

# Zebrafish as a model for haloperidol-induced catalepsy-like immobilization

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## Research Article

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# Abstract

This study investigated the validity of screening for antipsychotic-induced catalepsy-like immobilization in zebrafish (*Danio rerio*), as an alternative to the standard rodent model. To induce the desired symptoms, we used haloperidol, a typical antipsychotic that disturbs dopamine D2-receptors. In addition to observing swimming behaviors generally, we used the light and dark test to assess how drug exposure influences locomotive responses to those stimuli. We selected this test instead of the commonly used bar test for catalepsy in rodents, because fish cannot perform the necessary motions to participate in the latter. Normally, light attenuated activity and decreased locomotion, whereas darkness greatly increased activity levels in zebrafish. We confirmed that haloperidol had a dose-dependent inhibitory effect on activity; the highest dose of 10 mg/L almost stopped fish activity even in darkness. We did not observe any significant differences in heart rate or morphology across the control and treatment groups, whereas abnormal movements like rigid and erratic behaviors occurred in haloperidol-treated groups. Therefore, we found that immobilization and abnormal movements qualify as haloperidol-induced catalepsy. In conclusion, zebrafish appear to be a suitable model for antipsychotic-induced catalepsy-like immobilization.

## Introduction

Catalepsy (dampened reaction to stimuli and persistent posture maintenance) is a common symptom of catatonic schizophrenia that can also be directly induced with a typical antipsychotic, such as haloperidol [1–3]. Patients with catatonic schizophrenia are as likely to be involved in driving accidents from haloperidol-induced catalepsy as they are from illness-induced catalepsy [4–6]. In addition, abuse of psychotropic or antipsychotic drugs can induce adverse effects, including addiction, delirium, and even sudden death. Some case reports have even suggested that several antipsychotic medications may increase the risk of serious cardiac conditions, including ventricular arrhythmia, myocarditis, and death from cardiac arrest [7–13]. Thus, in fatal car accidents involving patients with schizophrenia exhibiting cataleptic symptoms, autopsies should attempt to determine whether the cause of death was the illness directly, drug-induced immobilization, or trauma from the crash. Several severe traffic accidents involving users of psychotomimetic drugs who experienced cataleptic rigidity have been confirmed worldwide.

Given the potential dangers, we require research identifying drug components that cause these side effects. Several assays have been developed that evaluate locomotor activities, startle latency [14–16], behavior modifications, and physiological conditions including myocardial necrosis. Typically, rodents are the preferred model for such studies, owing to their reliability in behavioral screenings and physiological responses [3, 17, 18]. However, during the past decade, zebrafish (*Danio rerio*) have become increasingly popular as a model for pharmacological or behavioral research testing diverse psychotropic drugs [19–22]. Zebrafish are easy to handle and more cost-effective than rats. Furthermore, zebrafish have some genetic phenotypes and specific proteins that are similar to humans [23].

Locomotion in zebrafish is usually evaluated using the variables of moved distance, moving velocity, and moving duration [24–28]. However, these three values are insufficient as evidence of abnormal behaviors, including catalepsy-like symptoms. When attempting to duplicate pharmacological symptoms, researchers should also concurrently measure immobilization, rigidity, effects on cardiopulmonary functions, and morphological changes [29–31]. In both rodent and zebrafish models, immobilization can be examined using moved distance, moving velocity, and moving duration. Only rodents, however, can participate in the bar test, used to evaluate rigidity. Therefore, researchers interested in the zebrafish as a catalepsy model need to identify appropriate alternatives. In this study, we selected the light and dark test for screening, and chose haloperidol as the drug to induce catalepsy-like immobilization [26, 32, 33].

The objective of this study was to determine whether zebrafish are an appropriate model for haloperidol-induced catalepsy. We used the light and dark test to evaluate zebrafish locomotion during acute drug exposure. Specifically, we investigated whether light or dark stimuli would affect drug-induced locomotive responses as a measure of catalepsy [34]. We also assessed zebrafish heart rate and morphological changes after exposure to different haloperidol concentrations. Our research is useful for advancing forensic toxicology, given that we are examining the toxic effects of antipsychotics on cardiopulmonary function and striated muscles, a link associated with sudden deaths [29–31, 36]. Our results will help reveal potential links between antipsychotics and unexpected physical dysfunctions that could trigger fatal accidents.

## Results

### Effects of haloperidol at different doses on total distance

There were significant effects on total distance ( $F = 38.54$ ,  $p < 0.0001$ ). Haloperidol (or HAL) at 1, 5 and 10 mg/L significantly influenced locomotor responses compared with the control (or CNTL). Haloperidol at doses of 0.5 mg/L induced mild decreases in locomotor activity, but the differences were not significant. Haloperidol at 10 mg/L exerted the strongest effect (lowest activity) compared with the control (the Tukey-Kramer test,  $**p < 0.01$  from CNTL + HAL group; Fig. 1).

