Original Research Article

Clomipramine Effects on Anxiety-Like Behaviors in Zebrafish: A Research Note

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ABSTRACT

Studies suggest that diagnoses of depressive and/or anxiety disorders have drastically increased, leading to increased use of antidepressants. More research is needed to understand whether and how antidepressants modulate anxiety. Proxy studies based on animal subjects help inform such inquiries. The current analysis tests the efficacy of the drug clomipramine, a tricyclic antidepressant, on anxiety-like behaviors in an animal model. The 55 adult (about 6-months old), mixed-sex, wild-type zebrafish were either part of the control group or given doses of 0.125, 0.25, 0.50, or 1.00 mL/L of clomipramine for 20 minutes. The novel tank test was applied to determine whether the drug had either anxiolytic (reduced anxiety-like behavior) or anxiogenic (increased anxiety-like behavior) effects based on the following six behavioral measures; distance traveled, mean speed, resting time, total time in the top, total distance in the top, and entries to the top. Findings show mixed dose-response outcomes. Mean speed decreased with dosage, while both time in the top and entries to the top increased with dosage. Yet the three remaining tests had inconsistent results. These findings represent preliminary animal-model observations requiring replication.

Keywords: Anxiety-like behavior; anxiolytic; anxiogenic; clomipramine; zebrafish; experiment

INTRODUCTION

The World Health Organization (WHO) suggests that one-third of the world's population is likely to develop an anxiety disorder at some point in their lives (1). Pro-oxidant and traumatic events are closely associated with anxiety (1). Constant stress without alleviation can foster anxiety (2, 3)[1]. Studies continue to search for viable treatments for anxiety-like behavior and other conditions like Obsessive-compulsive disorder **(**OCD) (4). Additional research is needed to evaluate the effectiveness of medical alternatives such as clomipramine, a tricyclic antidepressant used for symptoms of anxiety-like behaviors (5). In past studies, participants given clomipramine also showed a decrease in the Yale-Brown Obsessive Compulsive Scale (Y-BOCS), a scale used to measure obsessions and compulsions (5). Individuals who received clomipramine also reported minimal or substantial improvement on the Patient Self-Rating Scale in Protocol 59 (5).

In this study, clomipramine will be used to examine whether treatment produces anxiolytic (i.e., decreased anxiety-like behavior) and/or anxiogenic (i.e., increased anxiety-like behavior) effects in zebrafish[2]. The drug clomipramine is associated with greater anxiolytic effects compared to other psychotropic compounds. Moreover, it is common to use certain animals, such as zebrafish,

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as proxies in research because they have genetic similarities with humans (6-7). Zebrafish are often used in psychological studies to test the efficacy of drugs and analyze the effects of psychotropic compounds (6, 8). Moreover, they are used to study diseases and conditions and to test aggression, anxiety, learning, and memory because their genes are similar to those of humans (6). Studies have found that zebrafish experience withdrawal effects and anxiety-like behaviors or anxiogenic effects. These behaviors are observed before and after receiving a psychotropic drug compound alleged to ameliorate anxiety-like behavior. Anxiety/depressive-like behaviors can be inferred based on certain behaviors of zebrafish. For example, existing studies show that when zebrafish dwell at the bottom of a fish tank, they exhibit anxiety/depression-like behaviors (8). Zebrafish that remain at the top of the tank for an extended period are exhibiting anxiolytic effects (6, 7). Analyzing their behaviors helps establish the efficacy of the psychotropic compound to which they are exposed. This quantitative analysis is part of a larger experiment to assess whether there is a relationship between clomipramine exposure and anxiety-like symptoms in adult zebrafish (7).

It is hypothesized that adult zebrafish will exhibit anxiolytic behaviors (i.e., decreased anxiety-like behavior) after acute doses of clomipramine. Readers should note that the novel tank test using zebrafish measures anxiety-like behaviors rather than obsessions and compulsions and thus the implications of these findings to treat human OCD is not implied here. Yet findings of the effects of clomipramine on zebrafish are part of continued studies about possible ways to ultimately combat anxiety-like behavior in humans. This study investigates possible treatment for anxiety-like behaviors (7-8). Subsequent sections describe the study methods, research participants, procedure, analyses, and results.

