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(RESEARCH ARTICLE)

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Exposing zebrafish to both alcohol and nicotine for thirty minutes prior to a meal diminishes their inclination to seek food

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Abstract

The global issue of drug abuse, particularly involving alcohol and nicotine, influences individuals' appetite levels, potentially leading to fluctuations in body weight. This study employed the zebrafish model to investigate food-seeking behavior under the influence of alcohol, nicotine, or their combination, as well as after drug withdrawal. When food was easily accessible, no significant differences in food aggression were observed between the exposed and non-exposed fish groups, except when nicotine was present, which notably reduced aggression levels towards food. However, 30 minutes post-drug withdrawal, food cravings in the alcohol-nicotine exposed fish groups resembled those of the control group when food was readily available. Yet, when food searching activity was required, aggression toward food significantly decreased after drug withdrawal in the nicotine and nicotine-alcohol combined exposed fish groups. This finding could contribute to the reported weight loss in individuals using alcohol and nicotine together. Further research is warranted to elucidate the underlying mechanisms.

Keywords: Zebrafish; Alcohol; Nicotine; Co-abuse; Appetite Memory

1. Introduction

Alcohol and nicotine are widely abused substances and often co-abused in humans, as documented in various studies.[19, 12, 31] While both activate the dopaminergic system's final neural pathway, associated with pleasure and reward,[28, 23] they exhibit distinct behaviors toward food due to their unique characteristics [3, 20]. Under normal addictive conditions, they influence food behavior differently, although high doses may impair locomotor behavior and, in some cases, memory and learning. Nicotine, though highly toxic, rarely causes fatalities but can lead to central respiratory failure and arrhythmias.[24, 21] Conversely, acute alcohol intoxication often impairs memory and induces anxiety-like behavior, with an anti-appetite effect, similar to nicotine's.[3, 13, 6] Moderate co-abuse effect on feeding behavior remains unclear.

Zebrafish have emerged as a model for studying drug-induced behavioral changes, including hyperactivity, anxiety, appetite, and learning and memory.[25, 18, 26, 27, 17, 5, 22, 11] While alcohol-induced behavioral changes in Zebrafish have been extensively reviewed,[32, 8, 26, 27, 29] nicotine-induced changes are relatively recent.[14] However, the study of alcohol and nicotine's effects on feeding behavior using Zebrafish as a model remains scarce. Thus, this investigation aimed to assess food aggression levels in Zebrafish after exposure to alcohol, nicotine, or both.

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2. Materials and methods

2.1. Materials

Adult zebrafish (*D. rerio*), averaging 3 cm in length and weighing approximately 700 mg, were procured from a nearby aquarium store. Nicotine from Sigma-Aldrich (CAS Number: 5411-5) and alcohol from Merck (Product number 1009711000) were utilized. Prior to introducing the fish, aquarium water conditioning was conducted by adding 3 g of artificial instant ocean salts per liter of deionized distilled water. This treatment was performed on a large scale (200 L) and allowed to equilibrate for 3 days.

2.2. Zebrafish maintenance and selection

Healthy adult zebrafish were carefully chosen based on strict selection criteria and housed in spacious aquarium tanks measuring 45×60×25 cm. They were kept in preconditioned aquarium water with a conductivity of approximately 1,500 µs/cm and a pH of 7. The water temperature was maintained at 24 °C, and adequate oxygen levels were ensured by continuous aeration using an air pump. To maintain water quality, filtration was performed daily for 2 hours, supplemented by the addition of aquarium disinfectant, and 30 % of the tank water was replaced daily with Millipore-filtered water containing appropriate instant ocean salt. Under normal conditions, the fish were fed three times a day (at 10 am, 2 pm, and 6 pm) with a diet of bloodworms, sufficient to be consumed within 5 minutes. After 10 days of maintenance, healthy fish displaying normal swimming and feeding behavior, as well as typical morphological features, were selected for the study.

