Archives of Current Research International

Volume 25, Issue 7, Page 676-691, 2025; Article no.ACRI.139589
ISSN: 2454-7077

Rotenone-treated in the Early Life Stages of Zebrafish (Danio rerio) as a Model for Parkinson-like Motor and Non-motor Symptoms and Anxiety-like Behaviour

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/acri/2025/v25i71369

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://pr.sdiarticle5.com/review-history/139589

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Cite as: *Andrade, André Lucas Corrêa de, Renatta Priscilla Ferreira Silva, Priscila Rafaela Leão Soares, Thamiris Pinheiro Santos, Renata Meireles Oliveira Padilha, Paulo Eduardo da Silva Bastos, and Pabyton Gonçalves Cadena. 2025. "Rotenone-Treated in the Early Life Stages of Zebrafish (Danio Rerio) As a Model for Parkinson-Like Motor and Non-Motor Symptoms and Anxiety-Like Behaviour". Archives of Current Research International 25 (7):676-91. https://doi.org/10.9734/acri/2025/v25i71369.*

Original Research Article

Received: 10/05/2025
Accepted: 15/07/2025
Published: 18/07/2025

ABSTRACT

Background: Rotenone is a pesticide derived from a natural toxin present in plants and is used in agriculture and aquaculture to eliminate unwanted fish. The toxicity of rotenone in fish makes it difficult to determine an optimal dose that efficiently reduces dopaminergic neurons with low mortality.
Aims: To establish a zebrafish (*Danio rerio*) model for Parkinson-like symptoms by evaluating the developmental, teratogenic, and behavioural effects of rotenone, with a specific focus on non-motor and anxiety-like behaviours.
Study Design: An *in vivo* experimental study evaluating dose-response effects of rotenone on zebrafish embryo-larvae.
Methodology: The study was conducted at Laboratório de Ecofisiologia e Comportamento Animal (LECA), Universidade Federal Rural de Pernambuco (UFRPE), Recife, PE, Brazil, between August 2020 and July 2021. Zebrafish embryo-larvae were exposed to rotenone (5, 10, 15, and 20 µg/L) from 2 hours post-fertilisation (hpf). Developmental endpoints (epiboly rate, teratogenicity, mortality, morphometry) and behavioural endpoints (thigmotaxis, touch sensitivity, optomotor response) were analysed up to 144 hpf.
Results: Rotenone exposure increased mortality and teratogenicity in a dose-dependent manner, yielding an LC_{50} of 13.88 µg/L and an EC_{50} of 11.24 µg/L at 96 hpf. Significant reductions in head and body length were observed at all concentrations ($P < .05$), with a notable decrease of 6% in head length and 5% in body length even at the lowest dose (5 µg/L). Anxiety-like behaviour, indicated by a significant loss of thigmotaxis, was evident at 15 µg/L ($P = .013$) and 20 µg/L ($P = .001$), where the response rate dropped from 92% in controls to 68% and 63%, respectively. In contrast, touch sensitivity was only affected at the highest concentration (20 µg/L, $P = .009$), with the escape response rate dropping from 100% in controls to 85%. The optomotor response was unaffected.
Conclusion: Rotenone exposure in early-stage zebrafish effectively models key features of Parkinson's disease, including developmental defects and non-motor symptoms like anxiety-like behaviour. The 10-15 µg/L concentration range offers an optimal window for inducing these phenotypes, establishing a robust and high-throughput model for screening potential therapeutics.

Keywords: Parkinson's disease; anxiety-like behavior; animal model; rotenone; zebrafish.

1. INTRODUCTION

Parkinson's disease (PD) is the fastest-growing progressive neurodegenerative disorder in prevalence and disability among neurological disorders and has become a leading cause of disability worldwide (Zhu et al., 2024). PD may be due to the selective damage of dopaminergic neurons in the substantia nigra pars compacta. It has been observed that oxidative stress plays a major role in the pathophysiology of Parkinson's Disease (Aslam et al.,2021).

The most well-known symptoms are primarily based on motor features, such as a slowly progressive asymmetric resting tremor, cogwheel rigidity, and bradykinesia, although non-motor features, which include anosmia, constipation, depression and REM sleep behaviour disorder, can develop years before motor deficits (Simon et al., 2020). A proper understanding of these behavioural changes is crucial to understanding the pathophysiological mechanisms of PD and evaluating the efficacy and safety of new therapies (Asakawa et al., 2016). Motor, smell, and sleep alterations are already well known and addressed in animal models (Titova et al., 2017). On the other hand, behavioural changes, anxiety, and depression remain poorly studied and are limited to clinical studies due to the lack of vertebrate animal models (Wang et al., 2017).

A suitable animal model for performing this type of study is the zebrafish (*Danio rerio*), widely used as a model of neurological diseases and pharmacological tests (Basnet et al., 2019). It is

a freshwater fish belonging to the Cyprinidae of the order Cypriniformes. Their natural habitat is in Southeast Asian streams, rivers, or well-vegetated pools (Omar et al., 2023; Spence et al., 2008). Among the main advantages of this teleost are the small size of the larval and adult forms, transparent embryos, low maintenance cost, rapid external fertilisation, robust reproductive capacity, and large-scale quantitative methods of behaviour (Doyle and Croll, 2022). The genomes of humans and zebrafish are highly conserved, with 76–82% of human disease genes shared with the zebrafish (Çalışkan and Emekli-Alturfan, 2021). Another important factor for the use of zebrafish embryos is their suitability for the bioethical principle of the 3Rs (Cassar et al. 2020). The zebrafish animal model can be used in the Replacement of animals with higher taxonomic levels, such as mammals; to promote the Reduction of Mammalian use in the initial selection of pharmacological candidate compounds; and the possibility of Refinement of administration of the compounds to be tested by immersion in the aqueous environment, mitigating the use of invasive procedures (Cassar et al. 2020).

In addition to these characteristics, zebrafish are valuable in PD research because they embody the mechanisms for the neurotoxicity of PD-like inducing chemicals such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methyl-4-phenylpyridinium ion (MPP+), 6-hydroxydopamine (6-OHDA), paraquat, and rotenone (Bretaud, Lee and Guo, 2004; Kalyn et al., 2020). The use of these chemicals in zebrafish was able to cause motor changes and loss of dopaminergic cells in the different life stages of the animal (Khotimah et al., 2015; Melo et al., 2015; Wang et al., 2017; Kalyn et al., 2020). However, there are no studies that have investigated non-motor symptoms of PD in the early life stages of zebrafish, such as depression-like or anxiety-like behaviours using PD-like inducing chemicals (Doyle and Croll, 2022). Rotenone is one of these neurotoxins, which, like MPTP and MPP+, acts on the functional inhibition of the mitochondrial complex I, creating an environment of oxidative stress that results in the loss of dopaminergic neurons (Watabe and Nakaki, 2007).

