

Effects of Simultaneous Reinforcement of Endocannabinoid and Cholinergic Systems on Anxiety-Like Behavior in Mice

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Research

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Abstract

Objective

Anxiety behavior is regulated by different neurotransmitter systems. There has been no direct relationship between endocannabinoid and cholinergic systems on anxiety in previous studies. This study investigated the effects of each of these systems separately and simultaneously using Donepezil (Cholinesterase inhibitor) and URB-597 (endocannabinoid degrading enzyme inhibitor) on anxiety-like behavior.

Method

Eighty-eight male mice were divided into eleven groups (n=8) including control (saline), diazepam (0.3 mg /kg), URB-597 (0.1, 0.3, or 1 mg /kg), donepezil (0.5, 1 or 2 mg/kg) and the combination of the two drugs at low, medium and high doses. All treatments were injected intraperitoneally 30 minutes before the elevated plus maze test.

Results

Separate administration of URB597, donepezil or diazepam increased the number and time spent of open arms compared to the control group. Concurrent administration of URB and donepezil at low, medium and high doses did not change the number of open arms entries compared to the control group, but they reduced the number of entries to the closed arms.

Conclusions

These results suggest that strengthening any cholinergic or endocannabinoid system has anxiolytic effect similar to diazepam. However, the interaction of these two systems has fewer anxiolytic effects compared to the effects of each alone. It seems that these drugs alone may represent a strategy for the treatment of anxiety disorders.

Introduction

Anxiety is one of the most common disorders in psychiatry [1, 2]. According to World Health Organization statistics, about 275 million people suffer from the disorder, with an estimated prevalence of approximately 42 million worldwide each year [3]. This disorder ranks ninth among all diseases and holds the highest prevalence among all mental disorders worldwide [4]. Anxiety disorders are characterized by a variety of neuroendocrine and neurotransmitter systems in the neuroanatomical different areas [5].

Contradictory reports have been published on the role of the cholinergic system in anxiety [6, 7]. Therefore, administration of nicotinic or muscarinic antagonists decrease anxiety behavior induced by physostigmine [8] whereas enhanced cholinergic transmission, or activating M₁ receptors in the ventromedial prefrontal cortex induces anxiety [9] or gallamine (muscarinic receptor antagonist type 2)

showed no effect in the elevated plus-maze [7]. Moreover, it is interesting that depressed and bipolar populations showed that scopolamine was also effective on anxiolytic effects (decrease anxiety symptoms); however, rats and mice administered with scopolamine showed increased anxiety in standard behavioural tests [10].

Endocannabinoid system has emerged as an important neuromodulatory system in the brain involved in a variety of physiological processes including pain, memory, and mood [11, 12]. Although the physiological role of cannabinoid receptors (CB₁ and CB₂) has not yet been fully discovered, their wide distribution in different regions of the central nervous system indicates their significant role [13–15].

Anandamide is the most important and first endogenous cannabinoids that have been identified [16]. Anandamide is rapidly eliminated after releasing in two steps, including intracellular resorption and intracellular degradation of arachidonic acid and ethanol amide by fatty acid amid hydrolase (FAAH) [17]. A selective inhibitor of FAAH is URB-597, which enhances and prolongs the effect of endogenous and exogenous anandamide [18].

The results of several studies have suggested that the cannabinoid system participates in the modulation of anxiety behaviors through CB₁ receptor [19–21] but its effects are inconsistent and dose-dependent. According to distribution of the CB₁ receptor and acetylcholine in the prefrontal cortex, hippocampus, and amygdala of the mouse that overlap with anxiety responses are likely to interfere with this process as well [22].

Based on the previously described approach for the unclear role of the cannabinoid and cholinergic system in anxiety and their potential interference with this phenomenon, the aim of the present study was to determine the effect of coadministration of the cholinergic endogenous system (using donepezil, cholinesterase inhibitor) and the endocannabinoid (using URB 597, cannabinoid degrading enzyme inhibitor) on the anxiety model of the elevated plus maze in male mice.