### Effects of light and dark test on haloperidol-induced catalepsy

Significant effects were observed for the 1st light(on) ( $F = 6.028$ ,  $p < 0.0001$ ), 1st dark(off) ( $F = 23.83$ ,  $p < 0.0001$ ), 2nd light(on) ( $F = 3.895$ ,  $p < 0.01$ ), 2nd dark(off) ( $F = 35.99$ ,  $p < 0.0001$ ), 3rd light(on) ( $F = 2.796$ ,  $p < 0.05$ ), 3rd dark(off) ( $F = 35.85$ ,  $p < 0.0001$ ), 4th light(on) ( $F = 2.865$ ,  $p < 0.05$ ), 4th dark(off) ( $F = 32.74$ ,  $p < 0.0001$ ), 5th light(on) ( $F = 3.331$ ,  $p < 0.01$ ), 5th dark(off) ( $F = 32.09$ ,  $p < 0.0001$ ), 6th light(on) ( $F = 8.366$ ,  $p < 0.0001$ ), and 6th dark(off) ( $F = 22.34$ ,  $p < 0.0001$ ). Normally, zebrafish larvae exhibit hyperactivity in the dark and hypoactivity in light. We rotated between light and dark conditions every 5 min in 1 h trials. In comparison, light stimuli significantly altered the distance traveled in the control group. In the dark, control locomotor activity increased sharply, whereas light caused activity to drop. In the light, the distance traveled in 1st light(on) did not decrease in the 0.5 mg/L haloperidol group. By contrast, dark(off) conditions did not increase the distances traveled in 3rd – 5th in the 0.5 mg/L (Tukey-Kramer

test, \* $p < 0.05$ , \*\* $p < 0.01$  from CNTL + HAL group), 1st – 5th in the 1 mg/L (Tukey-Kramer test, \* $p < 0.05$ , \*\* $p < 0.01$  from CNTL + HAL group), 1st – 6th in the 5 mg/L (Tukey-Kramer test, \*\* $p < 0.01$  from CNTL + HAL group), and 1st – 6th in the 10 mg/L group (Tukey-Kramer test, \*\* $p < 0.01$  from CNTL + HAL group; Fig. 2, Table 1)

### **Effects of comparison between light and dark bands on haloperidol-induced catalepsy**

There were significant differences between light(on) and dark(off) in the control ( $F = 21.81$ ,  $p < 0.0001$ ), DMSO ( $F = 7.313$ ,  $p < 0.0001$ ), and 0.5 ( $F = 10.90$ ,  $p < 0.0001$ ), 1 mg/L ( $F = 9.230$ ,  $p < 0.0001$ ), 5 mg/L ( $F = 1.712$ ,  $p < 0.0001$ ), and 10 mg/L ( $F = 2.001$ ,  $p < 0.0001$ ) of haloperidol groups. Activity in the light bands seemed to decrease similarly regardless of the concentration gradient, but activity in the dark bands gradually decreased according to the concentration gradient. For the control (t-test  $t = 18.54$ ,  $df = 508$ ,  $p < 0.0001$ ), DMSO (t-test  $t = 19.35$ ,  $df = 525$ ,  $p < 0.0001$ ) and 0.5 mg/L (t-test  $t = 16.48$ ,  $df = 541$ ,  $p < 0.0001$ ), and 1 mg/L (t-test  $t = 15.35$ ,  $df = 555$ ,  $p < 0.0001$ ) of haloperidol groups, the total distances traveled between the light and dark bands were significantly different, but at 5 mg/L (t-test  $t = 1.679$ ,  $df = 531$ ,  $p = 0.0938$ ) of haloperidol, there was no significant difference. At 10 mg/L (t-test  $t = 4.549$ ,  $df = 509$ ,  $p < 0.0001$ ) of haloperidol, the light and dark bands appear to be significantly reversed (Fig. 3).

### **Effects of haloperidol on locomotion**

Locomotor activity decreased from the 1st light interval for the 5 and 10 mg/L haloperidol groups, whereas activity began to decrease at intervals from the 2nd light interval (Fig. 4a) for the remaining drug-treated groups. Locomotor activity noticeably decreased even in the 1st dark interval under 5 and 10 mg/L haloperidol, whereas decreased activity did not occur for the remaining drug-treated groups (Fig. 4b). We also found that 10 mg/L haloperidol notably induced rigid, erratic swimming [27] (Fig. 4c).

### **Time spent on upper and lower portions of the experimental tubes**

In our additional trial, we found that zebrafish tended to swim mainly near the surface. Under drug exposure, subjects remained on the bottom, but after 24 h in fresh water, subjects began to swim near the surface again (Fig. 5).

### **Haloperidol-induced heart rate (bpm) estimation and potential morphological modifications**

Heart rate did not change significantly in response to any dose of haloperidol ( $F = 1.017$ ,  $p = 0.4126$  from CNTL + HAL group; Fig. 6). Morphological changes, including rhabdomyolysis or spine curvature, were not observed even after exposure to haloperidol at 10  $\mu\text{g}/\text{mL}$  (Fig. 7). We found that 5 and 10 mg/L haloperidol induces catalepsy-like immobilization, but had less influence on heart rate or morphology.

## **Discussion**

The results of our light and dark test showed that light attenuated activity, whereas darkness facilitated activity in normal zebrafish. However, when exposed to haloperidol, subjects had no reaction to light or dark. At higher haloperidol concentrations (1, 5, and 10 mg/L), we observed significant decreases in locomotor activity but no effect on heart rate. Given this minimal influence, we concluded that haloperidol does not strongly affect cardiopulmonary functions. Although this result implied that automatic nerves

are not associated with the attenuation of locomotion in zebrafish, we have little insight regarding the involvement of  $\alpha 1$  adrenergic receptors. We found no signs of rhabdomyolysis and vertebral deformation. These findings suggest that haloperidol has distinct advantages over psychotomimetic drugs (e.g., methamphetamine) and psychoactive designer drugs (e.g., 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl) ethanamine), which induce complications like rhabdomyolysis [31]. These complications are associated with hyperactivity or hyperthermia, so antipsychotics that induce hypoactivity and immobilization are unlikely to have similar side effects.