METHODS AND MATERIALS

Participants

This study was conducted using adult (about 6-months old), naïve, mixed-sex, wild-type zebrafish (N=55) from Carolina Biological Supply (Burlington, NC). Zebrafish were kept in a filtered circulating system maintained at 26-28 ℃ and were housed in the colony room with a light cycle of 14h light: 10h dark per day (9). The study was performed under the guidelines of the Office of Laboratory Animal Welfare (OLAW) and the Animal Welfare Act of 2019. As detailed in the larger study (7), the zebrafish were given at least ten days to acclimate to the laboratory conditions before the experiment was performed. Other water parameters, feeding processes, and all husbandry protocols were designed in accordance and approved by the Indiana University School of Medicine-Northwest Institutional Animal Care and Use Committee (protocol IUSM-NW-IACUC-49) as documented in a larger study (7). Animals were monitored for general health parameters before and during the experiment; no adverse events were observed.

Materials

The drug of interest in this study is clomipramine purchased from Santa Cruz Biotechnology. Zebrafish were used as an animal model and recorded using a software laptop that uploaded videos to the software BehaviorCloud. Data were then analyzed using JASP software under the direction of the principal investigator of the lab.

Procedure

A controlled, between groups experimental design was conducted with one control and four clomipramine-dose groups to evaluate possible anxiolytic effects

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[1] *According to the National Institute of Mental Health (NIH), Obsessive-compulsive disorder (OCD) is defined as a mental health disorder characterized by repetitive, uncontrollable intrusive thoughts (i.e., obsessions) and ritual behaviors (i.e., compulsions). Research suggests that 2-3% of the United States adult population will be diagnosed with OCD in their lifetime. Antidepressants and other drug substances are then often used to ameliorate the symptoms. Although anxiety behavior is related to it, OCD is classified as a unique condition rather than a standard anxiety disorder and is not the focus of this study.*

[2] *The following four concepts are central to this study; anxiolytic, psychotropic, anxiogenic, and novel tank. Their definitions are provided below.*

a. Anxiolytic effects are the reduction of anxiety-like behaviors produced by psychotropic compounds.

b. A psychotropic substance is any drug that alters brain function, affecting mood, cognition, and behaviors.

c. Anxiogenic effects are produced by substances that increase anxiety-like behaviors.

d. The novel tank is a clear tank that houses the mixed-sex adult zebrafish for the experiment.

of clomipramine on zebrafish. In preparation for the experiment, the inventory of clomipramine was checked. The treatment and sacrifice spreadsheet were then prepared. The principal investigator of the lab prepared the concentrated drug that the research assistants used; they were all masked and randomized to reduce possible bias. The clomipramine was calculated and recorded in a lab notebook. Calculation was based on the number of fish receiving the drug. The clomipramine was then weighed, and the final volume of solution was determined for the concentrated drug. The drug was labeled, including its weight, the date, and the volume of system water to add. System water was added to the vial of clomipramine to make concentrated solutions. Treatment tanks were subsequently prepared. The diluted drug was inverted by hand and covered with parafilm. The control group consisted of a 1.00 L system water measured with a 1.00 L volumetric flask. Experimental tanks contained 1.00 L each of the diluted solutions.

The lab table was set with necessary supplies on sacrifice day. Home tanks were brought into the lab room to be fully acclimated 30 minutes before acute drug treatment. The zebrafish were chosen at random and carefully netted into the appropriate treatment tank. The tank was then covered with a lid. The net was cleansed with tap water between groups. After a 20-minute treatment, each fish was moved for the behavioral test. Zebrafish were subject to the novel tank test and recorded for 10 minutes. However, only the first six minutes were analyzed due to storage capacity and processing limitations.