Materials And Methods

5.1. Animals

Eighty-eight male mice (32- 40g) were purchased from animal house of the Hamadan University of Medical Science. The animals were maintained in groups of four per cage under a 12-h light/dark cycle (lights on at 7:00 a.m.) in a temperature-controlled room. All mice were allowed to adapt to the new environment and handling for at least one week before experiments. Animal protocols were approved by the Research Committee of the Hamadan University of Medical Sciences (IR.Umsha.REC.1394.200), and conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85 – 23, revised 1985).

Experimental groups

Eighty-eight male SWISS mice were randomly assigned to eleven groups (n = 8 for each group):

Control group (Group 1)

Mice received intraperitoneal saline before being placed on the maze.

URB-597 groups (Groups 2–4)

Mice received URB 597 (cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester) (Sigma, St. Louis, USA) intraperitoneally at (0.1, 0.3, or 1 mg/kg) 30 min before being placed on the maze. The medium dose was based on the previous study [23].

Donepezil groups (Groups 5–7)

Mice received (0.5, 1, or 2 mg/kg) donepezil (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally 30 min before being placed on the maze. The medium dose was based on the previous study [24].

URB-597 and Donepezil (U + D) (Groups 8–10)

Mice received low (L; 0.1 mg/kg) or medium (M; 0.3 mg/kg) or maximal (H; 1 mg/kg) doses of URB 597 and donepezil before test.

Diazepam group (Group 11): Mice received the intraperitoneal administration of diazepam at a dose of 0.3 mg/kg before being placed on the maze. All treatments were injected intraperitoneally 30 minutes before the elevated plus maze test.

The Elevated plus maze (EPM) test

The elevated plus maze test is a valid test for the determination of the anxiety-like behavior of various drugs or compounds [25]. The elevated plus maze test consists of four arms (two open arms and two closed arms; 51 cm long 10 cm wide) centered perpendicular to each other forming a plus shape and extending 50 cm apart. Each mouse was placed in the center of the maze and allowed to explore all arms freely for 10 minutes [25, 26]. After each test, the device was cleaned with 70% ethanol to eliminate residual odor. The main indicators for measurement in this test are number of open arm entry, number of closed arm entry, and time spent in open arm, time spent in closed arm, percentage of number of open arm entries to the closed arm (% OE/CE) and percentage of time spent on open arms to that spent on closed arms (% OT /CT).

Statistical Analyses

The data were analyzed by SPSS version 16.0 using one-way ANOVA. Tukey's test was performed to determine the effects of various treatments. Results were presented as mean \pm SEM for each group, and $P < 0.05$ was considered significance level.

Results

The number of entries to the open arms of the elevated plus maze

Comparison of the number of entries to the open arms of the elevated plus maze using one-way ANOVA showed a significant difference between groups [$F(10,77) = 13.88; P < 0.001$]. Tukey's test revealed that the number of open arms entries in the groups receiving different doses of URB-0.1 mg/kg ($P < 0.001$), URB-0.3 ($P < 0.001$) or URB-1 ($P < 0.01$) were significantly higher than the control group. Also, there was no significant difference in the number of open arms entries between groups receiving different doses of URB 597 (Fig. 1.A).

Tukey's test showed that the number of open arm entries in the groups receiving donepezil at doses of 0.5mg/kg ($P < 0.001$), 1mg/kg ($P < 0.01$) or 2mg/kg ($P < 0.05$) was significantly higher than that in the control group. The number of open arm entries in the mice given 0.5 mg donepezil was higher than that in the mice given 2 mg donepezil ($P < 0.05$; Fig. 1.A).