Regarding the number of repetitions of light stimulation, the data were unified with little variation, especially in the 3rd, 4th, and 5th light and dark bands, but the data were not stable at the 1st, 2nd, and 6th times. Therefore, it can be inferred that the data of the 3rd, 4th, and 5th times show the significant difference between the haloperidol groups and the control group. This is because the 1st and 2nd times vary owing to individual differences between groups, but the behavioral patterns are unified because the fish are acclimatized and their behavior is stable in the 3rd and subsequent times. In addition, as the behavior becomes unstable again from the 6th time onward, the reliability of the data after that is expected to decrease. Therefore, it is assumed that the most efficient and reliable light stimulation is in the 3rd, 4th, and 5th light stimulations which constitute six consecutive periods.

What is noteworthy in this result is that not only does the activity of the dark band gradually decrease according to the concentration gradient when comparing the distance traveled between the light band and the dark band, but also the activity of the dark band decreases to the same level as that of the light band at 5 mg/L. Furthermore, it is surprising that the phenomenon of the reversal of the amount of activity in the light and dark bands at 10 mg/L is confirmed.

This result clarifies not only the catalepsy symptoms of decreased activity and responsiveness to light stimulus but also unexpected behavior patterns as a result of increased haloperidol concentration. Importantly, this screening may change the current perception of haloperidol.

The low toxicity of haloperidol makes it less of a priority in psychiatric treatment research than other psychomimetic drugs because it is rarely linked to sudden deaths in conditions such as dementia [35]. However, some research suggests that the dangers of haloperidol may be underestimated. Schizophrenic inpatients under antipsychotic monotherapy had significantly worse performance in a driving simulator when they were treated with haloperidol than with other antipsychotic drugs [37]. However, when traffic accidents are associated with schizophrenia and antipsychotic drug treatments, driving dysfunctions are typically only attributed to illness-induced catalepsy. Our study suggests that antipsychotic drugs like haloperidol could cause exogenous catalepsy along with the endogenous catalepsy from schizophrenia, both of which could then combine to result in serious physical dysfunctions. Thus, we recommend that psychiatrists perform inquests into causes of driving-related accidents until the presence or absence of any correlation between drug intake and catalepsy is firmly established.

In summary, we demonstrated that the light and dark test is an appropriate substitute for the bar test in terms of evaluating catalepsy-like immobilization in zebrafish. Additionally, we demonstrated that

haloperidol only temporarily induced the locomotive disorders, supporting previous findings [38]. Moreover, haloperidol treatment had minimal effect on heart rate or morphology. Our study has important implications for the persistence of drug complications. The results of our study suggest that the zebrafish model could advance considerably and become useful in the behavioral screening for haloperidol-induced catalepsy.

## Conclusions

Although catalepsy is a typical symptom in catatonic schizophrenia, it can also be induced by the antipsychotic drug haloperidol. As with Parkinsonism, catalepsy appears to be possibly due to the disturbance of dopamine D2 receptors in the corpus striatum [3].

Several studies have attempted to duplicate and assess catalepsy-like motor functional disorders in zebrafish, but have met with little success in accurately replicating results from rodent models. Clearly, more research is necessary to evaluate catalepsy-like symptoms in both animal models.

In conclusion, this study confirmed that zebrafish appear to be a suitable and valid model for antipsychotic-induced catalepsy-like immobilization, as an alternative to the standard rodent model.

## Materials And Methods

### Animals

The Experimental Animal Committee of Tokyo Medical University approved all experiments (approval number: H30-0020, R1-0114, R2-0037). This study was carried out in compliance with the ARRIVE guidelines and also confirmed that all experiments were performed in accordance with zebrafish experimental guide. Adult zebrafish (*Danio rerio*; wild-type, purchased from Kamihata Fish Industries Ltd., Tokyo, Japan) were housed and raised in aerated breeding units at a density of 10 fish per liter, in water from a recirculating water system supplied with dechlorinated municipal tap water. The fish were maintained under conditions of pH 7.5–8.0, a conductivity of 300–500  $\mu\text{S}/\text{cm}$ , and a temperature of 26–28°C. Lighting was artificial, with 14 h of light (8 AM to 10 PM) and 10 h of darkness. Fish were fed with flake food once a day. To obtain embryos for the purposes of the present study, male and female zebrafish were paired in the evening, then fertilized embryos were collected from the mated zebrafish and placed in Petri dishes containing fresh water. These embryos were transferred to a 28°C incubator under a dark environment until 6–7 days post fertilization (6–7 dpf), During this time, the embryos were screened to assess overall health and dead embryos were removed daily.

### Drugs

Haloperidol (Sigma-Aldrich, St. Louis, MO) was diluted with 0.05% dimethyl sulfoxide (DMSO; Dojindo Laboratories, Japan) and sterile saline (vehicle). The drug is a traditional antipsychotic agent used primarily to treat schizophrenia and other psychoses [3, 18, 23, 28, 33] by relieving the symptoms of delusions and hallucinations commonly associated with schizophrenia. Haloperidol competitively blocks

post-synaptic dopamine D2 receptors, eliminating dopamine neurotransmission while partially inhibiting 5-HT<sub>2</sub> and  $\alpha$ <sub>1</sub>-receptors, but there is negligible activation of dopamine D1-receptors [39].

### **Catalepsy screening**

This study employed the light and dark test for catalepsy screening. Some previous studies have reported trials that evaluated locomotion with light stimuli using zebrafish [26, 33, 40, 41]. When evaluating abnormal behavior, more comprehensive insights on the characteristics of abnormal behavior can be gained not only by determining levels of activity but also by assessing normal behavioral patterns and changes in these patterns attributable to drug effects. Given that zebrafish tend to be characterized by higher locomotory activity in the dark than in the light, the light and dark test is often used to analyze changes in fish locomotor activity and behavioral patterns because of drug effects [42, 43]. Evaluations based exclusively on the parameters of distance moved, movement speed, and movement duration would provide an insufficient assessment of drug effects, and thus to address this deficiency, we also compared behavioral patterns using the repeated light and dark test.