2.3. Method for determining feeding behavior using:

2.3.1. Simple non-partition feeding tank

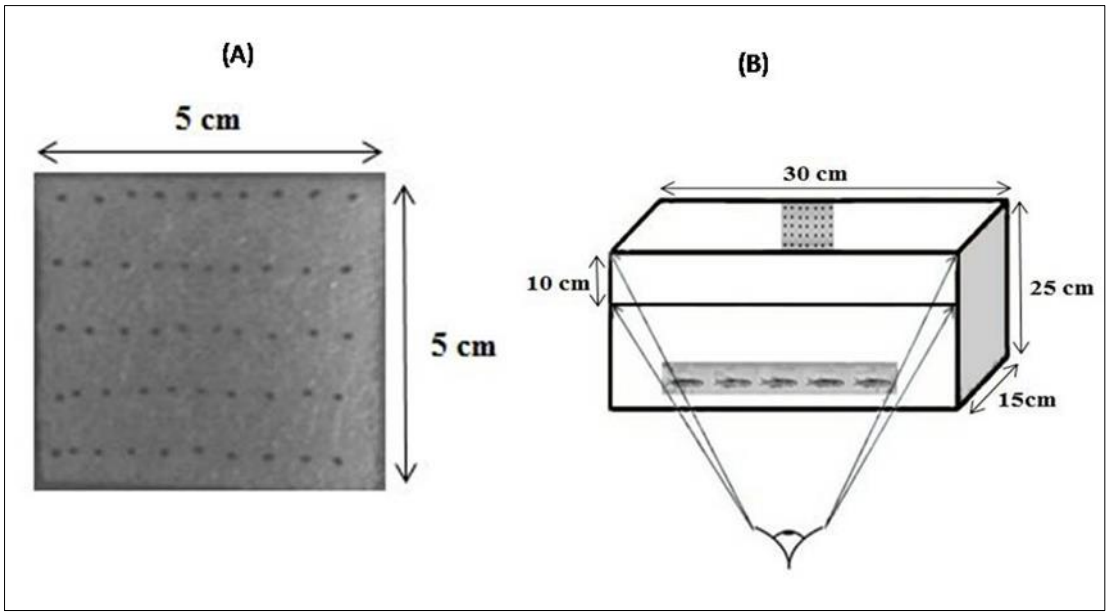

Figure 1 Schematic view of simple non-partition feeding tan

(A) Feeding was carried out through the food particles were attached to one side of thermo cool sheet (5×5 cm) with the help of soft tissue adhesive glue. This preparation was allowed to completely dry so that no food particles come out from the thermo cool sheet when introduced into simple non-partitioned feeding tank. (B) The size of the feeding tank was (30×15×25 cm) and a CCD camera of speed 25 frame/ sec was fixed from on the side of the tank in parallel to thermo- cool sheet, so striking of fish to the food particles could be directly record.

To assess feeding behavior, we employed a non-partition feeding tank measuring 30×15×25 cm, ensuring easy access to food. A (5×5 cm) thermo-cool square sheet, 5 mm thick, was utilized for food delivery and assessing aggression levels towards food. Food particles were affixed to one side of the sheet using non-toxic adhesive glue, ensuring no detachment during placement in the feeding tank. A CCD camera positioned parallel to the sheet facilitated direct recording of

feeding activity. The sheet was securely attached to the tank's side wall to prevent movement during feeding, allowing focused recording at a fixed point (Figure 1A and 1B).

This setup was crucial for accurately recording feeding activity; without it, food particles were easily dispersed by fish, hindering focused recording. Control fish underwent six days of training to acclimate to the feeding procedures. Their average feeding speed was recorded using the CCD camera after introducing the thermo-cool sheet carrying food particles as shown in figure 2.

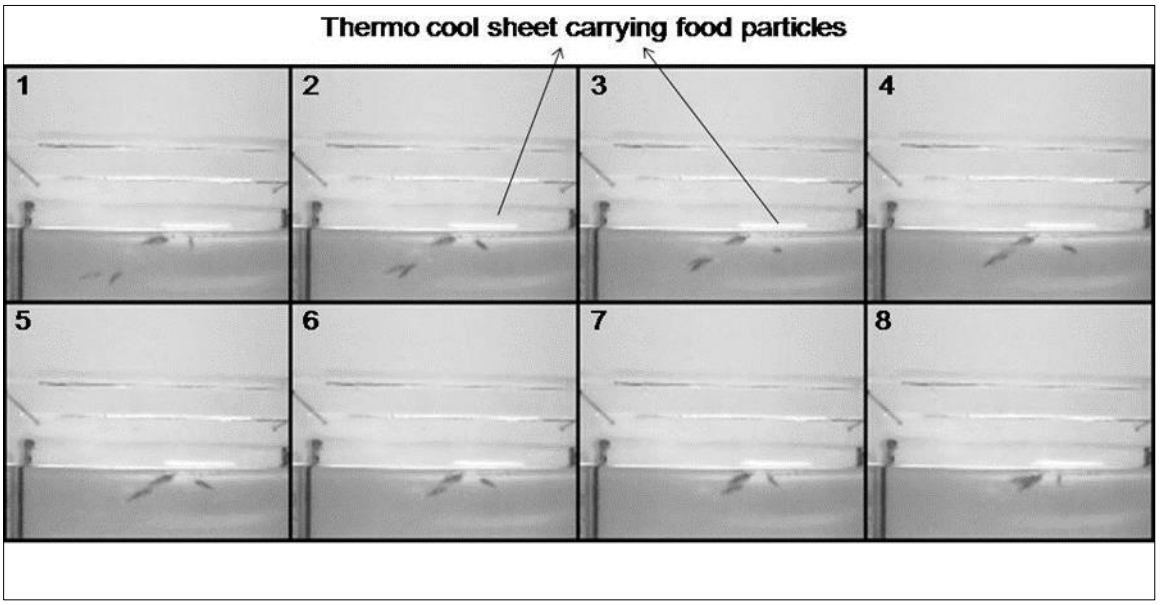

Figure 2 A representative feeding activity in non-partition feeding tank.

