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Alcohol Withdrawal at Different Points in Time Distinctly Affects *Wistar* Rats' Spatial Reference Memory

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ABSTRACT

Objectives: The consumption of alcohol by adults may lead to severe neurodegeneration and significant behavioral problems. An increase in the harmful effects of alcohol becomes aggravated after the development of alcohol dependence. Furthermore, the serious damage from chronic alcohol intake on the hippocampus has gained attention due to its role on learning and memory. Therefore, the study aimed to examine the retention of spatial reference memory during different points in time regarding alcohol withdrawal in *Wistar* rats.

Materials and Methods: The study has therefore administered alcohol to rats at gradually increasing doses from 4.5 to 12 g/kg/day in a binge-like manner using the intragastric intubation technique for six days followed by 24, 48, or 96 hours of alcohol withdrawal. To evaluate the effects of alcohol withdrawal, the alcohol-exposed rats have been tested regarding their spatial reference memory.

Results: An adverse effect from alcohol withdrawal on memory retention was observed in the 24-hour alcohol withdrawal group. This effect decreased at 48 hours of withdrawal, but reappeared at 96 hours.

Conclusion: The study's results suggest that alcohol withdrawal itself, even after a relatively short period of alcohol intake, may also adversely affect memory. Therefore, withdrawal therapy from alcohol should be performed in a controlled manner to protect the brain from extended alcohol withdrawal-induced spatial memory impairments.

Keywords: Alcohol withdrawal, spatial reference memory, *Wistar* rat, intragastric intubation

INTRODUCTION

Alcohol abuse is undoubtedly a major problem in human society. For several decades, chronic alcohol intake during adult life has been recognized to produce highly adverse effects on brain morphology due to severe neurodegeneration resulting in behavioral deficits. Some of these alcohol-induced adverse effects are directly related to alcohol intoxication, but some may be related with the product of the development of alcohol dependence. Alcoholics find both physical and psychological alcohol dependence to manifest itself upon withdrawing from alcohol in a form known as abstinence syndrome at different degrees of severity.

Animal studies have reported chronic alcohol consumption to damage on both the basal forebrain cholinergic system and hippocampus that structures are crucial in learning and memory (1, 2). A decrease in granule cells of the dentate gyrus, the synapses of the mossy fiber-CA3 pathway, the pyramidal neurons of the CA3 and CA1 sub-regions, and the local circuit interneurons are seen to have occurred in rodents treated with chronic alcohol consumption (3-5). Furthermore, chronic alcohol consumption has been observed to reduce the synthesis of protein in hippocampal neurons, especially in the CA3 sub-region (6). In addition, chronic alcohol treatment diminishes the intensity of the long-term potentiation (LTP) produced in the hippocampus

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(7), with a reduction in long-term depression (LTD) occurring in the hippocampal Schaffer collaterals (8).

At the behavioral level, a range of short-term memory tasks are disrupted by alcohol consumption in humans, including verbal list learning (9, 10), spatial learning, and pattern recognition (non-spatial tasks) (11, 12). Franke et al.'s study (13) used a complex elevated labyrinth and reported behavioral impairment regarding task performance in alcohol-exposed rats. As a result of chronic alcohol administration, the errors in long-term reference memory as well as the errors in trial-to-trial working memory had previously been observed as a decrease in the performance of radial maze spatial tasks (14, 15) - with working spatial memory that appearing to have been more affected than reference memory (16, 17). The effects of chronic alcohol consumption on cognitive functions and hippocampal cell loss are also exacerbated during the withdrawal phase (18, 19).

In light of these data, both chronic alcohol intake during adult life as well as alcohol withdrawal can be concluded to appear deteriorating effects on brain morphology, brain physiology, and behavior. However, some inconsistent results are found among experiments. The current studied with an animal model of human alcohol abuse to examine the effect of alcohol withdrawal at various points in time on memory retention in rats. In addition, the study aimed to dissociate the effects of early and late alcohol withdrawal with regard to testing spatial reference memory at different times in withdrawal.

MATERIALS AND METHODS

Animals

This study examined 4-month-old male *Wistar* rats provided by Ankara Serum-Production Facility (Hifzisihha, Ankara). The rats were housed at the Department of Biological Sciences at Middle East Technical University (METU). The animal room was set to a controlled 12 h light/12 h dark cycle with the temperature in the room being maintained at 22 ± 2°C, and the tests had being conducted during the light phase of the cycle. In addition, rats had free access to laboratory chow and water throughout the applications.

The study applied to the Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes (Official Gazette No. 28141) for all the animals during the experiments. All procedures involving animals were carried out in accordance with the ethical considerations obtained from the METU Ethics Committee (Decision No. 2009/15).