Rotenone is a pesticide derived from a natural toxin present in plants and is used in agriculture and aquaculture to eliminate unwanted fish (Doyle and Croll, 2022). It has a hydrophobic nature that allows easy penetration of neuronal cell membranes (Ott, 2006). The toxicity of rotenone in fish makes it difficult to determine an optimal dose that efficiently reduces dopaminergic neurons with low mortality (Kalyn et al., 2020). However, Melo et al. (2015) measured a half-maximal effective concentration (EC50) at 96 hpf for zebrafish embryos of 12.2 µg/L when evaluating the acute toxicity of rotenone in different fish species. In other studies, such as that by Khotimah et al. (2021), a growth reduction in embryos exposed to a concentration of 12.5 µg/L of rotenone was observed. Kalyn et al. (2020) found that zebrafish embryos were exposed to 50 nM of rotenone, producing a moderate reduction in locomotor activity and cardiac malformations. Other studies found PD-like symptoms in adult zebrafish exposed to 5 µg/L rotenone, including motor activity reduction (Khotimah et al., 2015), olfactory dysfunction, and anxiety-like behaviour (Wang et al., 2017). For this reason, it is necessary to describe the methodologies used in the zebrafish model that allows the assessment of larval behavioural parameters with developmental deficits caused by PD-like inducing agents. One of the ways to assess the developmental deficit of the zebrafish embryo is by observing the process of epiboly, the first morphogenetic event that occurs when cells spread over the yolk cell (Kimmel et al., 1995; Bruce and Heisenberg, 2020).

In addition to the zebrafish being suitable for the assessment of embryonic development, several mammalian and zebrafish behaviours are similar, suggesting evolutionary conservation of behaviours across species (Basnet et al., 2019). Chronic oral administration of rotenone in mice was shown to cause behavioural impairments (Doyle and Croll, 2022). One of the ways to assess behaviours in zebrafish larvae is by the thigmotaxis test, an index of anxiety-like behaviour, in which the zebrafish avoids the centre of an area and moves to the edge (Basnet et al., 2019). Visual dysfunction is another non-motor symptom of PD that negatively affects patients' quality of life, which has recently been described (Weil et al., 2016). The zebrafish model is capable of evaluating visual acuity by measuring the optomotor response (OMR). The OMR test is used to assess zebrafish visual and motor responses using an animation of a series of black and white lines that simulate movement, stimulating the larvae to follow the movement direction and align with the animation (Brastrom et al., 2019).

The development and behaviour of zebrafish are useful parameters to analyse drugs capable of protecting or mitigating the emergence of PD-like symptoms. In this study, adequate rotenone concentrations to induce PD-like symptoms in zebrafish embryos were tested and epibolic, morphological, behavioural, visual, and mortality effects were measured. We found that the zebrafish was a useful model to evaluate non-motor symptoms of PD.

2. MATERIALS AND METHODS

2.1 Zebrafish Maintenance

The experiments were carried out at the *Laboratório de Ecofisiologia e Comportamento Animal* – LECA, *Universidade Federal Rural de Pernambuco* - UFRPE. The protocols were previously approved by the ethics committee for the use of animals at the same University, with protocol number 3581030221. Adult zebrafish (*Danio rerio*, WT, nine months) were raised and maintained at 26.5 ± 1 ºC under a 14/10h light/dark cycle and fed twice per day with extruded commercial fish feed (40% crude protein) and brine shrimp (Westerfield, 2000; OECD 236, 2013). Adult' male/female zebrafish with a 4:2 ratio (Westerfield, 2000) were used for crossing in spawning aquariums developed in the laboratory (Patent BR 20 2016 017042 2). The eggs were collected 30 minutes after natural spawning, washed with distilled water in Petri plates, and unfertilized eggs and embryos showing irregular cleavage, injuries, or other malformations were discarded (Westerfield, 2000; Silva et al., 2019). Only embryos showing normal development of the blastula during the cleavage period (Westerfield, 2000) were used for experiments.

2.2 Rotenone Preparation and Exposure

Rotenone (lot # MKBZ2534V, CAS Number: 83-79-4, ≥ 95% purity) was purchased from Sigma (St. Louis, MO, USA), and all other reagents were of analytical grade. The rotenone stock solution was made fresh before the experiments and prepared by dissolving in 0.1% (v/v) dimethyl sulfoxide (lot # 85713, CAS Number: 67-68-5, ≥ 99.9% purity, Dinâmica, Brazil) and then diluted with the distilled water to obtain a final nominal concentration of 0.02 mg/mL. The stock solution was stored in Eppendorf tubes (2 mL) and maintained under refrigeration at 10 °C to be used on the following exposure days. Storing rotenone at a temperature of 9 ~ 10 ºC in a refrigerator prevents its rapid degradation (Summera and Connato, 2006).

The healthy embryos at 2 hpf (hours postfertilization) were exposed to 5, 10, 15, and 20 µg/L of rotenone, named ROT5, ROT10, ROT15, and ROT20 groups, respectively, and the control treatment with only 0.1% (v/v) dimethyl sulfoxide (DMSO group), that does not affect the studied parameters (Christou et al., 2020). The groups of each treatment had six replicates (15 embryos per replicate). Then the embryos were randomly incubated in sterile polystyrene test chambers with a capacity of 80 mL and kept at an incubator developed in the laboratory (Patent BR 10 2018 0150 4) with a controlled temperature of 26 ± 1 ºC and pH of 7.5 ± 0.5 (OECD 236, 2013; Silva et al., 2019). The polystyrene test chambers containing embryos and larvae were cleaned every day to remove dead embryos and chorions, with the replacement of rotenone concentrations.

2.3 Measurement of Epiboly Rate

To assess the epiboly rate, zebrafish embryos (n = 141) with 8 hpf (after 6 h of experimental treatment) were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH 7.1 for 24 h (Marrs et al., 2010; Cadena et al., 2020b). Subsequently, the fixed embryos were washed with PBS three times. Images were captured using a microscope (Bel model Solaris-T) with an attached camera (Bio-HDMI Model) for digital microscopy. The images were measured using ImageJ software (version 1.53e, 2021, National Institutes of Health, MD). To calculate the percentage of epiboly, the distance from the animal pole to the germ ring was measured (embryonic length), divided by the distance from the animal pole of the embryo to the vegetal pole (total length), and then multiplied by 100 (Fig. 1) (Cadena et al., 2020b; Sales-Cadena et al., 2021).