Image sequence showing the movement of fish toward thermo cool sheet carrying food particles.

For drug-stimulated fish, feeding speed was monitored in the presence of alcohol (210 mmol/l), nicotine (12 µmol/l), or a combination of both. Optimal drug concentrations were selected to minimize visual acuity effects, reduce anxiolytic effects, and ensure negligible mortality rates, based on previous studies.[16, 5]

Fish were initially treated with drugs for 45 minutes before introducing food particles, with recordings starting immediately thereafter for 15 minutes. In another experiment, adult fish were treated with addictive drugs for one hour in a separate tank. After this treatment, they were transferred to the feeding tank (without drugs) to allow for a 30-minute recovery period. Recordings commenced after introducing food particles.

Aggression levels towards food in control fish were compared with those under drug influence and after recovery, by counting strikes per minute to the thermo-cool sheet carrying food particles. Strikes were counted by reviewing recorded footage at reduced speed, ensuring accurate assessment.

2.3.2. T-maze partition feeding tank

T-maze tanks were constructed with opaque glass plates forming two arms and a neck (see Figure 3). A manual door controlled the opening from the neck to the arms. Small shielded holes with transparent glass were placed in the corners of both right and left arms to allow light to pass through. Low-intensity green and red lights were installed near these shield holes in each arm. A CCD camera connected to a computer was positioned above the tank to provide a top-down view of the entire setup.

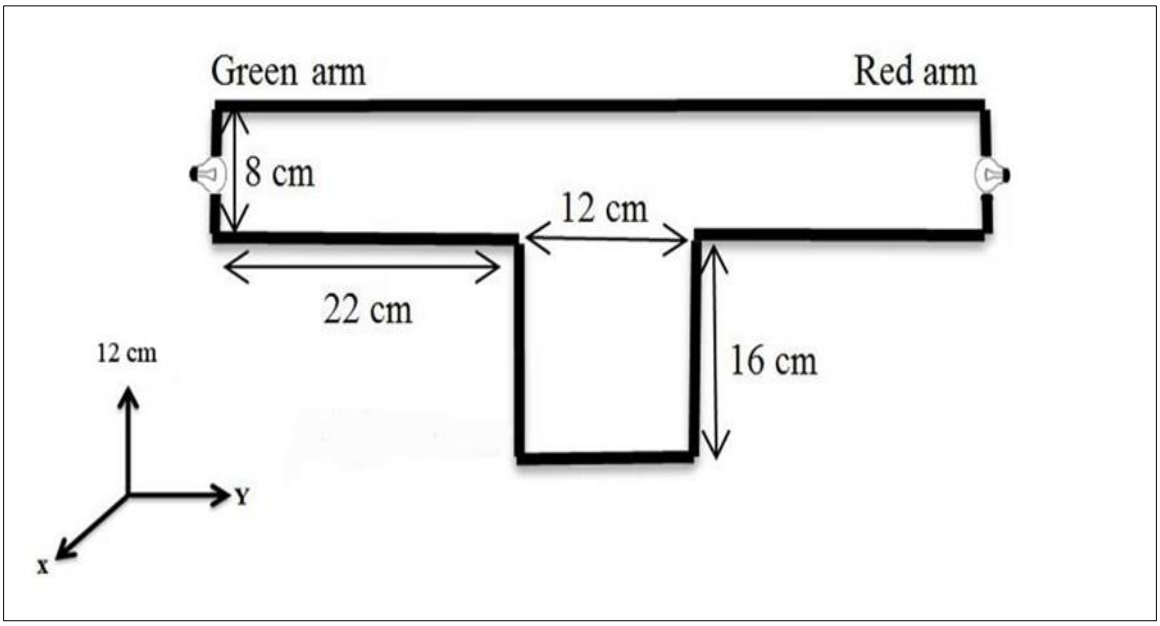

Figure 3 Schematic view of T-maze partition tank for assessing learning and memory behavior of Zebrafish. T-maze partition tank was constructed through the measurement pointed in model figure.

For recording movement of fish to the neck, right and left arms of the T-maze tank, a CCD camera was positioned on the top of the tank in such a way that it can view whole of the tank. The CCD camera was connected to computer placed outside of the observation room. During experimentation fish were first put in the completely opaque T-maze neck with the door closed. Such an arrangement prevents fish pre decision making of the direction of the arm where the food was placed. Whole of setup was placed in a closed room shield from human interference that was devoid of any external stimulation such as vibration and noise. The neck door was pull up mechanically from far away through the string attached to the door.

During experiments, fish were initially placed in the T-maze neck zone with the door closed, preventing them from seeing the area where food was located. The entire setup was placed in a behavior room devoid of external stimuli such as vibrations, noises, and human interference, with the observation room located outside. Fish behavior was monitored through live footage captured by the CCD camera and viewed on the computer. Feeding was initiated at the appropriate time by mechanically opening the neck door using a string attached to the door from the observation room.