Comparison of the number of entries to the open arms of the elevated plus maze between the control group and the URB 597 + Donepezil (U + D) groups at low, moderate or high doses showed no significant difference between the groups. However, this parameter in the U + D (L) treatment significantly decreased compared with the lowest doses of URB 597 ($P < 0.01$) or donepezil ($P < 0.05$) alone. Moreover, in the U + D (M)-treated group, the number of open arm entries significantly reduced compared to the groups given URB-0.3 ($P < 0.01$) or Donepezil-1 alone ($P < 0.05$). In the U + D (H)-treated group, the number of open arm entries was lower than that in the groups given the high doses of URB 597 ($P < 0.01$) or Donepezil ($P < 0.05$) alone. It was also found that the number of open arm entries in the diazepam group was significantly higher than that in the control group ($P < 0.001$; Fig. 1.A).

The comparison of the number of entrances to closed arms

The comparison of the number of entries to closed arms using one-way ANOVA showed a significant difference between groups [$F(10, 77) = 11.368; P < 0.001$; Fig. 1.B]. The Tukey's test results showed that the number of closed arm entries was significantly lower in URB-0.1 ($P < 0.01$), URB-0.3 ($P < 0.001$) or URB-1 ($P < 0.01$) groups than in the control group. Also, donepezil-0.5 ($P < 0.05$), donepezil-1 ($P < 0.001$) or donepezil-2 ($P < 0.001$) treatment showed significant decrease in this parameter compared to control group. The number of closed arm entries in the donepezil (2 mg/kg) group was lower than that in the donepezil-0.5 group ($P < 0.05$).

Figure 1.B illustrates the effect of cotreatment of U + D on the number of closed arms that in the U + D (L) group was significantly decreased compared to low dose ($P < 0.01$) or high dose ($P < 0.01$) URB treatment, as well as low dose ($P < 0.01$) or high dose ($P < 0.05$) Donepezil treatment. Also, significant decrease was also observed in the number of closed arms entries in U + D (M) compared to low dose ($P < 0.05$) or high dose ($P < 0.05$) URB treatment, as well as low dose ($P < 0.001$) Donepezil treatment, respectively. In addition, U + D (H) treatment lower than Donepezil drug ($P < 0.05$).

There were no significant differences between groups at low, medium or high dose of U + D. In addition, the number of closed arms entries in the diazepam receiving groups was significantly lower than the control group (Fig. 1.B; $P < 0.001$).

Total arm entries in Elevated plus maze test

Several parameters considered for mouse locomotive activity evaluation in EPM. The number of entries in closed + open arms and the time spent in closed + open arms represent the animals' locomotion status. As shown in Fig. 2, locomotor activity in U + D groups at low, medium, or high dose less than other groups [F (10, 77) = 12.036, $P < 0.001$, one-way ANOVA]. Tukey post hoc test showed that this parameter in U + D groups was significantly lower than the control ($P < 0.001$), URB-0.1 ($P < 0.001$), URB-0.3 ($P < 0.01$), URB-1 ($P < 0.01$), Donepezil-0.5 ($P < 0.001$), Donepezil-1 ($P < 0.01$), or Diazepam ($P < 0.001$) groups.

Time spent in open arms in the elevated plus maze

The results of one-way ANOVA revealed that between groups were significantly different in time spent in open arms [F (10, 77) = 6.253, $P = 0.001$; Fig. 3.A]. Tukey post hoc test revealed that time spent in open arms in URB-0.1 ($P < 0.001$) or URB-0.3 ($P < 0.01$) treatments was significantly more than control group. Also, the time spent in open arms in doses of donepezil-0.5 ($P < 0.05$), donepezil-1 ($P < 0.05$) or donepezil-2 mg/kg ($P < 0.001$) was significantly more than control group. This parameter was also decreased in the U + D (L) compared of URB-0.1 ($P < 0.01$) or donepezil-2 ($P < 0.01$) groups. In addition, in the U + D (H) group lower than the URB-0.1 ($P < 0.001$) or Donepezil-2 ($P < 0.001$) groups, respectively. Diazepam-treated mice showed significant increase the time spent in open arms compared to control group ($P < 0.001$; Fig. 3.A).