2.4 Analysis of Embryonic Development and Teratogenic Effects

Embryos and larvae (n = 450) were evaluated for developmental parameters daily between 24-96 hpf with an optical microscope with an attached camera to capture embryo and larva images. Mortality was recorded daily using the assessment of embryonic lethality protocol following the proposal by OECD 236 (2013). The half-maximal lethal concentration (LC50) and the half-maximal effect concentration (EC50) were

calculated from the data collected on mortality and teratogenic effects. Teratogenic effect endpoints were analyzed in vivo, including pericardial edema (PE), yolk sac edema (YSE), coagulation (CO), spinal deformation (SD), tail deformation (TD), absence of pigmentation (AP), absence of swim bladder (ASB), absence of somites (AS), and developmental delay (DD) (Fig. 2a-e) (OECD, 2013; Silva et al., 2019; Cadena et al., 2020a; Cadena et al., 2020b). The animal was considered affected if any of the teratogenic effects described above or mortality were observed. All these parameters were evaluated qualitatively, and the results were expressed as a percentage of affected animals (with teratogenic effects + dead animals).

2.5 Larvae Morphometry

Larvae morphometry (n = 374) at 144 hpf was performed after euthanasia using 1200 µg/L eugenol immersion (Strykowski and Schech, 2015, modified). The animals were euthanised after behaviour tests were completed. Larvae were fixed 4% PFA in PBS pH 7.1 overnight, and then, the larvae were washed 3 times with PBS. Fish were imaged in the dorsal view of the head (Fig. 2f) and lateral view of the body (Fig. 2g). The following parameters were measured on the dorsal view (Sales-Cadena et al., 2021): Head Length (HL); Eye Diameter (ED); and Head Width (HW) (Fig. 2f). In the lateral view, only the Body Length (BL) was measured (Fig. 2g). The measurements were made with ImageJ 1.53e software.

2.6 Evaluation of Behavioural Parameters

For the evaluation of behavioural parameters, larvae at 144 hpf (n = 374) from all treatment groups were transferred from the respective polystyrene test chambers to 48-well plates, and only one larva was placed in each well (Fig. 3). Larvae with teratogenic effects that could affect movement, for example, spine and tail deformation, or dead larvae, were not used in behavioural tests. The first behavioural test performed was the Thigmotaxis (TH test). This test evaluates anxiety-like behaviours in zebrafish by observing the percentage of larvae that swim close to the wall of the wells (Schnörr et al. 2012; Cadena et al., 2020a). After conditioning the animals from all treatments, the plates were placed on a white surface in a light environment. After 10 minutes of acclimatisation, the scan sampling observation method (Silva et al., 2019) was performed to obtain a dichotomous response from each animal: yes (for animals close to the wall) or no (for animals away from the wall) (Fig. 3a).

The second behavioural test performed was the Touch Sensitivity test (TS test) to evaluate the larval response to mechanical stimulation (Cadena et al., 2020a). This test was performed as soon as the TH test of the 48-well plate was done. Using the scan sampling method (Silva et al., 2019), the larvae from each well were touched on the tail. A dichotomous response was evaluated after touch: yes (for the larvae that exhibited escape behaviour) or no (for animals that did not exhibit escape behaviour) (Fig. 3b).

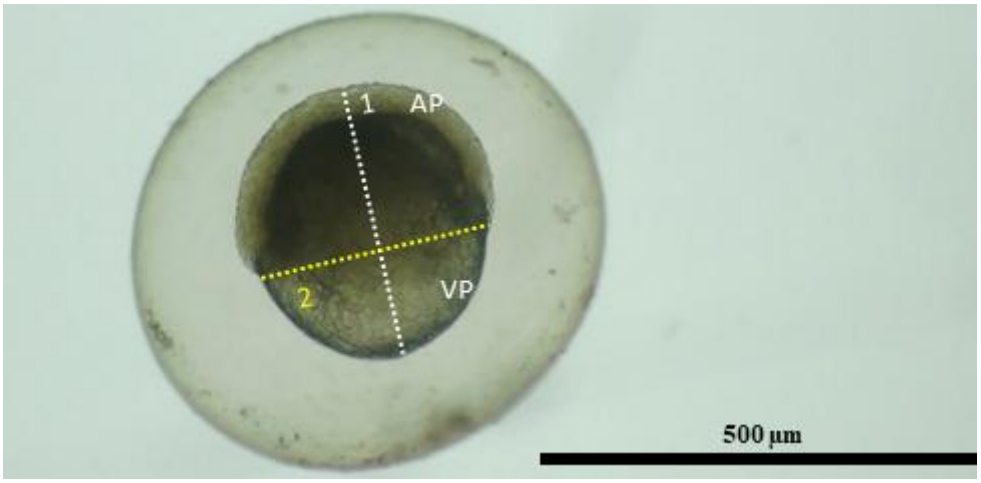

Fig. 1. Measurement of epiboly in zebrafish embryos
(1) White dotted line (total length): from Animal Pole (AP) to Vegetative Pole (VP); (2) Yellow dotted line (embryo length): from Animal Pole (AP) to germ ring

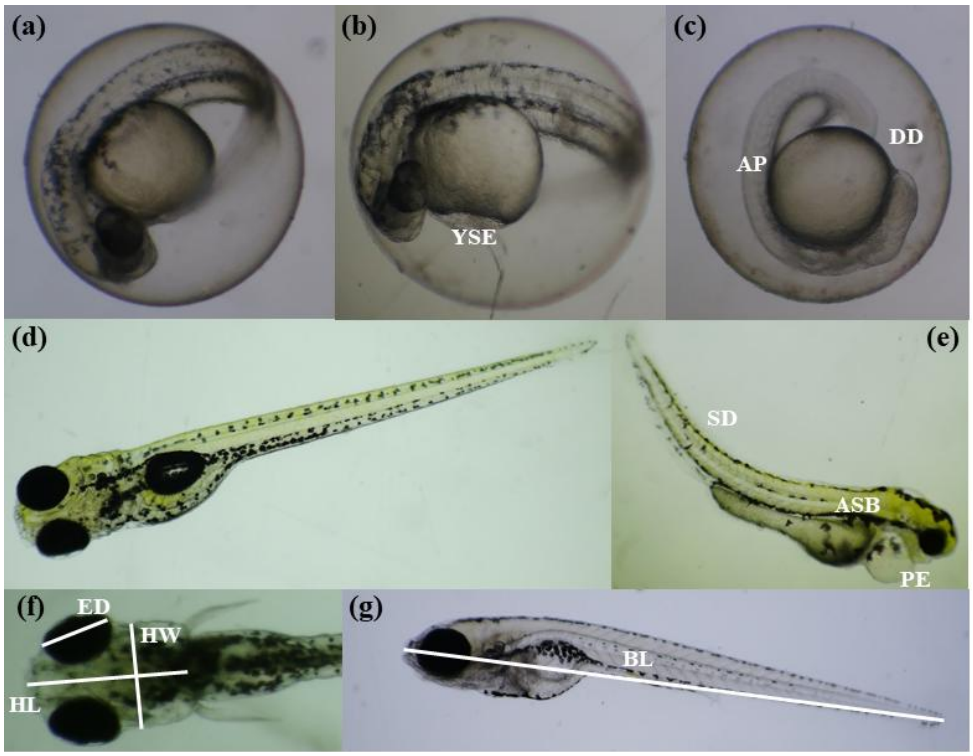

Fig. 2. Zebrafish embryos and larvae exposed to rotenone, main teratogenic effects (a-e) and morphometric measurements (f and g)