In our T-maze experiment, healthy fish were divided into four separate rearing tanks, with each group comprising 8 individuals (4 male, 4 female). Slight modifications were made to the feeding program and technique, reducing the feeding frequency from three times to twice a day. Instead of feeding at 10 am, feeding times were adjusted to 2 pm and 6 pm.

At 2 pm, feeding was conducted in the T-maze partition tank. Fish were introduced into the closed-neck area of the T-maze tank one hour before feeding. Instead of using a thermo-cool sheet carrying food particles, we utilized a transparent plastic sheet coated with non-toxic adhesive glue. A fixed amount of medium-sized dry liver particles was evenly distributed over the adhesive glue layer. The preparation stood for 1 to 2 hours to ensure proper attachment of all food particles. Subsequently, the food-laden plastic sheet was submerged in water and left for 30 minutes. Afterward, the preparation was removed from the water, excess moisture was carefully blotted with tissue paper, and the total weight of the preparation was recorded.

The prepared sheet was then clipped onto the side wall of the feeding arm of the T-maze tank, with the side containing food particles facing the water in the feeding tank. Figure 4 illustrates a representative cumulative feeding activity of well-trained control fish groups within 2 minutes after opening the neck door of the T-maze tank.

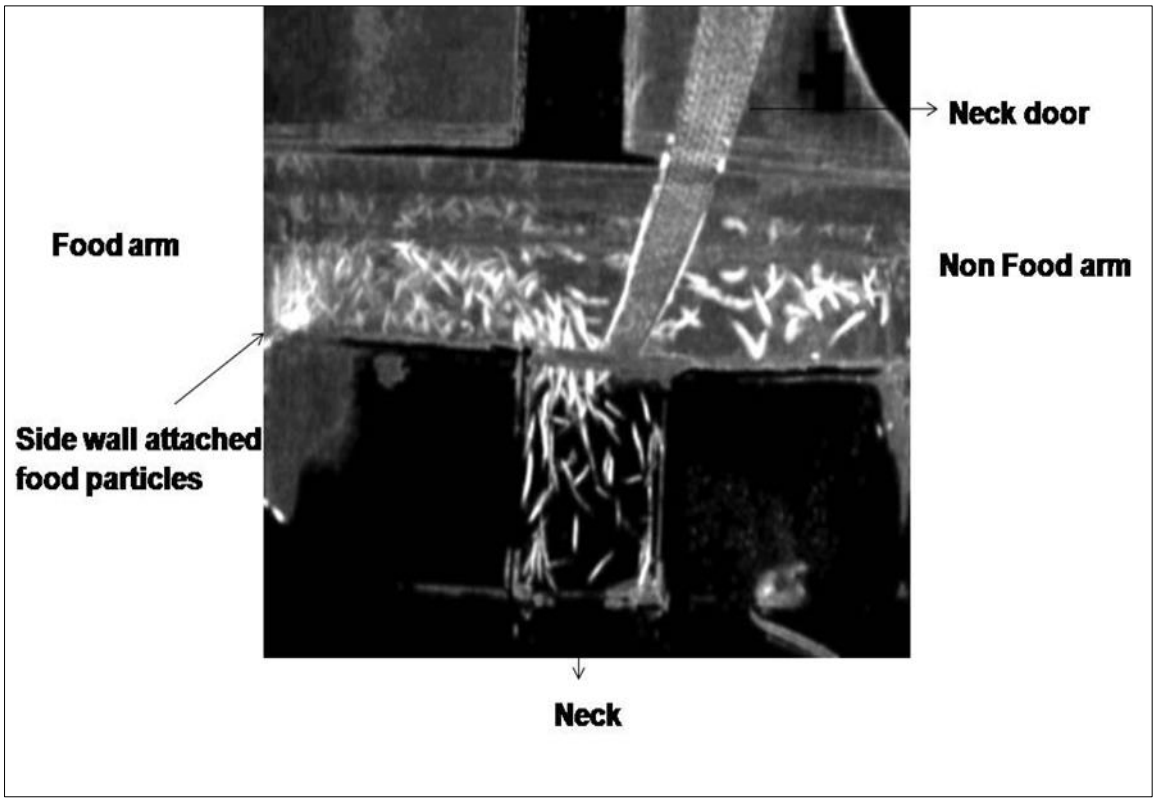

Figure 4 A representative accumulative feeding activity of well-trained control fish groups within 2 minutes after the opening of neck door of T-maze tank.

The high movement of fish toward food arms and feeding the sidewall attached food particles is visible while only few movements were seen toward non-food arm.

In assessing the withdrawal effects of drugs, fish were first introduced into a treatment tank for 1 hour. In this tank, fish were exposed to either alcohol at 210 mmol/l, nicotine at 12 µmol/l, or a mixture of alcohol and nicotine at the said concentrations. After this exposure, fish were removed from the treatment tank and placed into the closed-neck area of the T-maze tank. Feeding commenced 30 minutes after drug exposure ceased, initiated by opening the neck door of the T-maze tank. This treatment pattern was repeated for 5 consecutive days for all drug conditions, including control groups. Recordings began simultaneously with the opening of the T-maze neck door and continued for up to 15 minutes each day over the course of 6 days. The experiment was then continued for another consecutive 6 days.