The time spent on the closed arms of elevated plus maze

Figure 3.B indicated there were significant difference in the time spent in the closed arms of elevated plus maze between groups [F (10,77) = 19.579, $P < 0.001$; one-way ANOVA]. Post-hoc Tukey showed this parameter in U + D (L) mice decreased compared with donepezil-0.5 ($P < 0.01$) or donepezil-2 ($P < 0.001$) groups. In addition, in U + D (M) group lower than Donepezil-2 ($P < 0.01$) group. The U + D (H) group was significantly lower than all of Donepezil dosage ($P < 0.001$). Also, the time spent in closed arms in diazepam receiving groups was significantly lower than the control group ($P < 0.001$; Fig. 3.B).

The ratio of open arm entries/time to the closed arm entries/time

There was a significant difference in the percentage of open / closed arms entry between groups [F (10, 77) = 8.612, $P < 0.001$]. The U + D (H) treated mice spent significantly lower this ratio than donepezil-2 treated animals ($P < 0.01$; Fig. 4.A).

Results regarding the ratio of the time spent on open arm to that spent on closed arm time are shown in Fig. 4.B. One-way ANOVA showed that there was a significant difference in this ratio between groups [F (10, 77) = 4.927, $P < 0.001$]. Tukey's test showed that this parameter in the URB-597 + donepezil (L) was

lower than that in URB-0.1 ($P < 0.01$) or in URB-597 + donepezil (H) was lower than that in URB-0.1 ($P < 0.05$). This parameter was significantly higher in the diazepam group than in the control group ($P < 0.001$; Fig. 4.B).

Discussion

In this study, we investigated the effects of enhancement of endocannabinoid and cholinergic neurotransmitter system separately and simultaneously using donepezil (cholinesterase inhibitor) and URB-597 (endocannabinoid degrading enzyme inhibitor) on anxiety-like behavior. Key findings of this study included: (1) Injection of three doses of URB-597 (cannabinoid-degrading enzyme inhibitor) reduced anxiety. (2) Administering doses of 0.5, 1 or 2 of donepezil (cholinesterase inhibitor) decreased anxiety (3) Co-administration of donepezil and URB597 at low, medium and high doses did not affect the number of open arms entries, but reduced the number of entries to the closed arms.

The results of URB-597 injection showed that different doses of URB-597 increased the number and time spent of open arms, decreased the number and time spent of closed arms and increased the percentage the number and time spent of open arms to closed arms. Taken together, these results indicate the anxiolytic effect of this drug. However, there was no significant difference between the doses used but doses of 0.3 mg/kg and 0.1 mg/kg had more significant effect on some parameters than dose group 1mg/kg.

These results are consistent with the other results regarding a role in the modulation of anxiolytic effects of cannabinoids by type 1 receptor (CB_1) [19]. Another study showed contradictory result for example cannabinoid receptor agonists (CP55940, WIN55212-2) produced anxiolytic effects in mice only at low doses [20] whereas at higher doses had anxiogenic effect [21]. Other contradictions are the natural compound in the cannabis plant (Delta 9-tetrahydrocannabinol) that produced an anxiolytic effect in mice in the light-dark test [27] while in the same test had anxiolytic effect [28].

It has also been reported that WIN 55,212-2 drug in mice reduces anxiety by intervention in GABAergic transmission, whereas it increases anxiety through intervention in glutamatergic synaptic transmission [29]. The anxiolytic effects created by URB-597 are also similar to the anxiolytic effect of diazepam. According to the previous report, administration of URB-597 may interfere with GABAergic transmission [30].

The results regarding donepezil at doses of 0.5, 1, or 2 showed that it increased the number and the time spent of open arms and decreased the number and time spent of closed arms. The high dose of donepezil had no significant effect on the total number of mice entering the open and closed arms of the maze compared to the control group. The administration of different doses of donepezil also increased the percentage of open arms to closed arms and increased the percentage of time spent in open arms to closed arms.