(a) Zebrafish embryo of DMSO group at 24 hpf; (b) Zebrafish embryo exposed to 10 µg/L rotenone at 24 hpf; (c) Zebrafish embryo exposed to 20 µg/L rotenone at 24 hpf; (d) Zebrafish larvae of DMSO group at 96 hpf; (e) Zebrafish larvae exposed to 20 µg/L rotenone at 96 hpf. (f) Morphometric measurements of the head in dorsal view; (g) Morphometric measurement of the body in lateral view. Abbreviations indicate main teratogenic effects as AP – Absence of Pigmentation; AS – Absence of Somites; ASB – Absence of Swim Bladder; DD – Developmental Delay; CO – Coagulation; PE – Pericardial Edema; SD – Spinal Deformation; TD – Tail Deformation; YSE – Yolk Sac Edema. Abbreviations for morphometric measurements are BL – Body Length; ED – Eye Diameter; HL – Head Length; HW – Head Width

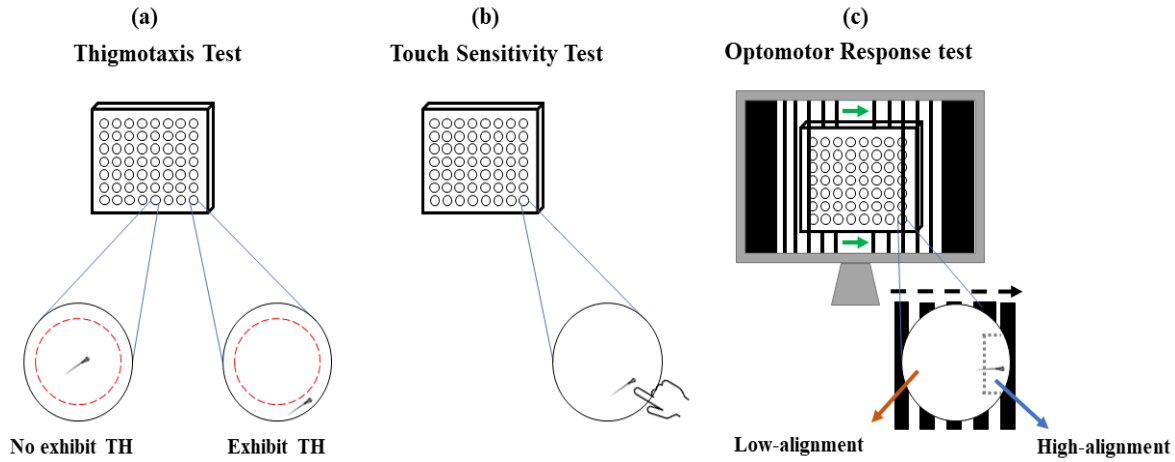

Fig. 3. Explanatory scheme of behavioural tests used to assess non-motor PD-like symptoms in zebrafish larvae exposed to rotenone at 144 hpf

(a) Method of collecting the behavioural response in the Thigmotaxis test, detailing which response zebrafish larvae may exhibit; (b) Method of collecting the behavioural response in the Touch Sensitivity test; (c) Method of collecting the Optomotor Response of zebrafish larvae

The Optomotor Response test (OMR test) was performed after completing the other two behavioural tests and consisted of obtaining data to assess whether the larva had its visual acuity affected by rotenone (Brastrom et al. 2019; Cadena et al., 2020a). The 48-well plates were placed on a horizontally oriented computer monitor on which a 40-second OMR animation was played and filmed by a smartphone camera with a top view (≈ 20 cm) of the 48-well plate. The animation consisted of an initial 5 s of solid white to note the initial larval position (IP), 30 s of alternating black and white bars from right to left to get a response, and 5 s of solid white to note the final larval position (MP). According to the position of the larva in the well and the moment of the video, the responses obtained were dichotomous: Larva in low-alignment, when it was in a region up to 70% away from the direction of the visual stimulus; Larva in high-alignment, when it was in a region up to 30% close to direction of the visual stimulus (Fig. 3c).

2.7 Statistical Analysis

The data collected in the experiments were presented as mean ± standard deviation (SD). Data on teratogenic effects, morphometry, TH, and TS tests were analysed by one-way ANOVA using Origin Pro Academic 2015 (Origin Lab. Northampton, MA USA). When the difference was significant, the means were compared using the Tukey test with $P < .05$. The OMR test results were analyzed by the McNemar symmetry test with $P < .05$. The effective concentrations (half-maximal effective concentration - EC_{50}) were calculated using the nonlinear allosteric decay function using a spreadsheet built in Microsoft Excel.

3. RESULTS AND DISCUSSION

The classic symptoms of Parkinson's Disease, which are related to motor disorders, are not the only ones to negatively affect the quality of life in patients. Several non-motor symptoms are reported as the first symptoms in PD patients, including behavioural changes such as anxiety and depression (Simon et al., 2020). Another feature of PD is that the treatments only address symptoms, and no treatment or medication to date can stop or delay the neurodegeneration process in PD (Doyle and Croll, 2022). Thus, it is necessary to carry out studies with animal models that can reproduce PD-like symptoms to help identify new treatments and drugs. Recent studies found that exposing zebrafish embryos to rotenone can induce PD-like motor symptoms (Kalyn et al., 2020). We examined whether rotenone induced behavioural and developmental changes in zebrafish and found delayed epiboly, morphometric changes, teratogenic effects, and behavioural changes. These parameters are reminiscent of PD-like symptoms. These endpoints could be used in drug screening experiments to identify candidate PD treatments.

3.1 Epiboly Rate

Exposure to different concentrations of rotenone in zebrafish embryos during the period from cleavage to mid-gastrulation (Kimmel et al., 1995) caused delays in embryonic development (Fig. 4). The DMSO group showed that the extent of yolk the cell covered by the growing embryo was not affected (Marrs et al., 2010; Cadena et al., 2020b). In the ROT5 group, the progress of epiboly at 8 hpf compared to the DMSO group was not affected, indicating low embryo toxicity of rotenone at this concentration. The epiboly rate of the ROT10, ROT15, and ROT20 groups was significantly reduced, but there were no differences in the progress of epiboly between these groups (Fig. 4). The toxicity of rotenone in the early stages of embryonic development, observed by the evaluation of epiboly rate, may be indicative of later teratogenic effects and other parameters.