The study employed a high-throughput tracking system (Danio Vision XT) and behavioral analysis software (Ethovision XT 11.5) to facilitate analysis of the behavioral responses of treated zebrafish [44]. This is currently rare among researchers using small organisms, but is becoming increasingly common for investigating pharmacological effects in zebrafish. The procedure adopted when using this device was as follows: after transferring the larvae from Petri dishes to an experimental microtiter plate, the plate was placed in a chamber that could be illuminated with bright lights (i.e., light-on periods) or infrared lights (i.e., darkness or light-off periods) using the associated software. The light intensity used during the experiments was 700 lx, whereas under infrared illumination, the intensity was 0 lx. During the periods in which measurements were obtained, the light stimulus was turned on and off at 5-min intervals (i.e., 5 min of bright light followed by 5 min of darkness), and the changes in movement patterns were repeatedly analyzed over six periods of alternating 5-min light and 5-min dark stimuli (i.e., a total measurement time of 1 h). Heart rate and morphological changes were also measured.

### **Experimental design**

Zebrafish larvae (6–7 dpf) were maintained in 96-well microtiter plates (IWAKI Co., Ltd., Tokyo, Japan; 1 larva/well) filled with 300  $\mu$ L E3 medium (n = 288). Locomotor activity was assessed after haloperidol treatments of 0.5, 1, 5, and 10 mg/L (16 larvae/group). Fish in each treatment group were then subjected to repeated light and dark stimuli to determine differences in locomotor activities and responses, examined under the alternating 5-min intervals of light and dark [42, 45]. Drug effects on subjects were measured using Danio Vision and Scope (Noldus, Leesburg, VA). DanioVision is a high-throughput system designed for studying locomotion in small organisms; it can simultaneously track up to 96 individuals. This experiment examined total distance traveled, responses to light stimuli, path of movement, and swimming behavior. This study collected heart rate data specifically. Morphological changes were determined under a microscope (Leica Application Suite X; Leica Microsystems, Switzerland), to determine the presence of muscular necrosis. Movement paths in the wells were delineated with red markings.

Hypoactivity in zebrafish can also be screened via assessment of time spent in the upper or bottom portions of a given vessel [24, 27], therefore we also assessed the time spent in the upper and lower portions of the experimental tubes.

### Statistical analysis

Data are presented as the means  $\pm$  SEMs (standard error of the mean) of at least three independent experiments. One-way ANOVAs were used for statistical comparisons of the recorded observational data, followed by pairwise post hoc comparisons using the Tukey-Kramer test. Statistical analysis was performed using GraphPad Prism 6 for Windows version 6.05. The data were analyzed using the Tukey-Kramer test, or Student's t-test at a confidence level of 95%.

## Declarations

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### AUTHOR CONTRIBUTIONS

### ADDITIONAL INFORMATION

### COMPETING INTERESTS

The authors declare no competing interests.

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## Table

**Table 1.** Results of light and dark responses

Haloperidol (mg/L)	1st. ON	2nd. ON	3rd. ON	4th. ON	5th. ON	6th. ON	(mm)
CNTL	66.54446	103.2868	71.03306	66.25666	66.10998	68.85615	
DMSO	57.57797	128.1369	74.42446	75.70519	59.71475	111.7412	**
0.5mg/L	109.3587	**	100.991	80.47347	89.92186	80.52053	75.99799
1mg/L	100.3901	88.8899	83.68288	80.70876	74.902	57.54582	
5mg/L	73.52189	79.04584	81.58211	84.53001	72.78159	77.91082	
10mg/L	71.68707	67.57038	52.91501	55.51472	46.49083	44.19802	

Haloperidol (mg/L)	1st. OFF	2nd. OFF	3rd. OFF	4th. OFF	5th. OFF	6th. OFF	(mm)
CNTL	409.3362	426.7172	410.0276	376.9898	358.6147	303.15	
DMSO	378.6062	403.335	367.1565	338.3295	328.7714	279.6826	
0.5mg/L	322.7775	346.3854	300.3713	*	271.718	*	244.0146**
1mg/L	281.1238	*	263.8356	**	255.4774	**	226.0294**
5mg/L	101.7565	**	106.5095	**	81.35495	**	81.74755**
10mg/L	65.89935	**	44.74797	**	35.66826	**	37.02889**
							35.00738**
							30.49957 **

Haloperidol-treated groups differed significantly from the vehicle control in response to light(on) and dark(off) stimuli. The graph is shown in Fig. 2. All values are expressed as means  $\pm$  SEM (n = 288). \*P < 0.05, \*\*p < 0.01 from CNTL + HAL group, Tukey-Kramer test.