Overall, donepezil as a cholinergic system, has anxiolytic effect. These results are consistent with previous studies in which administration of scopolamine (a muscarinic receptor antagonist) in mice produced anxiogenic effect [31, 32]. In contrast, administration of type 1 muscarinic (Xanomeline) [33] or nicotine receptors agonists has anxiolytic effect [34]. Further studies are needed to clarify the contribution of each of these receptors more precisely.

The results of coadministration URB-597 with donepezil at low, medium and high doses, did not affect the number of open arms entries, while reducing the number or spending time of closed arms entry. The anxiolytic effect observed in the simultaneous administration of the above drugs is lower than the anxiolytic effect of each drug alone. There is no study about interaction these systems in anxiety-like behavior. In memory studies showed the interaction between cannabinoid (WIN-2; a synthetic cannabinoid) and cholinergic systems (acetylcholinesterase inhibitor) on memory, showed that WIN-2 significantly decreased rates of hippocampal principal cells performance and that rivastigmine reversed these short memory deficits and normalized hippocampal discharge rates [35].

Conclusion

The present report confirms that in reduction anxiety-like behavior via acetylcholine and anandamide secretion with activation of cholinergic and endocannabinoid receptors. Operations of one neurotransmitter system alone reduce anxiety while interaction of the two systems have less effect on reduction in anxiety. Future studies include additional molecular and histological experiments designed to elucidate the mechanisms underlying interaction between these systems that is more effective on anxiety-like behavior.

Ethics approval and consent to participate

All animal experimental procedures were performed in accordance with the guidelines for proper conduct of animal experiments issued by the Ethics Committee of the Hamadan University of Medical Sciences, and performed according to *The Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health, United States (NIH Publication No. 85 – 23, revised 1985).

Consent for publication

Not applicable.

Availability of data and materials

The data are available for any scientific use with kind permission.

Declarations

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SS, AD, AK, and RS carried out the experiments, participated in the design of the study, performed the statistical analysis, and drafted the manuscript. SS participated in the design and coordination of the study. AD performed the behavioral tests. AK participated in the behavioral analysis. RS wrote the manuscript. All authors read and approved the final manuscript.