Time-lapse images (25 frames/second) were captured using the Perios program. These images were post-processed for movement tracking using either manual singles or multi-track plug-ins of the NIH ImageJ program. After each recording session, the transparent plastic sheet carrying food particles was carefully removed. Any excess water was blotted away with tissue paper, and the sheet was re-weighed. To calculate the percent of food consumption, the initial weight before feeding was subtracted from the re-weighed weight. The percent food consumption was then calculated using the following formula:

$$\text{Percentage of food eaten} = \frac{\text{weight of total food - weight of non-eaten food}}{\text{weight of total food}} \times 100$$

2.3.3. Data analysis

Statistical analyses were carried out using two-way ANOVA and graph fitting were done using Matlab software.

3. Result and Discussion

3.1. Analysis of feeding behavior in simple non-partition feeding tank

3.1.1. In presence of addictive drugs

Analysis of aggression towards food, measured by the frequency of strikes per minute in a simple non-partition feeding tank, revealed an exponential decrease with increasing feeding time, as illustrated in Figure 5. This decline in aggression towards food was observed despite minimal detachment of food particles from the thermo-cool sheet to the tank bottom, indicating a normal feeding pattern in Zebrafish.

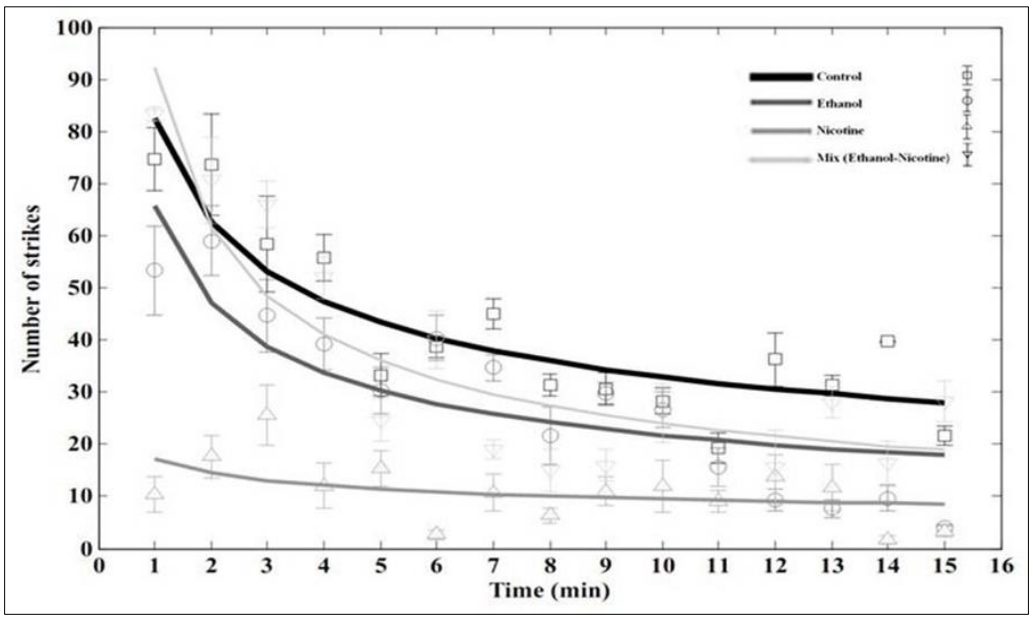

Figure 5 Average strikes per minute by Zebrafish to food particles in presence of addictive drugs.

Drug treatment conditions were as followed 210 mmole/I alcohol (0); 12 mole/I nicotine (A) and combine 210 mmole/1 alcohol-12 mole/I nicotine (V). Feeding was started after 45 minutes of drug treatment. A parallel control (D) was also conducted. Average strike per minutes to the fix thermo-cool shect carrying food particles up to 15 minutes was calculated by counting the number of strikes to thermo-cool sheet through feeding recorded movie. Each point represents the mean + SEM of counting per minute from 6 independent experiments.

However, under the influence of addictive drugs (alcohol at 210 mmol/l, nicotine at 12 µmole/l, and a combination of both), the pattern of aggression towards food markedly differed from the control. During feeding with drugs present, the number of strikes to food particles in the first 5 minutes appeared similar between the control and the combined alcohol and nicotine-treated fish ($F(1, 29)=0$, $p=0.9819$). However, in the subsequent 5-minute intervals, significant reductions in strikes per minute were observed in the combined alcohol and nicotine-treated group compared to the control ($F(1, 29)=5.37$, $p=0.0312$) and the last 5 minutes trended towards significance ($F(1, 29)=2.75$, $p=0.11$).