Epiboly is the first morphogenetic event during zebrafish development that is essential for normal development and coupled with gastrulation, which occurs from the late blastula (4.3 hpf) to the bud stage (10 hpf) (Bruce and Heisenberg, 2020). Zebrafish development at this stage of life is highly sensitive to chemical exposure. The chorion acts as a protective barrier to limit chemical exposure prior to hatching (Mandrell et al. 2012). However, we observed that exposure to rotenone at 10 to 20 µg/L from 2-8 hpf caused embryotoxicity, as reduced epiboly rate. In the literature, no evaluations of epiboly rate were found in zebrafish exposed to chemicals that have already been used for PD-like models. This may be related to the fact that the initial exposure time for PD-like induction in zebrafish normally occurs at 24 hpf (Kalyn et al. 2020). In order to evaluate the susceptibility of zebrafish embryos to rotenone, the epiboly rate proved to be adequate to confirm the influence of early stages of development. In addition, the epiboly rate can be a rapidly obtained endpoint used in the evaluation of new protective or treatment in the zebrafish PD-like model.

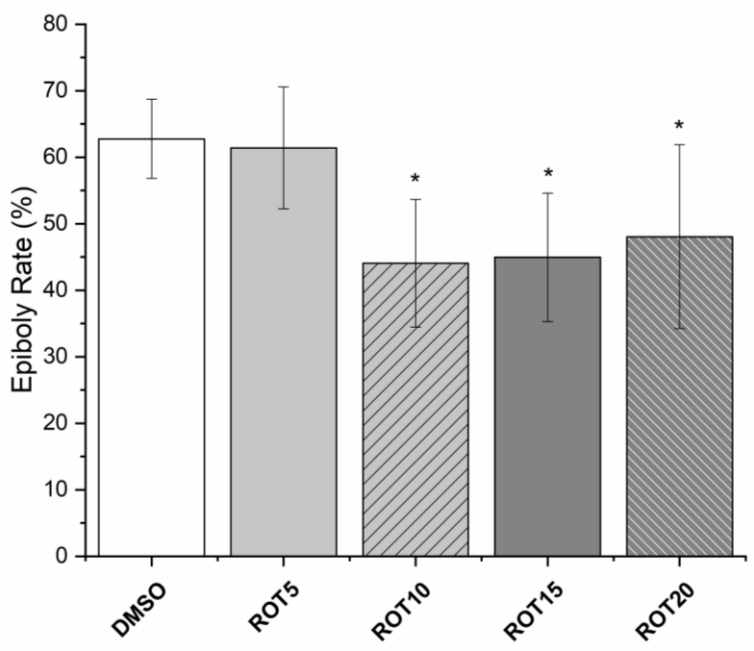

Fig. 4. Epiboly rate of zebrafish embryos exposed to rotenone between 2 to 8 hpf

*Epiboly rate was considered affected when * = P < .05. Each rotenone group was compared with the DMSO group by one-way ANOVA (F(4,141) = 23.904 P < .05) followed by Tukey's test (against DMSO group ROT5 P = .98, ROT10 P < .001, ROT15 P < .001, ROT20 P < .001). Legend of experimental groups: DMSO – 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively*

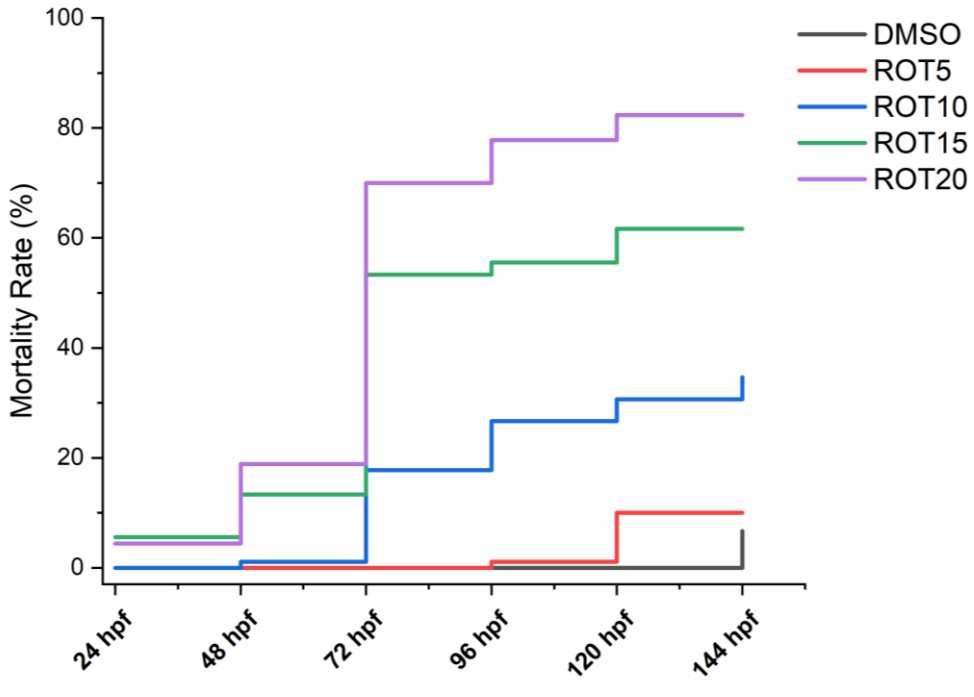

Fig. 5. The mortality rate of zebrafish embryos exposed to different concentrations of rotenone. Zebrafish embryos were exposed to rotenone at 2 hpf, and the mortality was recorded at 24 to 144 hpf after exposure

Experimental groups included: DMSO – 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively

3.2 Analysis of Embryonic Development and Teratogenic Effects

During the entire period between 24-144 hpf, little or no teratogenic effects and mortality equal to or below 10% (OECD 236, 2013) were observed in the individuals of the DMSO group (Figs. 5 and 6). The only group exposed to rotenone that did not exceed a mortality rate of 10% was ROT5, indicating low toxicity of rotenone at this concentration (Fig. 5), as observed for epiboly. Between 48-72 hpf, which coincides with the hatching period of the larvae (Kimmel et al., 1995), the mortality rate of the ROT10, ROT15, and ROT20 groups increased significantly, indicating that the chorion acted as a protective barrier against exposure to rotenone. At the end of 144 hpf, the ROT15 and ROT20 groups had a mortality rate above 50%.

Teratogenic effects were analysed to measure developmental effects on surviving animals exposed to rotenone. During the periods of 24-48 hpf (Fig. 6a-b), the most common effects were pericardial edema, absence of pigmentation, and developmental delay at 24 hpf in the groups ROT10, ROT15, and ROT20. Absence of pigmentation was observed at 24 hpf, but this effect was no longer observed in these groups at 48 hpf. From 72-96 hpf (Fig. 6c-d), there was additional mortality in the ROT10, ROT15, and ROT20 groups. Even with increased mortality, teratogenic effects were still observed; the most prevalent teratogenic effects in these last two periods were developmental delay, pericardial edema, and yolk sac edema observed in the ROT10, ROT15, and ROT20 groups with a consistent incidence proportional to the number of animals. Based on the observed data, the EC50 found for zebrafish embryos at 96 hpf was 11.24 µg/L, and the LC50 at 96 hpf was 13.88 µg/L.