Euthanasia

The zebrafish were euthanized after the novel tank test to comply with ethical guidelines to prevent suffering. As in humans, psychotropic substances can produce anxiogenic effects in zebrafish. To prevent prolonged exposure effects and ensure ethical treatment, zebrafish used in acute behavioral experiments are euthanized after an experiment. Euthanasia was conducted humanely following the guidelines of the NIH Office of Laboratory Animal Welfare. The euthanasia process was planned before the sacrifice day to prepare for the thoughtful transition of the fish. After testing, the zebrafish were euthanized with clove oil and non-denatured ethanol. The solution consisted of 1.00 mL of clove oil and 9.00 mL of ethanol in a 20.00 mL glass test tube. The solution was covered in parafilm and vortexed. The solution was then poured into a 1.00 L volumetric flask, and system water was added to reach the 1.00 L mark and stored at 4°C until the experiment. After the novel tank test, fish were netted and placed in 30.00 mL euthanasia solution. The zebrafish were then placed in ice. After 60 seconds elapsed from the introduction into the euthanasia solution, the opercular (i.e., gill) of each zebrafish was checked for lack of movement and unresponsiveness to tactile stimulation. Then the fish were removed with forceps from the solution and dried with Kimwipes. Each fish's body was placed in a labeled 1.50 mL microcentrifuge tube. The euthanasia solution was discarded, and the beaker was rinsed. When all samples were harvested, tubes were placed in a freezer box and stored at -20 °C.

Data Reduction

Once the behaviors of all fish were recorded, the video files were renamed with the appropriate subject number. The video files were then uploaded to OneDrive and BehaviorCloud. OneDrive securely stored the files, while BehaviorCloud stored and analyzed the data on animal behaviors. The data recorded for the overall analysis of the experiment were mean speed (cm/sec), total distance traveled (cm), and resting time or the total number of seconds that the subject was not traveling (sec). The data recorded for the zoned analysis of the experiment were total time in the top zone only (sec), distance traveled in the top zone only (cm), and top zone entries. Data were analyzed using a one-way Analysis of Variance (ANOVA) with a significance value of $p<0.001$ using JASP software. One-way ANOVA tests were performed based on a controlled, between groups experimental design with one control and four clomipramine-dose groups (mL/L). Means and standard deviations are provided. The F-statistic compares group means at ($p<0.001$) where F values much greater than 1 show that group differences are not random, but actually different. A Tukey post-hoc analysis was also performed to assess possible statistically significant results.

RESULTS

It was hypothesized that adult zebrafish would display anxiolytic (i.e., reduced anxiety-like) behaviors after exposure to acute doses of clomipramine. The hypothesis was tested based on an experiment that exposed zebrafish to either a control substance or different doses of clomipramine (0.125, 0.25, 0.50, or 1.00 mL/L) for 20 minutes. The zebrafish were then moved and examined using the novel tank test. Six tests were performed; 1) distance traveled, 2) mean speed, 3) resting time, 4) total

time in the top, 5) total distance in the top, and 6) entries to the top were recorded to assess whether clomipramine dose-dependently altered anxiety-like or surfacing-behaviors in adult zebrafish. Zebrafish exhibiting anxiety-like behaviors are hesitant to explore the novel tank, especially the top, resulting in lower distance traveled and mean speed, and higher resting time. Exploration at the top suggests lower anxiety-like behaviors and a willingness to explore novel environments. Greater time spent in the top and longer distance traveled in the top also indicate lower anxiety-like behaviors in zebrafish.

Test 1: Distance Traveled

There was a significant negative effect of the drug treatment on total distance traveled ($F(4,50) = 16.12$, $p<0.001$). This test indicated that zebrafish exposed to clomipramine traveled a shorter distance compared to the control group (Table 1). Moreover, according to a Tukey post-hoc test, zebrafish given 0.125, 0.50, or 1.00 mL/L traveled significantly less than the control group ($p<0.001$).