Alcohol-treated fish exhibited slightly lower strikes to food particles in the first and second 5-minute intervals compared to the control, with significant differences observed in the third 5 minutes ($F(1, 29)=29.87$, $p<0.001$). In contrast, nicotine-treated fish showed reduced appetite for food compared to the control ($F(1, 29)=26.69$, $p<0.001$), with varying degrees of suppression observed when combined with alcohol ($F(1, 29)=43.32$, $p<0.001$).

The higher number of strikes to food particles in the combined alcohol and nicotine-treated group compared to the nicotine-treated group suggests that alcohol may suppress the appetite-suppressing effect of nicotine, consistent with previous findings.[9] While alcohol is known to increase food appetite and is associated with obesity in humans, our study did not observe an increase in aggression towards food in the alcohol-treated fish compared to the control.

Instead, aggression towards food decreased with increasing feeding time, possibly due to the sedative effects of alcohol over time.[33, 34]

3.1.2. After withdrawing from addictive drugs

After 30 minutes of drug withdrawal, there was no significant change in the aggressive level towards food with continuous exposure to alcohol alone or in combination with nicotine, contrasting with previous observations. Comparison of strikes to food particle counts between the control and fish groups treated with either alcohol or nicotine alone showed similar results (p~ 0.2 to 1). However, alcohol-treated fish exhibited a higher tendency towards food during the first 5 minutes ($F(1, 29)=1.63$, $p=0.2158$) and second 5 minutes ($F(1, 29)=5.23$, $p=0.0332$) of feeding, declining in the third 5 minutes ($F(1, 29)=0$, $p=0.9867$). This partially elucidates the reported association between increased appetite and alcohol intake, especially exacerbated when alcohol was withdrawn 30 minutes before feeding.[4]

In contrast, nicotine typically suppresses appetite, but if fish were allowed sufficient time to recover from its effects, they showed similar tendencies towards food during the first 5 minutes ($F(1, 29)=0.15$, $p=0.6998$), marginally increasing during the second and last third 5 minutes ($F(1, 29)=1.14$, $p=0.3$). The stark increase in feeding after short durations of nicotine withdrawal or induction of nicotine withdrawal craving suggests a greater tendency towards food intake, consistent with findings of drastic weight increase after nicotine smoking cessation in chain smokers [15].

After 30 minutes of drug withdrawal, the fish group treated with combined alcohol and nicotine showed drastically reduced feeding compared to other groups (p~0.01 or less) during the first and second 5-minute intervals, with a slight increase during the last 5 minutes (Figure 6). This pattern resembled the feeding observed in the presence of nicotine alone. Previous studies indicated that alcohol greatly suppresses the effect of nicotine, but the nicotinic effect reappears only after complete alcohol metabolism, suggesting that alcohol already ingested by the fish can suppress nicotine-dependent nicotinic receptor channel activity. However, with time and the high rate of alcohol metabolism, the suppressing effect of alcohol may decline, activating the nicotine-bound nicotinic receptor channel and resulting in lower food intake, similar to observations in its absence.

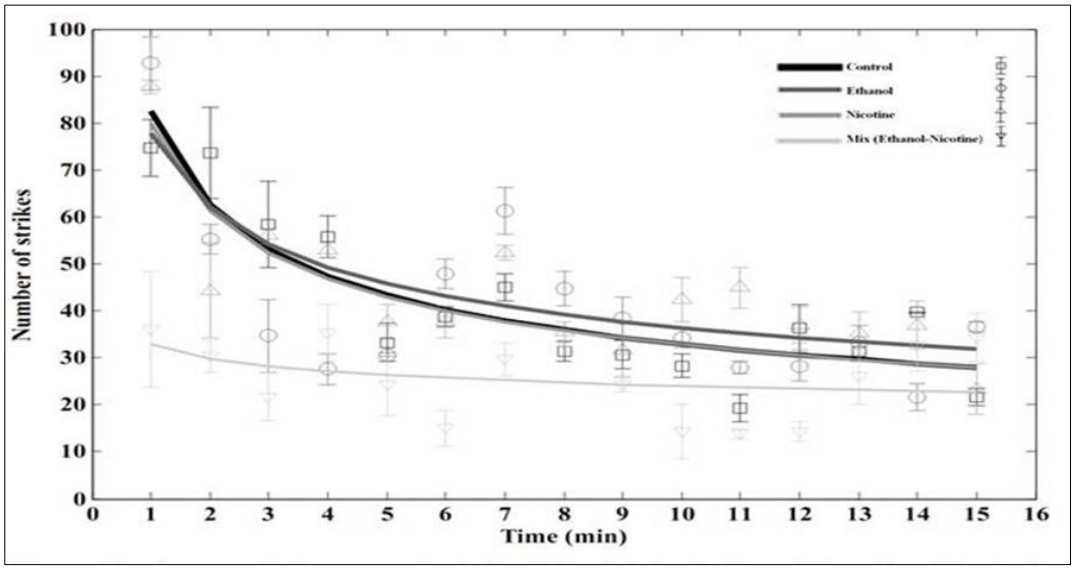

Figure 6 Average strikes per minute by Zebrafish to food particles after 30 minutes withdrawal from addictive drugs.