Place Learning (Acquisition Training) in the Morris Water Maze

Rodents were commonly tested for long-term spatial learning and memory using a Morris water maze (MWM). The tank was 150 cm in diameter and 60 cm high, filled with water that has been colored with food dye. The temperature of water was maintained at 23°C by an automatic heater. The animal in the MWM was recorded for measurements of distance, time, and velocity for finding the invisible platform using a computer-controlled video system (EthoVision, Noldus Information Technology, Holland). The maze was divided into four quadrants on the computer screen through imaginary lines. A transparent Plexiglas portable platform (11×11 cm) was positioned 2 cm below the surface of the water in the center of one of the quadrants. The rats were able to swim and climb onto the platform to get out of the water. During the entire experimental period, multiple extra-maze cues remained immobile in the experimental room.

In accordance with classical MWM training (20), the study conducted six daily sessions of four trials to each animal. During the session, the rats were facing the pool wall and were placed into the MWM from one of four starting quadrants (N, S, E, W) that were pseudo-randomly selected. The trial ended once the rat climbed onto the invisible platform or spent a maximum of 60 seconds in the water. Inter-trial intervals were limited to 5 minutes. After each session, the mean latency was calculated for each rat. The learning criterium for the classical MWM training was the latency of reaching the hidden platform in 10 seconds or less. At the end of the sixth session, the rats were grouped that all three groups showed equal levels of performance at the beginning of the alcohol administration.

Alcohol Administration

To control the alcohol dosage delivered to the rats, binge drinking was applied through the intragastric intubation method. Alcohol administration started the day after the sixth session of MWM training. The alcohol was administered to the three alcohol groups (the AW24 group with a 24-hour alcohol withdrawal period (n = 8), the AW48 group with a 48-hour alcohol withdrawal period (n = 7), and the AW96 group with a 96-hour alcohol withdrawal period (n = 8)) by using an intragastric feeding needle (18ga, 3 in, Stoelting Co. USA) to deliver alcohol directly to each rat's stomach. The alcohol dosage was increased daily, starting with 4.5 g and going up to 12 g of alcohol per kg of body weight. The alcohol administration continued for six consecutive days. Laboratory chow and water were available *ad libitum* for these animals. The behaviors of the alcohol-treated rats were rated after each treatment in accordance with the Majchrowicz protocol (21). The maximum tolerated alcohol dosage was determined based on their ratings. To prepare the alcohol, distilled water was mixed with ethyl alcohol (99.8%, Merck) to form a 25% v/v solution. The intubation control group (IC, n = 7) consumed sucrose solution whose caloric value was calculated with respect to the alcohol's caloric content to observe potential intubation-related stress effects. In addition, an intact control group (C, n = 7) was also present that underwent no treatment whatsoever. A strict daily time schedule was followed for the rats' protocol for alcohol consumption. This schedule had allowed to the rats obtain the solutions in three equal doses at 10:00 a.m., 1:00 p.m., and 4:00 p.m. To protect the animals' stomachs, a 50 mL mixture of water and milk were given to each rat after the last alcohol dose.

Blood Alcohol Concentration (BAC)

A separate group of rats received alcohol treatment but no behavioral testing (n = 3) and was used for measuring BAC. Under

ether anesthesia, blood samples from the alcohol-consumed animals were collected in tubes containing EDTA through an intracardiac puncture. The blood was taken 3 h after the last intragastric intubation on the sixth day of alcohol administration (22). At room temperature, a Biolabo alcohol assay was performed to determine the BAC of the serum samples obtained by placing them in a centrifuge at 1,000 rpm for 10 minutes.

Probe Trials: A Memory Retention Test

The study used the probe trial to gain insight into the strength of rats' acquired responses as well as indirectly in regard to their spatial memory. Therefore, a 60-second probe trial was performed after completion of alcohol consumption. During the probe trial, the platform was removed from the maze, and a circle with a 40 cm diameter (annulus 40, A40) was set up on the computer monitor so it surrounded the original platform site. The total durations of time the rats spent in each quadrant as well as in the A40 circle were recorded. The probe was applied based on the groups 24, 48, or 96 hrs after the last dosage of alcohol.

Statistical Analyses

Using all variables, group means were calculated along with the standard errors for the mean (SEM). The study also used the statistical package program SPSS to conduct the repeated measures ANOVA, with the treatment being the independent variable and the sessions/trials being the repeated measures. One-way ANOVA and the post-hoc Tukey test were performed to analyze the probe data.

RESULTS

Determining BAC

The alcohol-administered rats were measured as having a BAC of 605.67 ± 36 mg/dL (range between 569-641 mg/dL) 3 hours after the last administration of alcohol (12 g/kg/day).