Table 1. *Distance Traveled (cm) for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L).*

Treatment	Mean	SD	N	F
0 Control	1065.307	428.036	11	16.12
0.125	786.605	181.724	11	
0.25	507.131	148.034	11	
0.50	358.362	167.682	11	
1.00	424.261	167.845	11	

Test 2: Average Velocity/Mean Speed

In this test, there was a significant main effect of the drug treatment clomipramine on mean speed ($F(4,50) = 6.01$, $p<0.001$). Zebrafish given the treatment had a reduction in swimming speed compared to the control group. According to a Tukey post-hoc test, treatment groups of 0.50 mL/L, 1.00 mL/L, and 0.25 mL/L had a significant decrease in mean speed when compared to the control group ($p<0.001$).

Test 3: Resting Time

There was an insignificant main effect of the drug treatment on resting time ($F(4,50) = 3.00$, $p=0.027$). According to the Tukey post-hoc test, zebrafish freezing or immobility did not significantly differ based on the dosage of clomipramine received when gauged at ($p<0.001$). Overall, clomipramine treatment groups did not have an increase in resting time compared to the control group.

Table 2. *Average Velocity/Mean Speed (cm/s) for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L)*

Treatment	Mean	SD	N	F
0 Control	5.490	1.790	11	6.01
0.125	4.398	0.550	11	
0.25	3.894	0.850	11	
0.50	3.826	1.258	11	
1.00	3.360	0.478	11	

Table 3. *Resting Time (s) for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L)*

Treatment	Mean	SD	N	F
0 Control	39.221	43.207	11	3.00
0.125	27.861	16.808	11	
0.25	56.941	71.177	11	
0.50	103.011	79.726	11	
1.00	51.202	39.532	11	

Test 4: Total Time in Top

There were significant positive effects of the drug treatment related to time in top, ($F(4,50) = 88.6$, $p<0.001$). According to a Tukey post-hoc test, there were significant results that indicated clomipramine dose-dependently altered anxiety-like behaviors in zebrafish. Moreover, zebrafish that received a 1.00 mL/L dosage of clomipramine spent more time at the top compared to zebrafish in the control group and other treatment groups.

Test 5: Total Distance in Top Zone

There was a significant main effect of drug treatment on distance in the top zone (cm) ($F(4,50) = 6.44$, $p =$

<0.001). Clomipramine treatment groups, excluding the 0.50 mL/L dosage, had an increase in distance to the top of the tank compared to the control group. According to a Tukey post-hoc test, zebrafish that received a 0.50 mL/L dosage of clomipramine had a significant decrease in distance at the top of the tank compared to the control group. Zebrafish that received a 0.125 mL/L dosage of clomipramine had a significant increase in distance at the top of the tank compared to zebrafish in the control group.

Table 4. *Total Time in Top for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L)*

Treatment	Mean	SD	N	F
0 Control	83.169	48.217	11	88.6
0.125	300.296	70.913	11	
0.25	334.646	25.399	11	
0.50	349.463	11.096	11	
1.00	355.598	5.458	11	

Table 5. *Total Distance in Top (cm) for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L)*

Treatment	Mean	SD	N	F
0 Control	310.372	215.157	11	6.44
0.125	635.112	190.723	11	
0.25	427.932	149.626	11	
0.50	300.643	153.844	11	
1.00	387.231	167.372	11	

Test 6: Entries to Top

There was a significant main effect of clomipramine on entries to the top ($F(4,50) = 9.07$, $p<0.001$). All clomipramine treatment groups had fewer entries to the top compared to the control group. According to a Tukey post-hoc test, zebrafish receiving a 0.50 mL/L dosage and 1.00 dosage of clomipramine had significantly fewer entries to the top compared to the control group.