The exposure of zebrafish embryos to rotenone caused teratogenic defects and mortality, affecting ROT10, ROT15, and ROT20 groups, which were the same groups that showed delayed epiboly. Similar results were found by Melo et al. (2015), showing a lack of pigmentation and developmental delay in embryos at 24 hpf that were exposed to 10, 20, 40, and 80 µg/L rotenone beginning after fertilisation. At 48 hpf, the ROT10 group did not have a significantly higher rate of affected animals than the DMSO group, and the pigmentation defect was not observed at 48 hpf and subsequent observation periods, indicating that pigmentation was delayed, not blocked. However, our results are different from those observed by Melo et al. (2015), particularly the appearance of pericardial edema after 72 hpf in the three groups exposed to the highest concentration of rotenone. In 24 and 48 hpf, the increasing mortality produced higher proportions of affected embryos with increasing rotenone concentrations. Yolk sac edema was first observed at 72 and 96 hpf, and the developmental delay and pericardial edema effects persisted at these stages. Kalyn et al. (2020) exposed zebrafish to 50 nM rotenone starting at 72 hpf and observed cardiac edema. The developmental mechanism of rotenone's effects remains unknown.

Fish in embryonic and larval stages are known to be sensitive to xenobiotics (Çalışkan and Emekli-Alturfan, 2021), as observed in our epiboly and affected animal analysis. However, the 5 µg/L concentration of rotenone was not able to cause other adverse effects, besides morphometry, in the zebrafish embryos tested. Bretaud et al. (2004) exposed zebrafish 24 hpf embryos to 5 and 10 µg/L rotenone, and no teratogenic effects were observed at either concentration. In contrast to our results, 10 µg/L rotenone exposure induced teratogenic effects, but 5 µg/L exposure had no observed effects.

Adult zebrafish exposed to 2 to 5 µg/L rotenone for 28 days were able to induce locomotor changes and showed non-motor PD-like symptoms (Khotimah et al., 2015; Wang et al., 2017). The sensitivity of adult zebrafish to rotenone may be due to the long exposure duration and greater absorption by the gills, intestine, and skin. Due to its lipophilic structure, rotenone easily crosses the blood-brain barrier, enters the central nervous system and accumulates in mitochondria (Ünal et al., 2020). The rotenone doses causing effects are quite narrow, something that was also observed in our study (Melo et al. 2015). Therefore, we measured EC50 in order to find an ideal concentration to induce PD-like symptoms in zebrafish embryos without causing high mortality rates, but causing several observable and measurable effects on development, behaviour, and locomotor activity. In our study, an EC50 of 11.24 µg/L was observed for zebrafish embryos exposed to rotenone, like the EC50 of 12.2 µg/L measured by Melo et al. (2015).

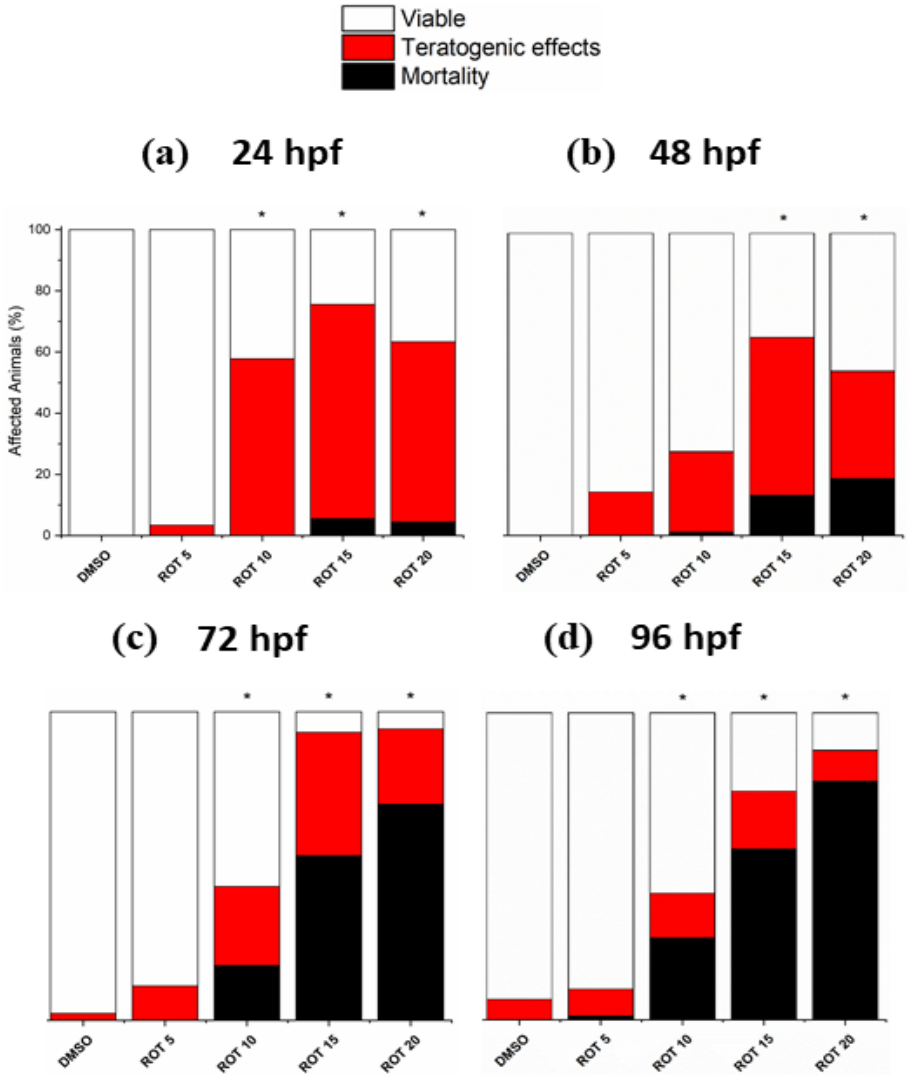

Fig. 6. Per cent of affected animals between 24 to 96 hpf after rotenone embryonic exposure. Significant increases in the percentage of affected animals were considered when * = *P* < .05

For 24 hpf experimental group was compared with the DMSO group by one-way ANOVA (F(4,29) = 7.867 P < .05) followed by Tukey's test (against DMSO group ROT5 P = .1, ROT10 P = .026, ROT15 P = .002, ROT20 P = .012). For 48 hpf one-way ANOVA (F(4,29) = 5.378 P < .05) followed by Tukey's test (against DMSO group ROT5 P = .905, ROT10 P = .47, ROT15 P = .004, ROT20 P = .023). For 72 hpf one-way ANOVA (F(4,29) = 84.226 P < .05) followed by Tukey's test (against DMSO group ROT5 P = .68, ROT10 P < .001, ROT15 P < .001, ROT20 P < .001). For 96 hpf one-way ANOVA (F(4,29) = 32.095 P < .05) followed by Tukey's test (against DMSO group ROT5 P = .99, ROT10 P = .007, ROT15 P < .001, ROT20 P < .001). Legend: DMSO – 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively.