## Figures

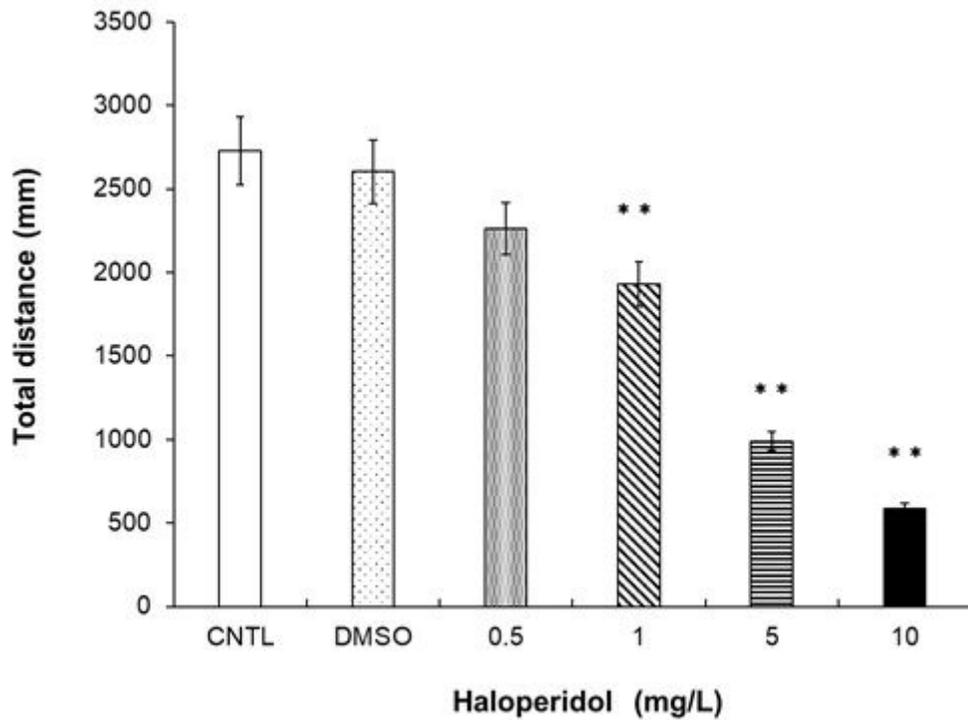


Figure 1

Effects of haloperidol at different doses on total distance. Groups treated with 0.5 mg/L had slightly lower ambulation distance but did not differ significantly from control, whereas 1, 5, and 10 mg/L significantly decreased ambulation distance compared with control group. Vehicle control and DMSO-treated fish did not differ (data not shown). All values are expressed in means  $\pm$  SEM ( $n = 288$ ). \*\* $p < 0.01$  from CNTL + HAL group, The Tukey-Kramer test.

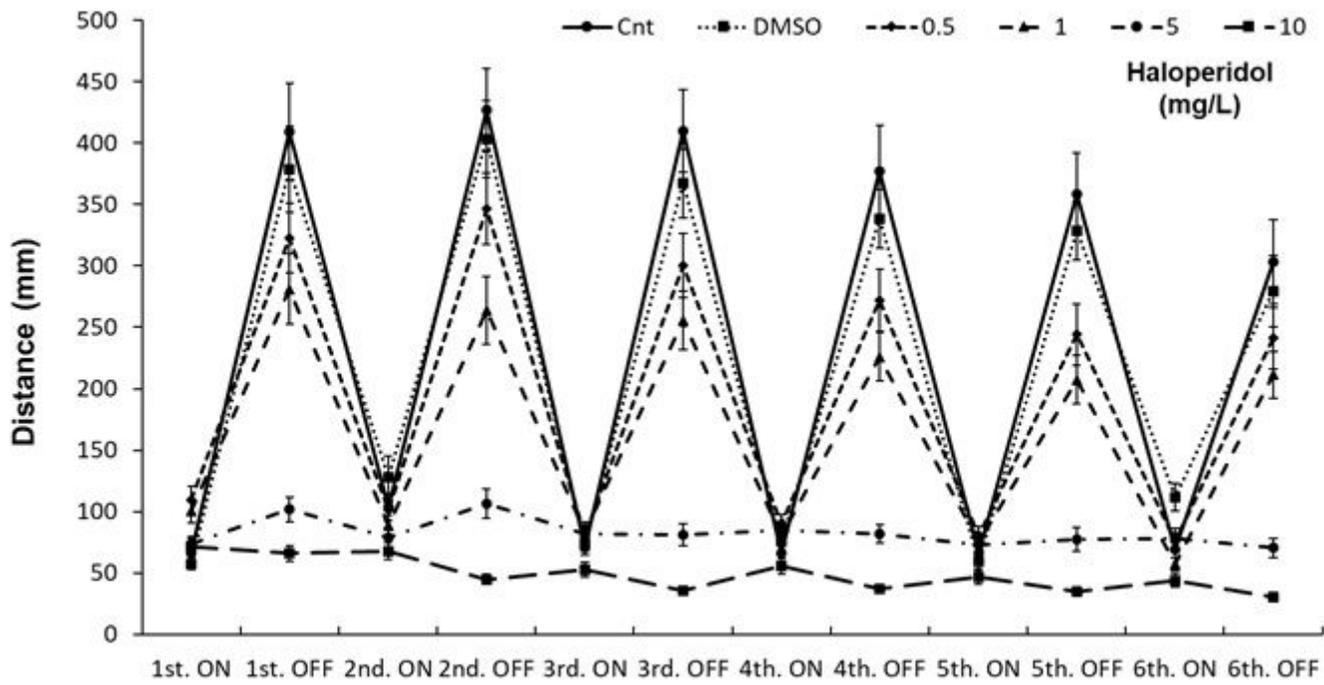


Figure 2

Effects of the light and dark test on haloperidol-induced catalepsy. We rotated between light(on) and dark (off) conditions 6 times. Light stimuli significantly altered the distance traveled in the control group. In the dark, control locomotor activity increased sharply, whereas light caused activity to drop. The data and results are shown in Table. 1. All values are expressed as means  $\pm$  SEM (n = 288).