Table 6. *Entries to Top for Five Experimental Groups Based on One-way ANOVA and Tukey Post-hoc Tests: N=55: n=11 (one control and four clomipramine-dose groups mL/L)*

Treatment	Mean	SD	N	F
0 Control	22.818	15.892	11	9.07
0.125	14.545	7.967	11	
0.25	8.727	4.315	11	
0.50	5.273	3.608	11	
1.00	4.455	2.296	11	

DISCUSSION

This study observed the effects of clomipramine on six zebrafish behaviors; 1) distance traveled, 2) mean speed, 3) resting time, 4) total time in the top, 5) total distance in the top, and 6) entries to the top. Zebrafish were either part of the control group or different clomipramine treatment groups. Zebrafish behaviors were recorded during the novel tank test and uploaded to BehaviorCloud to be analyzed. The data were uploaded to JASP for statistical analyses using a one-way ANOVA test. The results of this study offer partial support of the hypothesis and suggest that clomipramine has some anxiolytic-like effects (i.e., decreased anxiety-like behaviors) on the zebrafish animal model.

The predicted dose-response relationships occurred in three of the six tests. Mean speed generally decreased with dosage, total time in the top increased with dosage, and there were fewer entries to the top as dosage increased. However, irregular patterns were noted for the three remaining tests. Specifically, lower and higher dosages did not produce uniformly expected behaviors for the tests of distance traveled, resting time, and total distance in the top. This means that clear anxiolytic responses were not apparent across the six tests.

The top dwelling behaviors of the zebrafish suggest possible anxiolytic effects of the drug clomipramine. Latency to begin exploring the top of the tank is an indicator of possible stress- and anxiety-like behaviors. The treatment groups had shorter latency to enter the top compared to the control group. Many entries to the top suggest that zebrafish are not ready to explore the top of the tank, as exhibited by the control group. As dosing increased, entries to the top decreased. Although treatment groups had a greater distance in the top zone compared to the control group, the results of distance in the top zone in treatment groups were erratic. Zebrafish

given a clomipramine dosage of 0.125 and 0.25 mL/L had a greater distance in the top zone compared to the higher treatment groups. Moreover, as dosing increased, time in the top zone increased. These top dwelling behaviors of the clomipramine treatment groups suggest possible anxiolytic effects of the drug compound clomipramine. However, because results from three of the six tests did not support the hypothesis, it will be important to replicate this study to determine whether more consistent findings emerge as well as replicate the experiment based on the limitations described below.

Several additional limitations of this study should be noted. A mixed zebrafish model was used here, which excluded separate results of both female and male zebrafish. Also, the zebrafish were not stressed before the novel tank test was administered. Thus, additional research is needed to test the efficacy of clomipramine using both female and male zebrafish who experience stress pre-test and based on longer novel tank duration. Specifically, the duration of the novel tank test lasted only 20 minutes, were recorded for 10 minutes and only analyzed during the first 6 minutes. The decision to analyze only the first six minutes of the ten-minute recording due to storage and processing limitations represents a methodological limitation, as novel tank behavior can change over time and possible changes in zebrafish behaviors over a longer period could not be detected. Thus, the decision to analyze only part of the behavioral recording should be rectified in a future study. The time constraints and lack of stress can affect research negatively and potentially alter the results as well. In addition to the absence of a sex-stratified analysis, other limitations here include acute rather than chronic dosing, the limited sample size per group, and lack of drug toxicity or sedation. This study is considered an initial step in preparation for future studies in which these limitations can be addressed.

Like any drug, there are side effects, but clomipramine exhibits more anxiolytic effects in zebrafish and in humans. Patients administered clomipramine had a 40% to 45% mean improvement on OCD symptoms, in which Y-BOCS scores were highly decreased compared to the unchanged control group scores (5). These results suggest anxiolytic-like effects of clomipramine in certain tests and correlate with the results of an earlier study (5). Yet zebrafish that received clomipramine in this study exhibited anxiolytic-like behaviors and anxiogenic-like behaviors. Future studies that expose zebrafish to clomipramine are warranted (7).