Drug treatment conditions for hour were as followed 210 mmole/I alcohol (0); 12 mole/I nicotine (A) and combine 210 mmole/1 alcohol-12 mole/I nicotine (V). Feeding was started after 30 minutes withdrawal from drug treatment. A parallel control (D) was also conducted. Average strike per minutes to the fix thermo cool sheet carrying food particles up to 15 minutes was calculated by counting the number of strikes to thermo cool sheet through feeding recorded movie. Each point represents the mean ‡ SEM of counting per minute from 6 independent experiments.

3.2. Analysis of feeding behavior in T-maze partition feeding tank

To address concerns about memory abnormalities associated with drug consumption, T-maze partition feeding tank experiments were conducted. Food was provided in the green arm, illuminated with a green light, while the red arm, illuminated with a red light, remained devoid of food. During the 6-day training period, fish were released from the T-

maze neck simultaneously, allowing them to choose between the green and red arms. Control fish consistently moved towards the green arm during this training period (see Figure 7A). Interestingly, this movement pattern persisted even when the location of the green arm was altered. When food was placed in the red arm, movement was notably slower compared to when it was in the green arm. However, with increased training time, movement towards the red arm also increased, suggesting that fish had learned to associate the red arm environment with food.

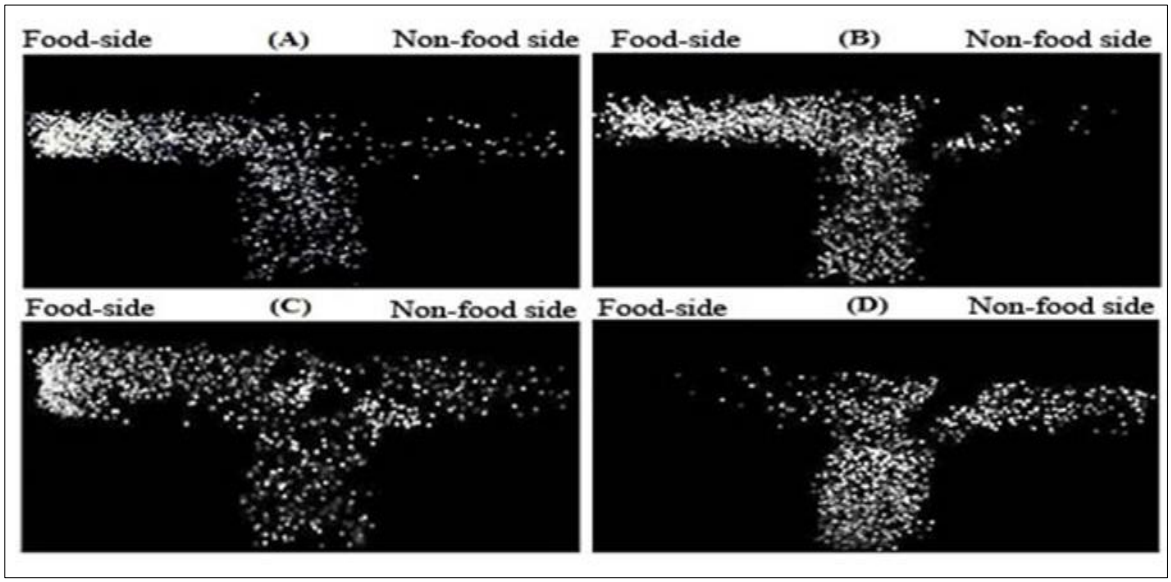

Figure 7 Total distribution of 8 adult Zebrafish (4 male, 4 female) in the different zones of T-maze partition tank during 15 minutes feeding period after 30 minutes recovery from addictive drug treatment.

Drug treatment conditions for hour in separate tanks were as followed 210 mmole/I alcohol; 12 mole/I nicotine and combine 210 mmole/1 alcohol-12 umole/I nicotine. The drug treated fish were then subjected to close neck of T-Maze tank for recovery up to 30 minutes. The recording of fish movement towards food arm was started soon after opening of closed neck door. In order to estimate the total distribution area, the recorded movie was subjected to NIH Image program to track the head position of each fish frame by frame through dot manual tracking process. The stack movie containing the position of dot in each frame was then finally subjected to Image sum slice (frame) z-projection to get total distribution in each zone of T-maze during 15 minutes of feeding starting from opening of neck door of T-maze tank (zero minute. (A) control; (D) alcohol (C) nicotine and (D) alcohol and nicotine combined.