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Figures

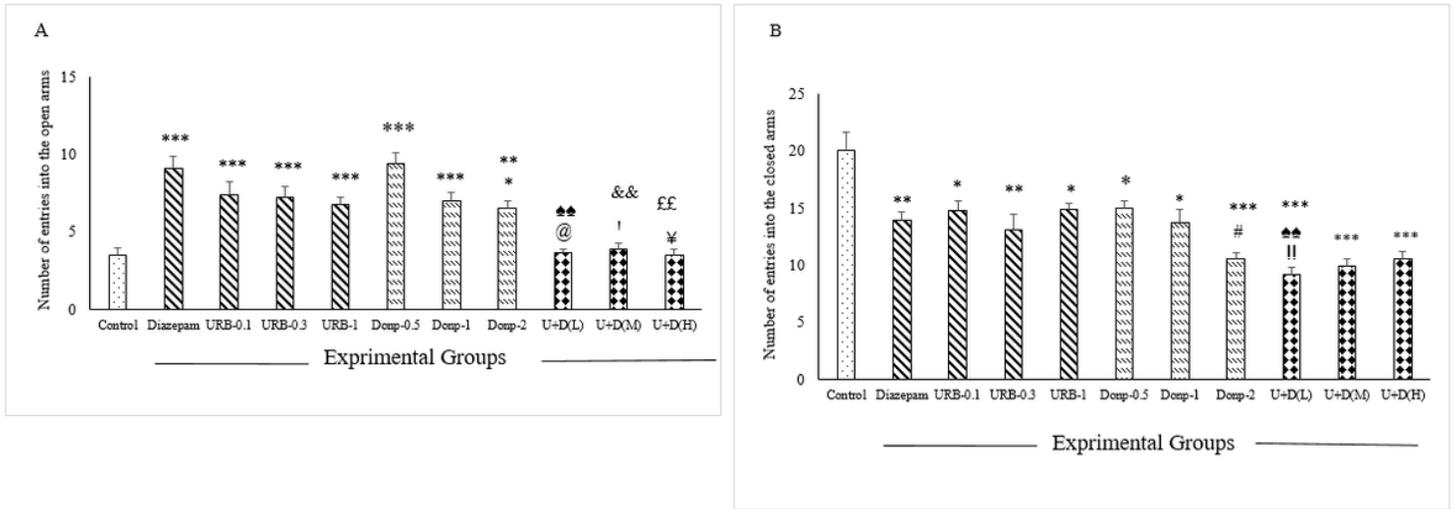


Figure 1

Comparison of the number of entrances to the (A) open arms or (B) closed arms of the elevated plus maze between control (saline), URB597, donepezil, diazepam groups or URB-597+ Donepezil treatment (U+D) at low (L), medium (M), and high (H) levels, at 10 min. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ URB vs control; # $P < 0.05$, 2mg/kg vs 0.5 mg/kg Donepezil group; ♠♠ $P < 0.01$ U+D (L) group vs lowest doses of URB-597 or Donepezil (@ $P < 0.05$), U+D (M) treatment vs URB-0.3 (&& $P < 0.01$) or Donepezil-1 groups (! $P < 0.05$, !! $P < 0.01$), U+D (H) treatment vs the high doses of URB-597 (££ $P < 0.01$) or Donepezil groups alone (¥¥ $P < 0.05$); Mean \pm SEM are shown.