It is also important to consider how these findings engage existing studies on zebrafish experiments based on exposure to antidepressants. For example, novel tests based on acute exposure to amitriptyline found similar outcomes to this current study. Specifically, one study showed that increased exposure to amitriptyline among zebrafish resulted in increased time to the top and decreased top entries – paralleling existing research (10). Although more detailed in terms of sample size and zebrafish diversity as well as observed outcomes, such studies, like this project, add to knowledge about the effects of psychotropic compounds on zebrafish. They also illustrate the salience of developing other novel experimental models (10). In addition, the current study examined the efficacy of clomipramine doses in zebrafish, whereas another previous experiment investigated clomipramine using other animal models (11). The effects of clomipramine were analyzed on tree shrews, which are considered behavioral models for humans. Some of the evaluated behaviors associated with social defeat were decreased body weight, anhedonia (i.e., loss of desire), and fatigue (11). The tree shrews were divided into three groups of six: naive, subordinate + saline, and subordinate + clomipramine. Only the subordinate + saline and subordinate + clomipramine groups underwent the social defeat phase and received either 50 mg/kg of clomipramine or 0.9% saline per day for four weeks. Compared to the current study, the results were similar. The effects of clomipramine suggested that while some depression and anxiety related behaviors may be ameliorated, others may be exacerbated. The increased top dwelling behaviors observed in zebrafish administered clomipramine, together with the sucrose preference and recovered locomotor activity observed in tree shrews suggested that the drug alleviates behavioral indications of depression- and anxiety-like behaviors. While the decreased locomotor activity in zebrafish suggests anxiogenic effects, the decreased body weight gain in tree shrews suggests severe chronic stress. When interpreted in a human behavioral model, these symptoms may parallel fatigue, sedation, and weight loss, signifying potential adverse effects to clomipramine.

Lastly, although the findings from previous and this current studies suggest that clomipramine can both ameliorate and exacerbate behavioral indications of depression and anxiety, other results were not consistent. For example, research was performed using four groups of male sprague-dawley rats: the normal or control group, the CMS (i.e., chronic unpredictable mild stress) group, the saline group, and the CMI group (12). Unlike the current study, all groups, excluding the normal group,

were exposed to chronic unpredictable mild stress for 12 weeks. The saline group received saline treatment, whereas the CMI group received 5 mg/kg of clomipramine diluted in saline during the final four weeks. After treatment, the rats underwent an open field test, elevated plus-maze test, and forced swim test. The outcomes were consistent with the hypothesis. Because the rats were exposed to stress, the normal group was expected to perform better than the stress groups. Compared to the partial support found in this current study, outcomes found that clomipramine treatment significantly ameliorated anxiety- and depression-like behaviors in the test rats without evidence of adverse behavioral effects. The CMI group exhibited reduced immobility time and increased time struggling in the forced swim test, as well as increased time in the open arms of the elevated maze, suggesting reduced anxiety-like behaviors and increased exploratory behaviors compared to the other groups exposed to chronic unpredictable mild stress. Although the current study suggests anxiolytic effects, the reduced speed, reduced distance, and freezing/immobility in the clomipramine treatment groups indicated anxiogenic-like effects, which may represent adverse behavioral side-effects of the drug. Although their findings were based on sprague-dawley rats rather than zebrafish, they suggest the need to replicate the current study and expose zebrafish to stress prior to experimentation. Overall, as research on this topic grows, so will knowledge about the use of psychotropic compounds in the treatment of anxiety-like behaviors in humans.

CONCLUSION

As reported by the World Health Organization (WHO), one in three people will be diagnosed with an anxiety disorder at some point in their lifetime. These outcomes suggest the need for antidepressants (1). Clomipramine has been hypothesized to possibly help ameliorate anxiety-like behaviors (6-7). This current study endeavors to test the efficacy of clomipramine and its effects on anxiety-like behaviors in a zebrafish model. The zebrafish in this study experienced more top exploration compared to the control group, which suggests potential anxiolytic-like effects or reduced anxiety-like behaviors in the animal zebrafish model. Yet three tests had inconsistent results. Other studies testing antidepressants have results congruent with the results found in this study (5, 8, 11). These findings, although inconclusive, reflect preliminary animal-model observations requiring replication.

Animal Ethics

The Indiana University School of Medicine-Northwest Institutional Animal Care and Use Committee provided approval and oversight of this work (protocol IUSM-NW-IACUC-49).

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest related to this work.

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