3.2.1. In presence of addictive drugs

Table 1 Effect of addictive drugs on percent food consumption by adult Zebrafsh

	A		**B**	
Group	**Mean**	**SEM**	**Mean**	**SEM**
Control	81.83	+/- 2.54	81.83	+/- 2.54
Ethanol	80.83	+/- 2.18	80.83	+/- 2.87
Nicotine	32.16	+/- 1.87	36.33	+/- 2.17
Mix (Ethanol-Nicotine)	72.83	+/- 3.01	4.83	+/- 0.60

In the case of fish groups treated with alcohol alone or in combination with nicotine, their movement in the T-maze remained random throughout the training period, persisting even after 6 days and regardless of changing the feeding direction from green to red (figure not shown). This response suggests that the feeding of fish with alcohol, either alone or in combination with nicotine, resulted in impairments of memory. Conversely, the nicotine-treated fish group consistently moved towards the food within the first five minutes, regardless of the arm color (figure not shown). This indicates that nicotine treatment enhances memory of food location comparatively faster than the control group of fish. However, their movement subsequently decreased drastically, and the fish began to move erratically towards both

arms, including the neck region. The cessation of movement towards the food arm suggests that despite having high memory, the decreased appetite induced by nicotine predominates, causing them to move away from the direction of food.

The results represent percent food consumption by 8 adults Zebratish during 15 minutes of feeding with fixed amount of food. The results were given in mean + SEM of 6 independent of Zebrafish feeding experiment both (A) in presence of drugs and (B) after 30 minutes withdrawal from addictive drugs.

A comparison of the amount of food consumed between the control, alcohol, nicotine alone, or in combination-treated fish groups showed that the control group consumed a relatively greater amount of food, followed by the alcohol alone or in combination with nicotine, with the lowest consumption observed in the nicotine-treated fish (see Table 1.A).

3.2.2. After withdrawing from addictive drugs

After withdrawing the drugs for 30 minutes and subjecting the fish to the T-maze partition tank, a complete change in movement patterns towards the food direction was observed. The alcohol-treated fish group readily gravitated towards the food arm (Figure 7B), while the group co-treated with alcohol and nicotine moved away from food preference (Figure 7D). In contrast, the nicotine-treated fish group maintained their movement pattern even after 30 minutes of drug withdrawal, similar to observations made in the presence of nicotine (Figure 7C).

Furthermore, comparing the amounts of food consumed revealed that the alcohol-treated fish group exhibited increased food consumption after withdrawal, comparable to the control group (Table 1B). Notably, the alcohol-treated group displayed a longer feeding period than the control, suggesting an increase in food-searching activity even after drug withdrawal. This observation partly supports the notion of increased obesity or overweight in individuals consuming alcohol.[33]

Conversely, the amount of food consumed after nicotine withdrawal showed a slight increase but was far less compared to the control. This observation contrasted greatly with food intake in the non-partition tank, where aggressive intake was nearly the same as the control after drug withdrawal. The reduction in food-searching activity in the T-maze partition tank among nicotine-treated fish may indicate a decrease in appetite or other unclear mechanisms.

In the case of the group co-treated with alcohol and nicotine after drug withdrawal, the total amount of food consumed was significantly less compared to the control and even compared to consumption in the presence of nicotine alone. This phenomenon mirrored observations in the non-partition tank after drug withdrawal from combined alcohol and nicotine treatment. One possible mechanism for this observation may involve the delayed activation of nicotinic receptors previously suppressed by alcohol.[7, 10] However, the substantial reduction in food-searching activity after drug withdrawal from co-treated alcohol and nicotine, even compared to the presence of nicotine alone, suggests that the mechanism may not solely be attributed to late nicotinic receptor activation but could involve alcohol-induced physical or memory impairment.[2]

Additionally, nicotine-dependent improvements in cognitive functions, such as selective visual attention, motor task planning, sensory perception, and working memory, are believed to result from synchronized neuronal firing of hippocampus networks.[1] Alcohol, as an antagonist to ionic channels,[30] may reactivate numerous ionic channels upon withdrawal, including N-methyl-D-aspartate receptor channels, in addition to late nicotinic channel activation. However, the extent of nicotine-dependent synchronization in the absence of alcohol and after its withdrawal remains unclear. The substantial reduction in food-searching activity after withdrawing drugs in the combined alcohol and nicotine-treated group may be associated with partial nicotinic receptor activation before complete recovery from nicotine-alcohol-induced memory impairment.[2] Consequently, based on these experimental findings, it can be predicted that individuals co-addicted to alcohol and nicotine may experience a greater loss of body weight after short periods of drug withdrawal, particularly when subjected to food-searching activity, compared to those using nicotine alone.

4. Conclusion

Based on findings, when food was easily accessible, no significant differences in food aggression were observed between the exposed and non-exposed fish groups, except when nicotine was present, which notably reduced aggression levels towards food. However, 30 minutes post-drug withdrawal, food cravings in the alcohol-nicotine exposed fish groups resembled those of the control group when food was readily available. Yet, when food searching activity was required, aggression toward food significantly decreased after drug withdrawal in the nicotine and nicotine-alcohol combined

exposed fish groups. This finding could contribute to the reported weight loss in individuals using alcohol and nicotine together. Further research is warranted to elucidate the underlying mechanisms.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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