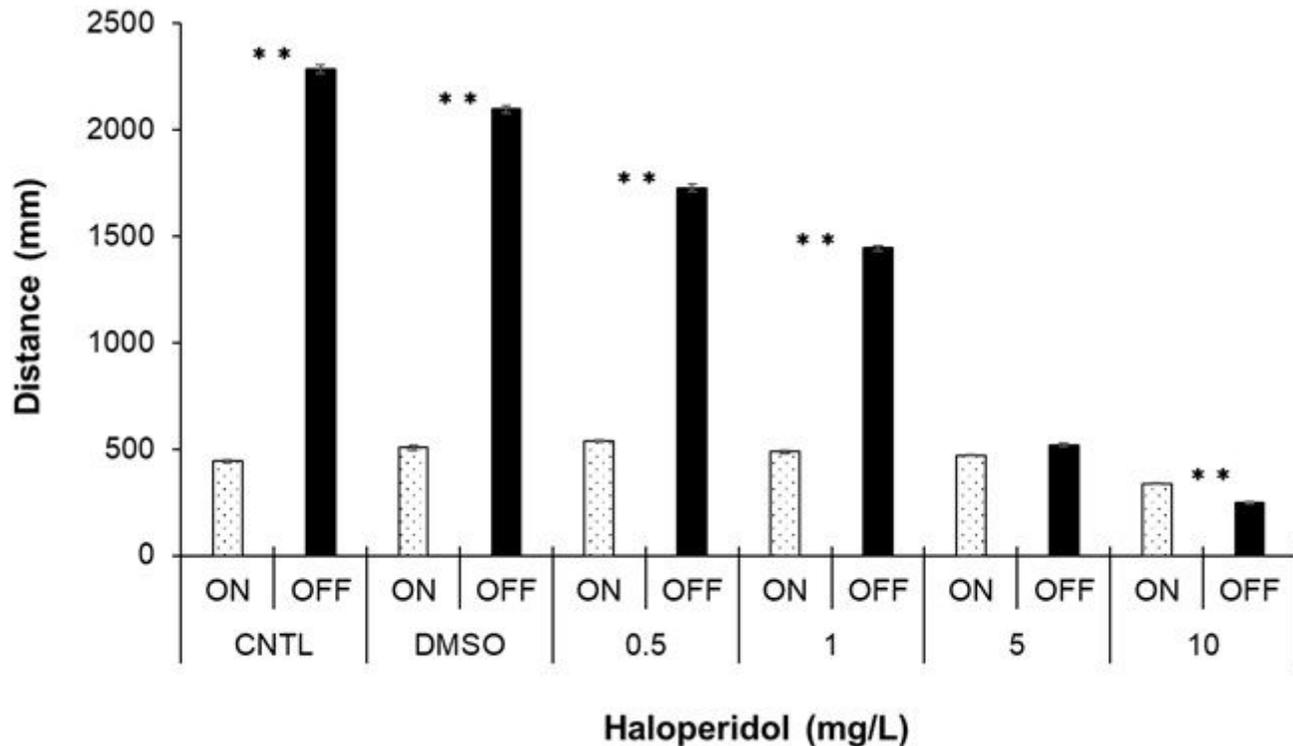
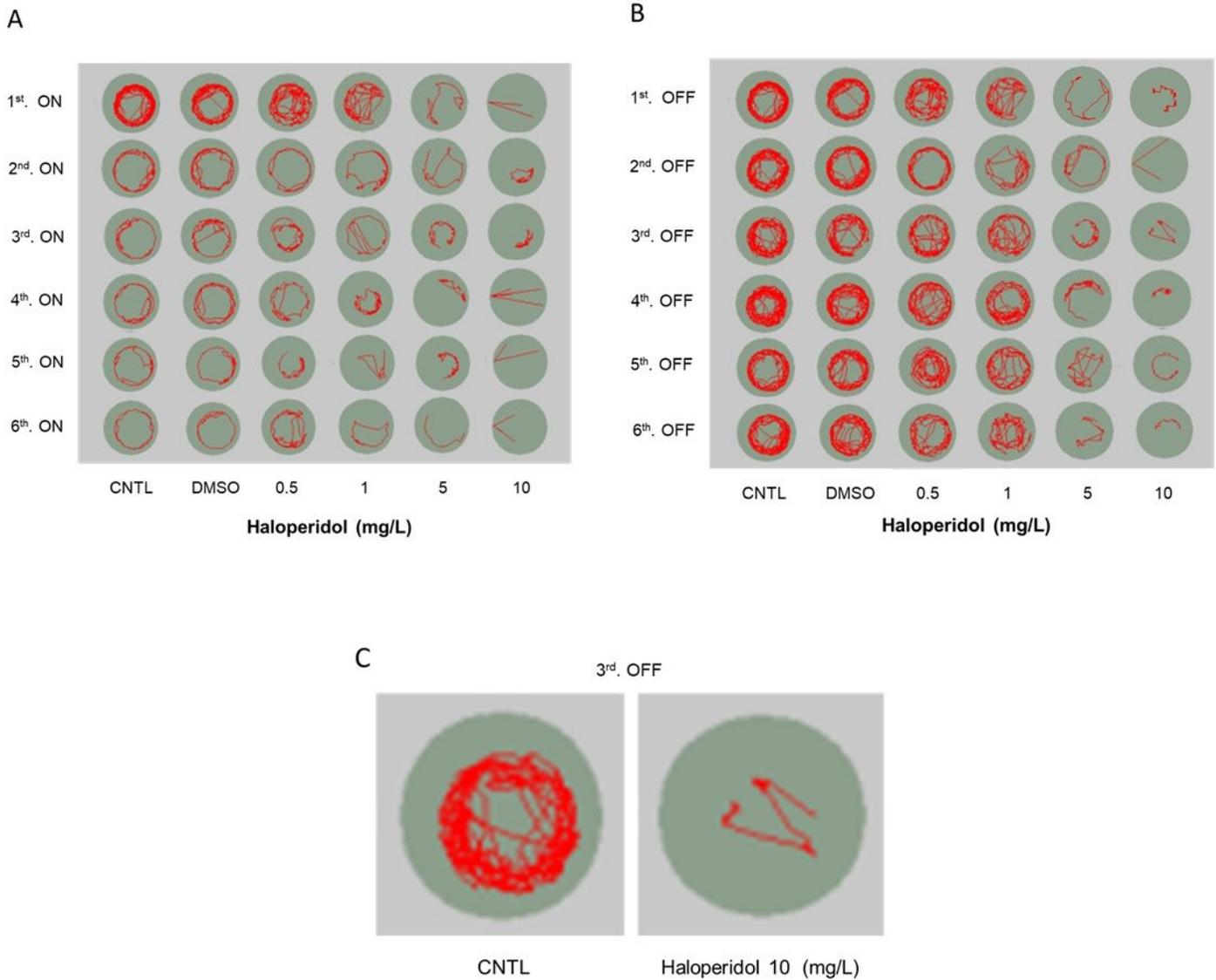