Classical MWM Training

According to two-way repeated measure ANOVA, the day effect ($F_{(5, 120)}$ = 62.70, $p \leq 0.001$) was found significant regarding escape latency in the water maze. During the whole training period, the latency to get out of the water by climbing onto the invisible platform decreased for all animals. No differences were observed among the groups ($F_{(15, 120)} = 0.87$, $p = 0.60$). The learning criterium was achieved by all rats on the fourth day of the MWM learning period (Figure 1).

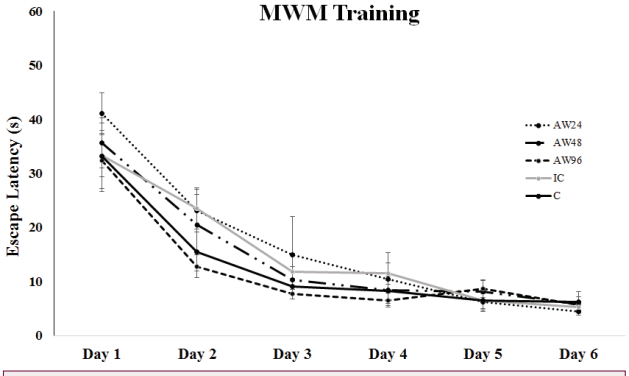

Figure 1. Comparison of the learning performance of rats by mean swim latency to reach hidden platform calculated for six days of MWM training, Error bars denote SEM.

Probe Trials After Alcohol Administration

Animal performance was assessed based on the percentage of time spent in the platform quadrant. According to the one-way ANOVA, a significant difference among groups was obtained regarding the percentage of time spent in the platform quadrant ($F_{(4, 34)}$ = 8.135, $p < 0.001$). In addition, the percentage of time spent in the platform quadrant for the AW24 and AW96 groups were significantly lower according to Tukey's test (p = 0.024 and p = 0.045, respectively) compared to the IC group. The AW48 group performed significantly better than the other AW groups (p = 0.001), approaching the performance of the control groups (Figure 2).

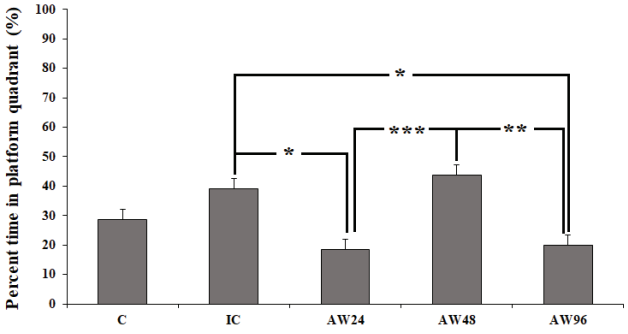

Figure 2. Percent time which was spent in the platform quadrant on the 60-s probe trials in each treatment group independently. Error bars denote SEM. The degree of significance was denoted as *for p≤0.05, ** for p≤0.01, *** for p≤0.001.

As seen in Figure 3, a significant change in the amount of time spent in the A40 circle occurred among the groups ($F_{(4, 34)}$ = 3.885, p = 0.012). Compared to the control groups, the decrease in time spent in A40 was not significant for the AW24 and AW96 groups

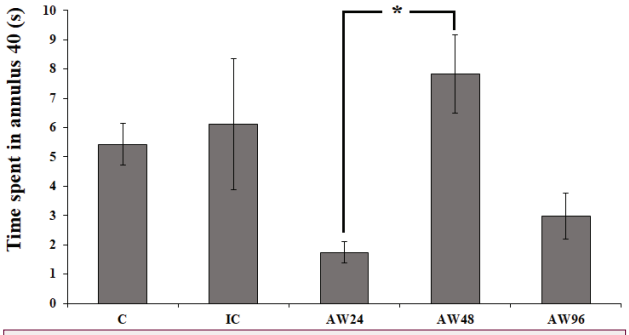

Figure 3. Time in A40 ± SEM calculated for each treatment group independently. The degree of significance was denoted as *for p≤0.05.

according to the post-hoc comparison tests (Figure 3). However, the AW48 group was observed to have spent significantly more time than the AW24 ($p = 0.014$) and AW96 ($p = 0.063$) groups in the platform quadrant.

As seen in Figure 4, the ratio of the time spent in the platform quadrant (NE) compared to the time spent in the opposite quadrant (SW) varied significantly among the groups ($F_{(4, 34)} = 5.035$, $p = 0.003$), with the ratio being highest in the AW48 group ($p = 0.018$ for control; $p = 0.008$ for AW24; $p = 0.004$ for AW96).

