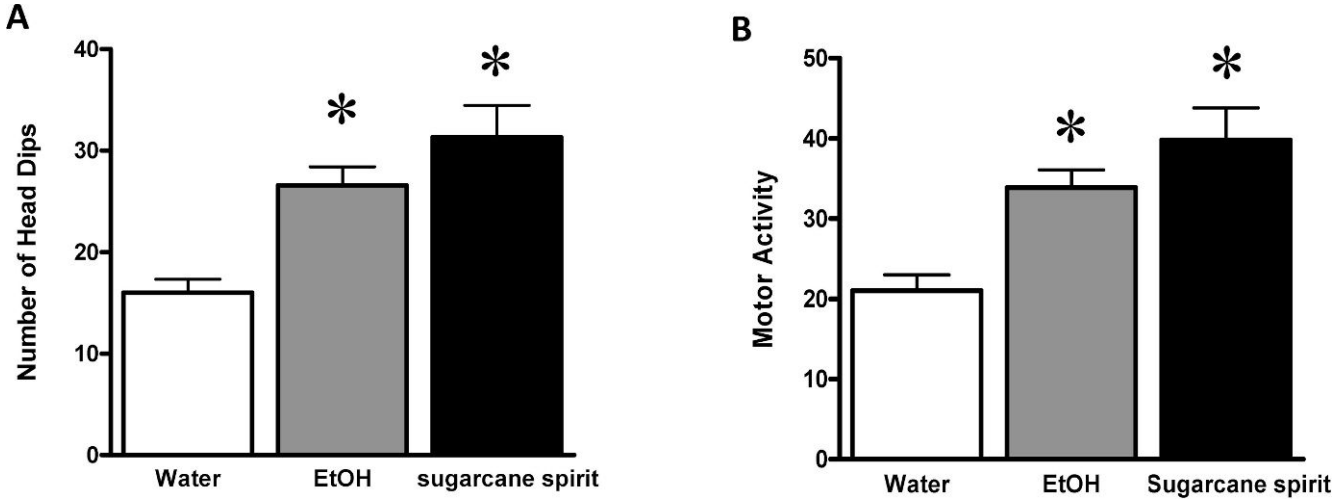

Figure 4 - Hole board test. A. The number of head dips. **B**. The motor activity, as expressed by the number of quadrants crossed during the time on the board. The data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the controls (water).

DISCUSSION

The major finding of this study is that chronic sugarcane-spirit consumption induced liver injury and anxiolytic-like behavior in mice. To our knowledge, this is the first study to report that sugarcane spirit elicits anxiolytic-like responses. In addition, the sugarcane spirit increased water consumption compared to the ethanol.

Many studies have shown anxiety-like behavior in mice after alcohol withdrawal.[5,6] In the present study, however, we investigated the behavioral effects caused by the chronic consumption of ethanol or sugarcane spirit while the mice were still intoxicated. Our findings demonstrated that the chronic consumption of sugarcane spirit or ethanol elicited anxiolytic-like effects, which were measured in the elevated-plus maze and hole-board tests.

In the elevated-plus maze, the normal exploratory behavior favors the closed arms, and this tendency to stay in the closed arms of the maze can be enhanced by compounds that increase the aversion toward the anxiety-provoking open arms (i.e., anxiogenics). By contrast, the administration of anxiolytic compounds reduces the natural aversion to the open arms and promotes exploration in them.[16,17] In the elevated-plus maze test, therefore, mice that spend more time in the open arms are less anxious, given that rodents are extremely aversive to open areas. We found that both ethanol and sugarcane spirit were able to significantly increase the time spent in the open arms, which indicated an anxiolytic effect in this test. In addition, the ratio of the time spent in the open arms to the total time spent in both the closed and open arms was significantly increased in the mice consuming ethanol or sugarcane spirit. Similar anxiolytic-like effects have been reported by Correia et al.[6] in mice chronically exposed to ethanol. Interestingly, the initial level of anxiety may determine the effects induced by ethanol. For example, Spanagel et al.[18] have reported a positive correlation between the initial level of anxiety and ethanol intake in Wistar rats. They tested drug-naïve rats in the elevated-plus maze to determine their initial level of anxiety and classified them as anxious or non-anxious prior to subjecting the rats to an oral ethanol self-administration procedure. Spanagel et al. have also demonstrated that moderate doses of ethanol (0.5-1.5 g/kg, i.p.) dose-dependently produce anxiolytic-like effects when tested in the elevated-plus maze. The blood ethanol levels achieved by oral self administration were similar to those obtained by i.p. injection, which suggested that the rats drank sufficient amounts of ethanol to produce anxiolytic effects. We further confirmed the anxiolytic effects induced by sugarcane spirit or chronic ethanol consumption using the hole-board test. In our experiments, sugarcane-spirit or ethanol consumption produced an increase in the number of head-dipping events, which has been proposed as an index of anxiolysis.[14,15] In addition, sugarcane spirit increased motor activity in the hole-board test, which suggested that motor activity is not compromised by chronic sugarcane-spirit consumption.

Serum AST that is elevated compared to serum ALT has been proposed as an indicator that ethanol has induced organ damage, and an AST/ALT ratio >1.5 is highly suggestive of alcohol-induced liver injury.[11,12] Interestingly, the biomarker data for this study indicated that both ethanol and sugarcane spirit affected the AST/ALT ratio. This result suggests that the consumption of ethanol and sugarcane spirit for six weeks initiated a process of liver injury and that the sugarcane spirit was more toxic than the ethanol. The explanation for the difference between ethanol and sugarcane spirit in producing liver injury over the six weeks of treatment may be attributable to the other components present in sugarcane spirit, such as organic molecules (higher alcohols, acids, esters, aldehydes, and ketones) and inorganic species (Ca, Fe, Mg, K, and Na).[9] Ethanol is known to produce liver fibrosis, steatosis and hepatotoxicity in mice.[19] These liver alterations, however, are dependent on the experimental paradigm. In our experiments, the AST/ALT ratio in the mice consuming ethanol was higher than 1.5, which suggested liver injury. Whether ethanol would eventually induce the same AST / ALT ratio as sugarcane spirit in this protocol is unclear, and further investigations are necessary.

Another important finding was that the mice consuming sugarcane spirit had higher water intake than the mice consuming ethanol, despite there being no difference between the total ethanol consumption and the sugarcane-spirit intakes. In folk culture, sugarcane spirit has been known to induce powerful hangovers, which are characterized by several symptoms (e.g., headache, tremulousness, nausea, diarrhea, fatigue combined with decreased occupational, cognitive, or visual–spatial skill performance, as well as increased vasopressin release and thirst).[20] Thus, the increased water intake in the sugarcane-spirit group may be attributable to a more intense hangover. The hangover induced by sugarcane spirit may involve compounds other than ethanol, such as higher alcohols and aldehydes. Importantly, there was no significant difference in the food intake or body weight among groups. Because ethanol consumption can possibly lead to malnutrition, a limitation of our study was the lack of an isocaloric group. Malnutrition did not seem to be a factor, however, because the animals drinking ethanol had body weights and food intakes similar to those of the animals that drank only water.

In conclusion, this study showed that the chronic consumption of sugarcane spirit produces liver injury and anxiolytic-like effects in mice. The greater AST / ALT ratio induced by the sugarcane spirit, which is suggested liver injury, may have been caused by the other organic compounds present in the sugarcane spirit, while the anxiolytic-like effects seemed to be caused by both the ethanol and the other compounds present in sugarcane spirit. The mechanisms by which sugarcane spirit leads to more rapid liver injury than does ethanol should be examined in future investigations.

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