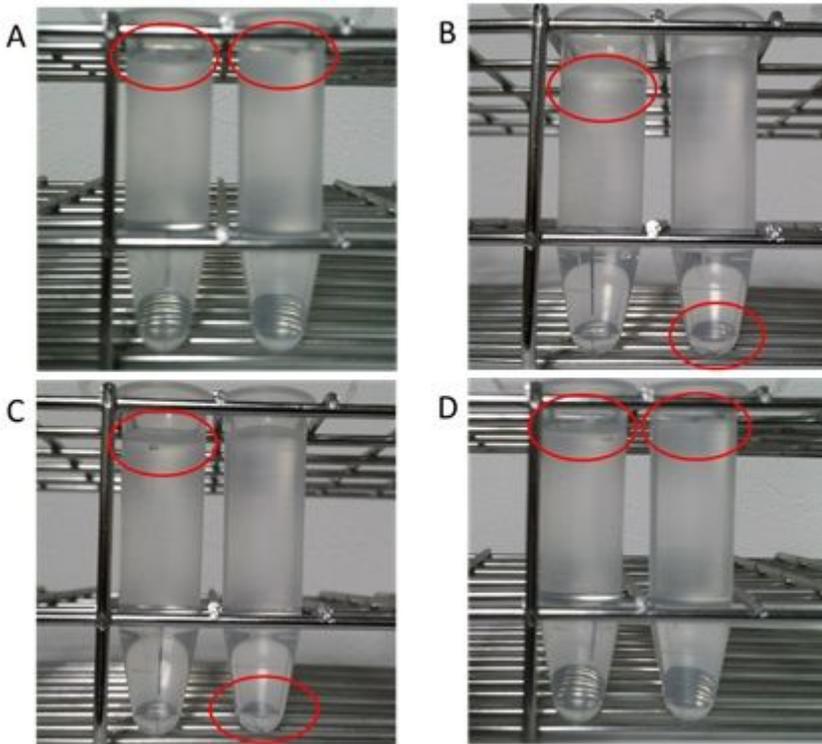
Figure 3

Comparisons between total distances traveled in light and in dark bands. The control, DMSO and groups treated with 0.5-1 mg/L had significant differences between the light(on) bands and the dark(off) bands, whereas the light band of 5 mg/L did not differ significantly from the dark band. By contrast, at 10 mg/L haloperidol, the light and dark bands appear to be significantly reversed. All values are expressed in means  $\pm$  SEM (n = 288). \*P < 0.05; \*\*, P < 0.01, Student's t-test.



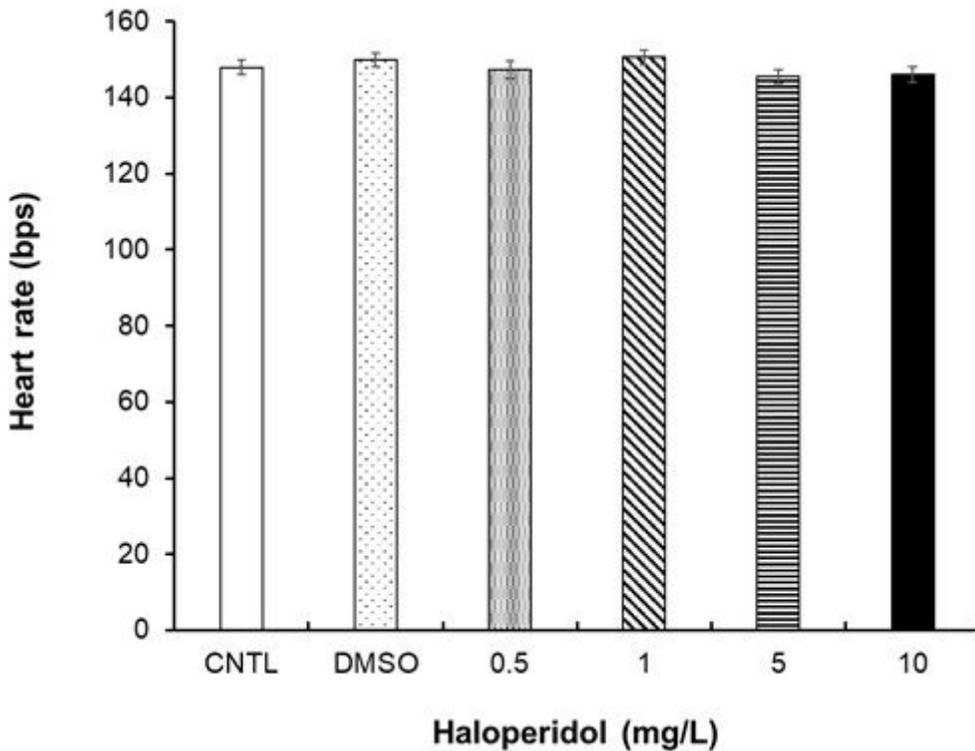
**Figure 4**

Effects of haloperidol on locomotion. Red markings in circles represent tracking of locomotion (a-c). We did not observe a notable decrease in locomotion under light(on) conditions among all groups except in the first interval (a). Decreased locomotion occurred in the 5-10 mg/L groups in the dark(off) conditions (b). High doses (10 mg/L) of haloperidol caused movement disorders in zebrafish, which exhibited rigid, erratic swimming in the 3rd dark condition compared with the vehicle control sample (c).