3.3 Morphometric Analysis

Morphometric changes in the head length, head width, and body length were observed in zebrafish larvae at 144 hpf (Tukey test p > 0.05) in all groups exposed to rotenone (Table 1). The measurement of the eye diameter did not show significant differences (*P* > .05) between the groups exposed to rotenone and the DMSO group. The only parameters changed in zebrafish exposed to the lowest concentration of rotenone (ROT5) among all the analyses performed were the significant reduction in head length, head width, and body length. The morphometric changes observed in the ROT5 group indicate that even at low concentrations, rotenone was able to disrupt the development of the animals tested, although no significant changes were observed in the other parameters evaluated in the ROT5 group.

Table 1. Morphometric measurements of zebrafish larvae exposed to rotenone at 144 hpf

	Head Length (mm)	Head Width (mm)	Eye Diameter (mm)	Body Length(mm)
DMSO	0.803 ± 0.050	0.546 ± 0.041	0.358 ± 0.022	3.704 ± 0.203
ROT5	0.755 ± 0.060*	0.514 ± 0.041*	0.361 ± 0.159	3.560 ± 0.101*
ROT10	0.770 ± 0.050*	0.528 ± 0.033*	0.342 ± 0.021	3.631 ± 0.181*
ROT15	0.775 ± 0.030*	0.526 ± 0.029*	0.343 ± 0.016	3.538 ± 0.147*
ROT20	0.764 ± 0.029*	0.509 ± 0.025*	0.349 ± 0.014	3.525 ± 0.128*

*Data are expressed as the mean ± SD. * Significant difference (P < .05) from the DMSO group. For head length each experimental group was compared with the DMSO group by one-way ANOVA (F(4,374) = 13.772 P < .05) followed by Tukey's test (ROT5 P = 0, ROT10 P < .001, ROT15 P = ,018, ROT20 P < 0.001). For head width each experimental group was compared with the DMSO group by one-way ANOVA (F(4,374) = 12.638 P < .05) followed by Tukey's test (ROT5 P < .001, ROT10 P = .013, ROT15 P = ,04, ROT20 P < .001). For eye diameter each experimental group was compared with the DMSO group by one-way ANOVA (F(4,374) = 0.881 P < 0.05) followed by Tukey's test (ROT5 P = 0.1, ROT10 P = .63, ROT15 P = ,88, ROT20 P = .92). For body length each experimental group was compared with the DMSO group by one-way ANOVA (F(4,374) = 17.174 P < .05) followed by Tukey's test (ROT5 P < .001, ROT10 P = .03, ROT15 P < ,001, ROT20 P < .001). Legend for exposed groups: DMSO - 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively*

Changes in TH and TS behaviours due to rotenone exposure were observed in larval zebrafish at 144 hpf. In the TH test, the larvae move close to the wall in the wells, as expected in healthy larvae, in the DMSO group, with less than 10% of the animals away from the wall of the well during the tests (Fig. 6a). In contrast, rotenone caused significant behavioural changes (Fig. 6a) in the groups with the two highest concentrations of rotenone (ROT15 and ROT20). This result may indicate that rotenone can alter neuronal activity, causing changes in anxiety-like behaviour. In the TS test, which examines the ability to respond to touch and the display of escape behaviour, only larvae of the ROT20 group showed significant reductions in the escape response (Fig. 6b). The OMR visual stimuli test showed no change in this parameter (Fig. 6c).

3.4 Evaluation of Behavioural Parameters

Changes in TH and TS behaviours due to rotenone exposure were observed in larval zebrafish at 144 hpf. In the TH test, the larvae move close to the wall in the wells, as expected in healthy larvae, in the DMSO group, with less than 10% of the animals away from the wall of the well during the tests (Fig. 7a). In contrast, rotenone caused significant behavioural changes (Fig. 7a) in the groups with the two highest concentrations of rotenone (ROT15 and ROT20). This result may indicate that rotenone can alter neuronal activity, causing changes in anxiety-like behaviour.

In the TS test, which examines the ability to respond to touch and the display of escape behaviour, only larvae of the ROT20 group showed significant reductions in the escape response (Fig. 7b). The OMR visual stimuli test showed no change in this parameter (Fig. 7c).

Behaviours like thigmotaxis are evolutionarily conserved by a wide variety of species, including rodents, fish, and humans (Basnet et al., 2019), which can be used to assess anxiety-like conditions. Thigmotaxis is one of the most commonly used behavioural parameters in preclinical studies employing rodent models, in which animals are placed in a novel environment and, by default, strongly avoid the centre of that environment while trying to move close to the edges (Schnörr et al. 2012). In our thigmotaxis tests, larvae were placed in an unfamiliar environment. Fish showing apparent teratogenic defects or impaired swimming is not evaluated. Thigmotaxis were reduced by the two highest concentrations of rotenone (15 and 20 µg/L). Previous studies using zebrafish PD-like models did not analyse anxiety-like behaviours, a non-motor symptom of PD. Wang et al. (2017) evaluated anxiety-like behaviours in the PD-like model of adult zebrafish and found that behavioural symptoms were associated with decreased levels of dopamine.

Only 20 µg/L of rotenone reduced the response of larvae to physical stimuli and subsequent escape behaviour (14.55% compared to the DMSO control). The reduced sensitivity may be due to the neurotoxic action of rotenone and may indicate a mild locomotion defect (Kalyn et al., 2020). TH and TS behavioural defects were produced in larvae exposed to the highest concentrations of rotenone, even in animals that did not have apparent teratogenic effects.