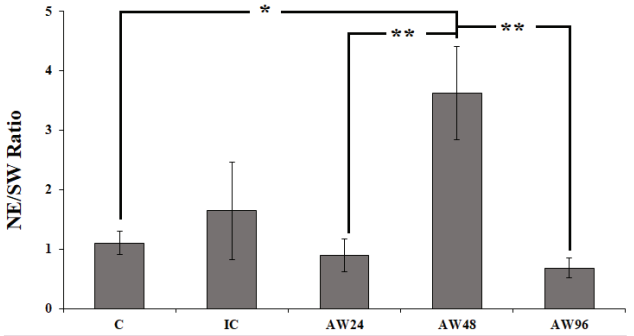

Figure 4. Total time spent ratios in the platform quadrant (NE) to the opposite quadrant (SW) for each treatment group independently. The degree of significance was denoted as *for p≤0.05 and ** for p≤0.01.

DISCUSSION

The literature has a few studies that have examined the effects of alcohol withdrawal on memory retention over different withdrawal time periods. Hence, the present study has been designed to examine whether alcohol withdrawal affects on memory retention and retrieval. According to the application of the experimental protocol (up to 12 mg/kg alcohol administration), exposing rats to binge-like alcohol dosages for 6 days produced an average BAC of 605.67 mg/dL. The memory retention was disrupted 24 hours after the last alcohol consumption, which may be indicative of an early withdrawal symptom. Interestingly, the memory performance was restored 48 hours after the last alcohol consumption, reaching the control levels. However, alcohol withdrawal dramatically deteriorated the memory performance of rats during the 96th hour of withdrawal.

The late effect of alcohol withdrawal has also been noted in previous studies. For example, researchers found remarkable alterations in memory retention tasks between alcohol and control groups when retraining was conducted a year after the learning acquisition time (16). Chronic alcohol intake has also been found to adversely affect the working spatial memory in adults more than their reference spatial memory when performing hippocampus-dependent cognitive tasks (13-15, 23). In addition, mild effects are seen to have occurred on spatial working memory after short-term alcohol intoxication (26 days of a liquid diet containing alcohol followed by 17 days of no alcohol) (24). According to another study, all alcohol-dependent patients showed impaired free memory recall on their first day of withdrawal over a battery of behavioral tests. However, their verbal memory ameliorated on the seventh day of withdrawal, then deteriorated again on the 14th day of withdrawal (25). Accordingly, the study suggested different brain regions engaged in different memory functions to be affected at different time periods by alcohol withdrawal. The prefrontal cortex functions have been postulated as being primarily required for short-term memory while the medial temporal lobe functions are required for long-term memory. The frontal lobe is also believed to be more affected by early alcohol withdrawal than the medial temporal lobe (25, 26).

A correlation also exists between alcohol withdrawal and dysregulation of stress hormones. The level of corticosterone in rats after chronic alcohol consumption changes 24 hours after withdrawal. Animal studies have confirmed the acute rise in adrenocorticotropic hormone (ACTH) and glucocorticoids after drinking alcohol in nonhuman primates and rodents. Neuroendocrine tolerance toward alcohol is thought to result from repeated intoxication and withdrawal cycles (27, 28). Therefore, the current study found the differences in the memory performance in the different withdrawal periods to perhaps also be related to the different levels of stress hormones.

In addition, the lack of difference at the 48-hour alcohol withdrawal group is related to the hypothesis that a compensatory process may mitigate alcohol's potential damaging effects on the brain. For instance, alcohol-exposed rats may compensate neuronal death-related functional deficits by adapting their synaptic structure to moderate alcohol doses over a relatively long period of time. This notion has been supported by the ratio of mossy fibers-CA3 synapses being shown to be maintained and the percentage of mossy fibers plasmalemma occupied by synapses to also increase while hippocampal CA3 pyramidal and granule cells gradually are lost after consuming alcohol for 6-12 months (5, 29). Interestingly, the results from these previous studies revealed the reduced formation of new contacts, suggesting a compensatory process. Moreover, the compensatory process was seen to have broken down in rats when they consumed alcohol in a liquid diet over 18 months. Another possibility is that moderate alcohol consumption might result in increased adult neurogenesis (30). Hippocampal dentate gyrus (DG) and forebrain subventricular zone (SVZ) are two important regions of the brain that undergo adult neurogenesis. Hippocampal neurogenesis also appears to be important in learning and memory development (31). While unusual adult hippocampal neurogenesis may produce negative effects on brain circuitry in a healthy brain, this may be advantageous in the alcoholic brain.

In summary, different mechanisms are suggested to be active at different points in time with regard to alcohol withdrawal. Therefore, designing a treatment for alcohol abuse and

disorders and time-dependent treatment strategies should be included in order to prevent time-dependent damages to the alcohol-addicted brain.

Ethics Committee Approval: Ethical approval was obtained from the ethical committee of the Middle East Technical University (Decision No. 2009/15).

Peer-review: Externally peer-reviewed.

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