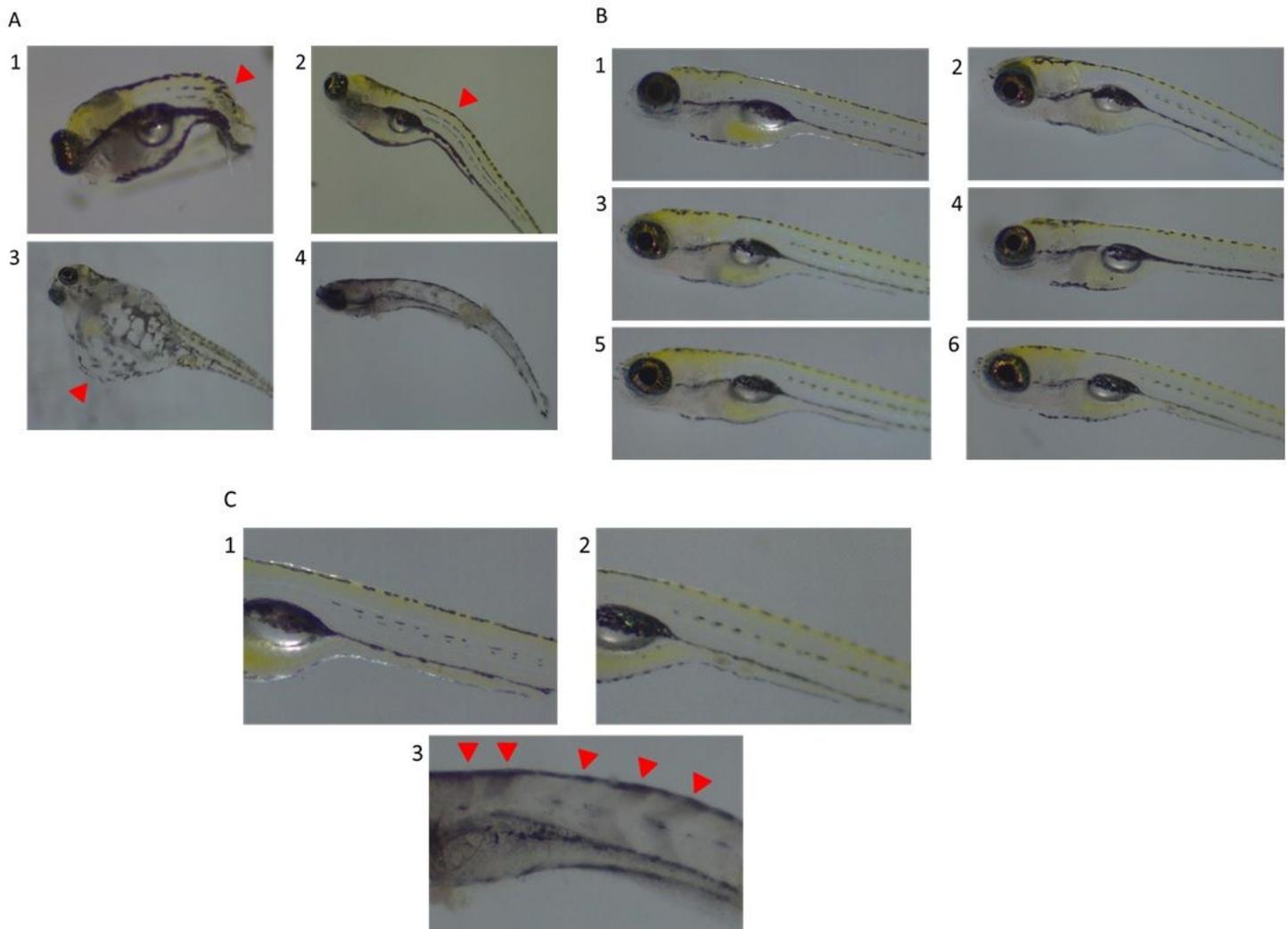
**Figure 5**

Time spent on upper and lower portions of the experimental tubes. Zebrafish tended to swim near the surface before drug treatment (a). However, at 60 min (b) and 120 min (c) of exposure to haloperidol, subjects moved to the bottom, whereas control fish did not. After spending 24 h in fresh, untreated water, fish in haloperidol groups began to swim near the surface again (d).



## Figure 6

Haloperidol-induced heart rate (bpm) estimation. Haloperidol did not significantly alter heart rates compared with control. All values are expressed as means  $\pm$  SEM (n = 96).  $p > 0.05$ , The Tukey-Kramer test.



## Figure 7

Microscopic images of potential haloperidol-induced morphological modifications. (a). Morphological abnormal modifications (arrow heads). Vertebral deformation (a-1, 2), Abdominal deformation (a-3), Postmortem change (a-4). In this study, we observed the microscopic images of haloperidol-treated zebrafish bodies (b-c). Control as normal body (b-1), DMSO (b-2), 0.5 mg/L (b-3), 1 mg/L (b-4), 5 mg/L (b-5), 10 mg/L (b-6). Haloperidol treatment did not alter morphology significantly from vehicle control. The striated muscular changes did not occur in even 10 mg/L compared with control. Control as normal striated muscle (c-1), 10 mg/L (c-2), Rhabdomyolysis (arrow heads) as the sample image (c-3).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [1.tif](#)