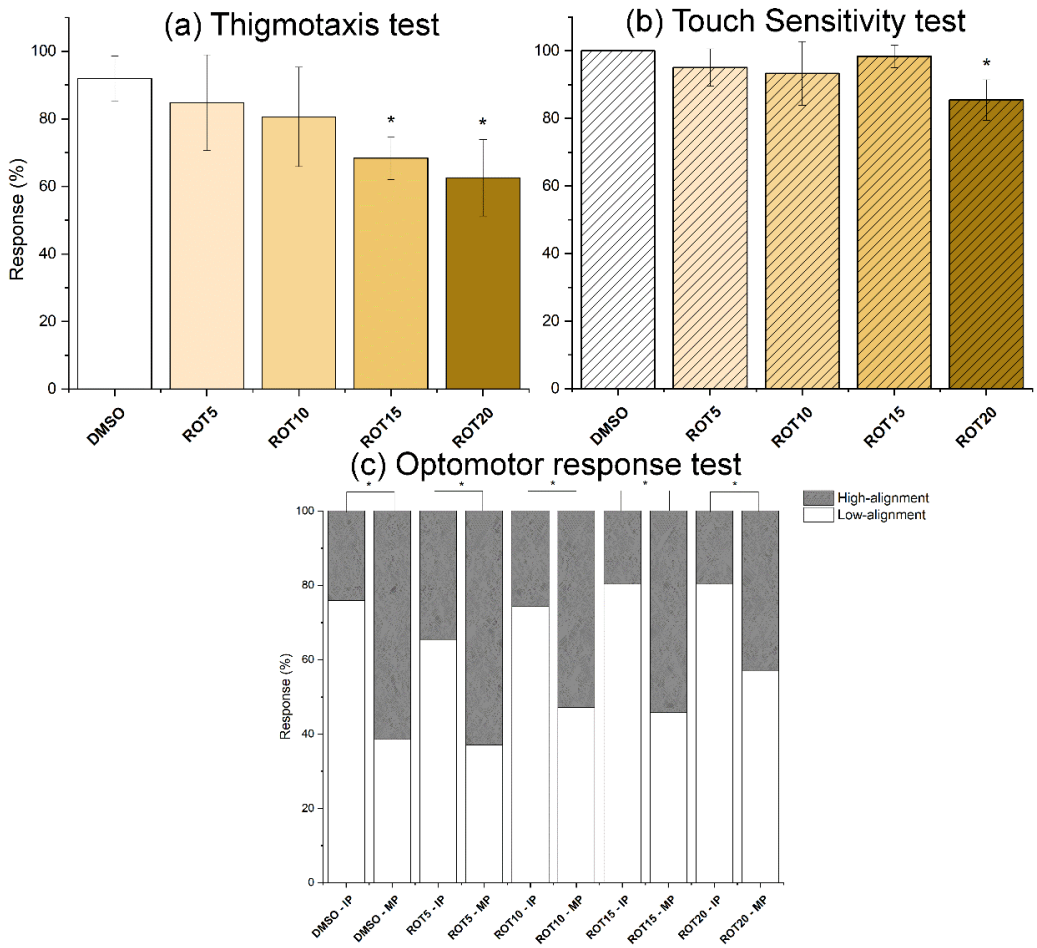

Fig. 7. Zebrafish larvae exposed to high rotenone concentrations showed reduced thigmotaxis and touch sensitivity response

*(a) The thigmotaxis test evaluates anxiety-like behaviours in zebrafish larvae. The percentage response from each group was compared with the DMSO group by one-way ANOVA (F(4,34) = 6.165 P < .05) followed by Tukey's test (ROT5 P = .67, ROT10 P = .26, ROT15 P = .013, ROT20 P = .001); (b) Touch Sensitivity test evaluate the larval response to mechanical stimulation. The percentage response from each group was compared with the DMSO group by one-way ANOVA (F(4,12) = 4.366 P < .05) followed by Tukey's test (ROT5 P = .63, ROT10 P = .41, ROT15 P = .99, ROT20 P = .009). (c) The optomotor response of the zebrafish as a percentage of the total and compared using the McNemar test of symmetry on the same group between the initial compared with middle positions. * = P < 0.05. (Initial position against middle position: DMSO P < .001; ROT5 P < .001; ROT10 P < .001; ROT15 P < .001; ROT20 P < .001. Initial position against final position: DMSO P < .001; ROT5 P < .001; ROT10 P < .001; ROT15 P < .001; ROT20 P < .001). Legend: DMSO – 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively.*

Table 2. Summary of observed effects caused by different concentrations of rotenone on the parameters evaluated in zebrafish PD-like model

	Development	Affected Animals		Behavioral Tests			Morphometric Measurements		
	Epiboly Rate	Teratogenic	Mortality	TH	TS	OMR	Head	Eye	Body
DMSO	NO	NO	NO	NO	NO	NO	NO	NO	NO
ROT5	NO	NO	NO	NO	NO	NO	+	NO	+
ROT10	+	+	NO	NO	NO	NO	+	NO	+
ROT15	+	+	+	+	NO	NO	+	NO	+
ROT20	+	+	+	+	+	NO	+	NO	+

+ = positive; NO = Not observable. Legend for exposed groups: DMSO - 0.1% (v/v) Dimethyl sulfoxide; ROT5, ROT10, ROT15, and ROT20 – 5, 10, 15 and 20 µg/L of rotenone, respectively

The OMR test evaluates visual and motor activities in zebrafish larvae. This assessment is useful since many patients with PD have sensory dysfunctions, such as changes in visual perception (Weil et al., 2016). Rotenone causes retinal degeneration in rats (Sasaoka et al., 2020). Zebrafish larvae exposed to 6-OHDA had optomotor damage at 7 dpf (Bevenutti et al., 2018), producing locomotor defects and sensory impairment. In our study, all groups exposed to rotenone responded normally to the visual stimulus, indicating that the rotenone concentrations tested were not sufficient to affect animals in this assay, even in the groups that demonstrated alterations in the TH and TS tests.

The rotenone effects were concentration-dependent. Concentrations between 10 to 15 µg/L are recommended (Table 2), in which mortality effects are not too severe, but there are significant measurable defects. These rotenone concentrations will be suitable for evaluating drug treatments for symptoms in the zebrafish PD-like model.

The use of zebrafish exposed to rotenone as a PD-like model has advantages and disadvantages at different life stages. The adult zebrafish are more sensitive to rotenone (Ünal et al., 2020), inducing PD-like symptoms with lower concentrations and producing motor and non-motor effects with low mortality (Khotimah et al., 2015; Wang et al., 2017; Andrade et al., 2022). Adult zebrafish have a wider variety of behavioural and locomotor symptoms than larvae (Wang et al., 2017). However, adult zebrafish require prolonged rotenone exposure (28 days) for PD-like induction, and it is more costly and difficult to test similar numbers of adult animals, as compared to larvae. Thus, the early stages are useful for drug screening, and the adult stage is useful to evaluate the therapeutic effects of drugs.

4. CONCLUSION

The use of zebrafish at early life stages exposed to rotenone is a powerful PD-like animal model. Rotenone induced toxic effects in embryos during development, producing abnormal morphometric parameters and anxiety-like behaviours that mimic non-motor PD symptoms. Optimal rotenone concentrations of 10-15 µg/L produced developmental and behavioral changes with moderate mortality. This PD-like animal model will be useful for screening drugs and gaining insights into PD therapeutics.

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ETHICAL APPROVAL

All procedures involving animals were conducted in accordance with the ethical standards of the institution and national guidelines. The study protocol was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal Rural de Pernambuco (Protocol number 3581030221).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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