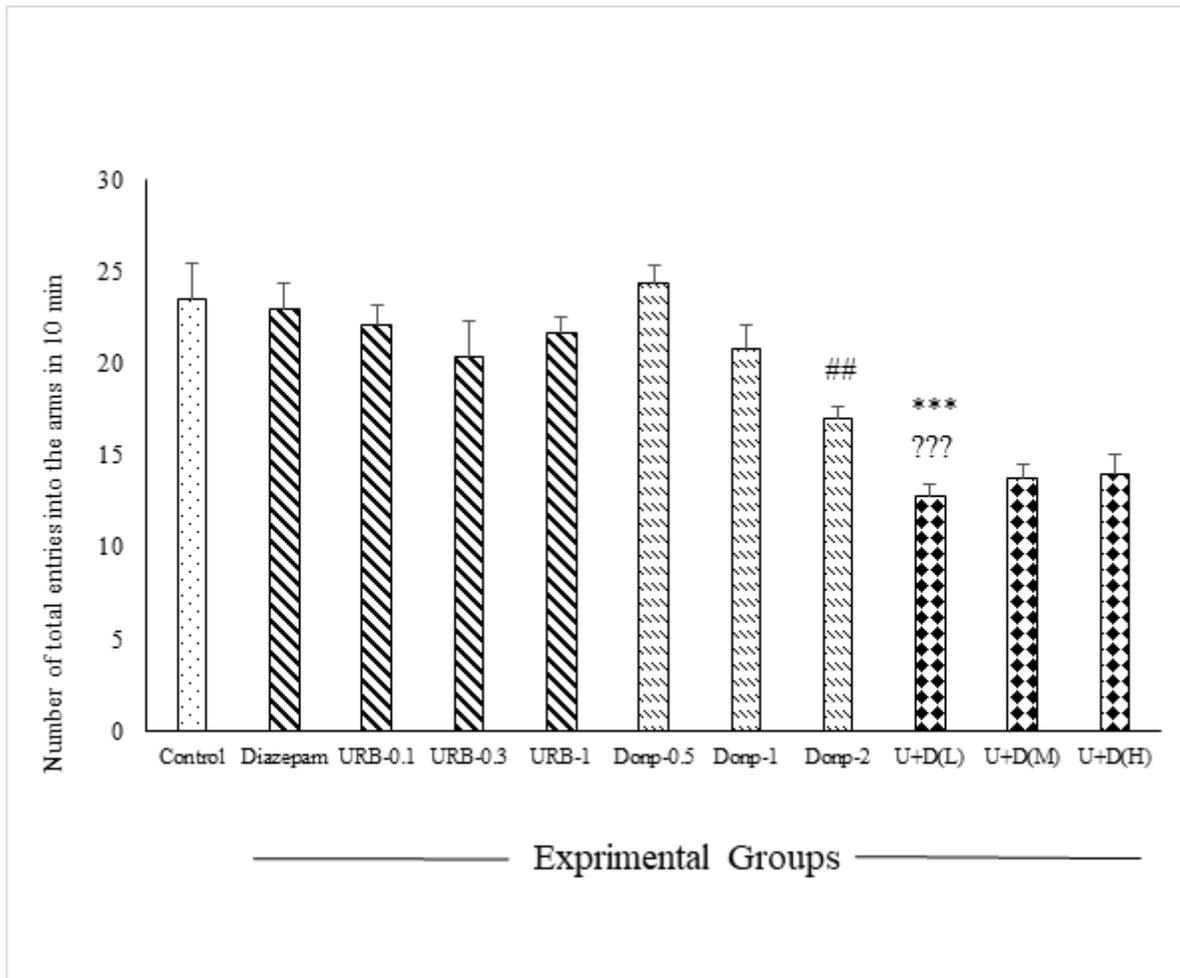


Figure 2

Comparison of total arm entry in Elevated plus maze test between control (saline), URB597, donepezil, or diazepam groups or URB-597+ Donepezil treatment (U+D) at low (L), medium (M), and high (H) levels, at 10 min (*P < 0.05, **P < 0.01, and ***P < 0.001 vs control, ? P < 0.05, ??P < 0.01 and ??? P < 0.001 U+D groups vs other groups). Mean ± SEM are shown.

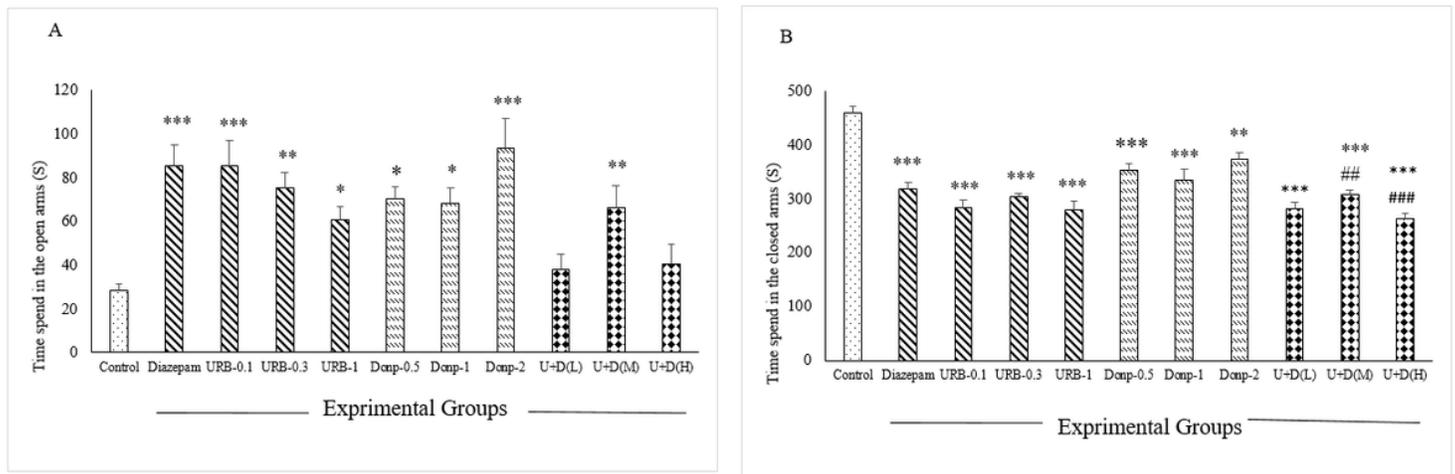


Figure 3

Comparison of time spent in (A) open or (B) closed arms in the elevated plus maze between control (saline), URB597, donepezil, or diazepam groups or URB-597+ Donepezil treatment (U+D) at low (L), medium (M), and high (H) levels, at 10 min (*P < 0.05, **P < 0.01, and ***P < 0.001 vs control, ♠♠P < 0.01 U+D (L) group vs URB treatment, !!P < 0.05 and P < 0.01 Donepezil treatment.). Mean ± SEM are shown.

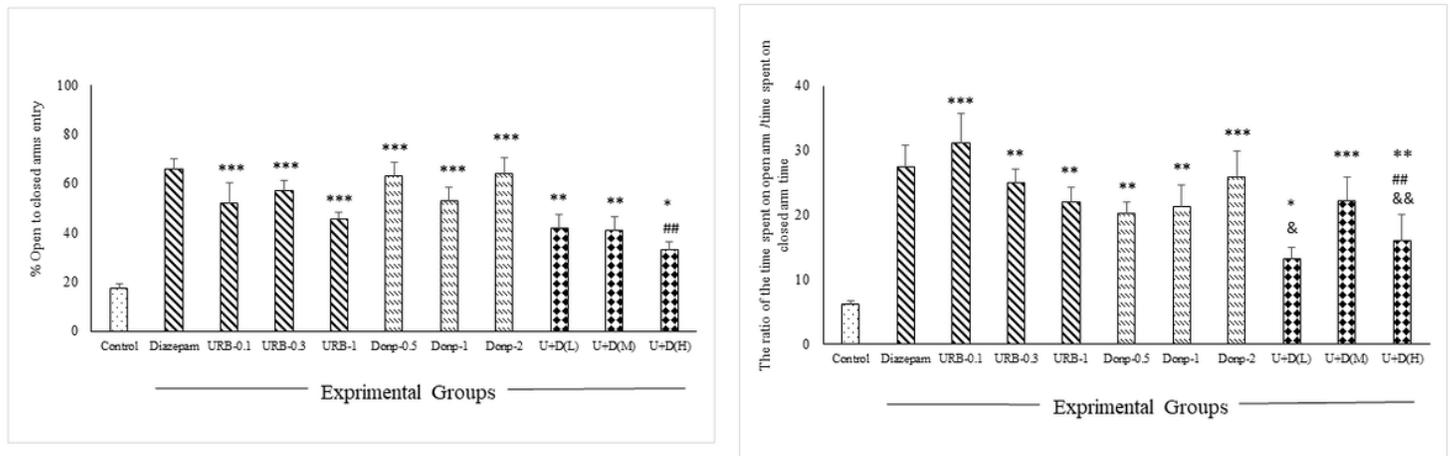


Figure 4

Ratio of percentage of (A) open to closed arms entry or (B) time elapsed between open arms to closed arms in the elevated maze plus between control (saline), URB597, donepezil, or diazepam groups or URB-597+ Donepezil treatment (U+D) at low (L), medium (M), and high (H) levels, at 10 min. ** P < 0.01 and *** P < 0.001, * P < 0.05 at Comparison with control group. ## P < 0.01, The U+D (H) treated mice vs donepezil-2 group. &P < 0.05, or &&P < 0.01, URB-597+donepezil (L) or URB-597+donepezil (H) groups vs URB-0.1. Data are presented as mean ± SEM.