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Zebrafish and *Artemia salina in vivo* evaluation of the recreational 25C-NBOMe drug demonstrates its high toxicity

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ABSTRACT

The NBOMe (N-2-methoxybenzyl-phenethylamines) family of compounds are synthetic hallucinogens derived from the 2C series. Although this family of compounds has been responsible for multiple cases of acute toxicity and several deaths around the world, to date there are few studies. These compounds act as potent 5-HT_{2A} receptor agonists, including the hallucinogen 25C-NBOMe (2-(4-chloro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine). In this study, we first evaluated the toxicity of 25C-NBOMe in two animal models: *Artemia salina* and zebrafish using the lethality test of Meyer et al. (1982) modified for *Artemia salina* and the Fish Embryo Toxicity test (FET) for zebrafish (*Danio rerio*). Subsequently, we determined the behavioral and morphological effects using different concentrations of the 25C-NBOMe. As a result, we found that this substance is highly toxic according to lethality tests in both animal models. We also observe that this hallucinogen induces alterations in swimming and motility patterns in *Artemia salina*. Similarly, there were alterations in the motor response to a stimulus, as well as abnormal development in the zebrafish. The developmental effects of zebrafish suggest a teratogenic potential for 25C-NBOMe. Therefore, these findings are correlated with side effects, such as motor response abnormalities and muscle deterioration, clinically reported for consumers of this recreational drug. Finally, although recent studies are addressing the neurotoxicity and cardiotoxicity of 25C-NBOMe in cell cultures, to the best of our knowledge, this is the first *in vivo* report for 25C-NBOMe related to toxicological parameters and their global effects on development. Therefore, it could represent an advance in the study of the substance that contributes to the understanding of the effects on behavior and development in humans.

1. Introduction

Hallucinogens of the NBOMe family were initially synthesized for research purposes to study the 5-HT_{2A} receptor. However, their introduction to recreational use has made evident different negative and adverse effects including in some cases, death [1]. This family of hallucinogens consists mainly of 25C-NBOMe, 25I-NBOMe, and 25B-NBOMe. Since June 2012 several fatal and non-fatal intoxication cases caused by the NBOMe family have been reported [2]. By the year 2017 at least 10 deaths of this substance consumers were recorded. In Colombia, an informative alert was issued in 2013 about a new substance sold fraudulently as lysergic acid diethylamide (LSD) which was being commercialized in a paper stamp. Up to date, three deaths and significant growth in their consumption have been reported in the country [3]. Unlike LSD, NBOMe hallucinogens can cause death. Additionally, since they are distributed in unknown concentrations, they could be more harmful than LSD.

Some aspects of the toxic effect of 25B-NBOMe like survival rate and the muscle effects have been evaluated but no study has been made for any other derivatives [4]. Specifically, 25C-NBOMe is under

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investigation due to adverse reactions attributed to it, such as sublingual numbness, levitation perception, involuntary motor activity, psychedelic effects, rhabdomyolysis, cardiac function problems, and, in some cases, death. However, the underlying cellular mechanisms for these effects are unknown as well as the long-lasting side effects reported from consumers of this substance [2].

Pharmacologically, 25C-NBOMe acts as a potent 5-HT_{2A} receptor agonist, due to an N-benzyl substitution which increases its affinity. Computational modeling studies indicate that the benzyl ring is stabilized by aromatic stacking with the amino acid Phenylalanine-339 (Phe339) in the transmembrane domain 6 (TM6), increasing the affinity for the receptor [5]. Additionally, the presence of the methoxy group and chlorine (Cl) on the benzyl ring enhances the activity of the compound and increases lipophilicity respectively, allowing greater mobility in the Central Nervous System (CNS) [6].

The 5-HT_{2A} receptor (coupled to G proteins) performs functions in the CNS as a mediator in cognitive processes, memory, hormone secretion, and affective disorders. Thus, it is also associated with the primary effects of various hallucinogenic drugs [7]. The final action of the agonist is mediated by the activation of the G protein receptor signaling cascade which is conserved among vertebrates [8].

After the appearance of 25C-NBOMe as a drug of consumption, different studies have been developed in which the effect of this molecule has been determined at the cardiological and neurological level. Among these studies, it has been found that 25C-NBOMe affects the viability of cell lines used to determine neurotoxicity [9]. Similarly, it was determined that 25C-NBOMe has cardiotoxic effects in rats [10]. Thus, the use of different animal models, considered standard for toxicology like *Artemia salina* and zebrafish, is necessary for understanding the effects of 25C-NBOMe. *Artemia salina* is widely used for toxicological evaluations allowing to categorize in discrete toxicity levels any substance. On the other side, FET has been established as a standard for substance evaluation allowing to determined lethality and teratogenicity in a vertebrate embryo. Furthermore, FET could be used as an accessible and inexpensive biological test to evaluate the adverse effects of different chemicals on the nervous system [11].

Moreover, the use of zebrafish as a model in research has increased due to its high reproductive capacity, small size, external fertilization, rapid development, and short life cycle, which allows studying its embryonic development [11]. Also, zebrafish has been widely used as a model due to 70 % genome homology with genome human. Thus, it preserves neuroanatomical and neurochemical pathways in its brain suitable for psychoactive substances studies [12]. For example, the blood-brain barrier acquires functionality during embryonic development in both zebrafish and humans [13]. On the other hand, although the zebrafish heart is a two-chambered organ, it recapitulates the human heart and general cardiac function. At the same time, it leads to similar electrocardiographic patterns as humans. Due to this similarity between the two species, the zebrafish has been adopted as a model for neurological and cardiac physiology [14]. However, it is important to keep in mind specific differences between these species.

Therefore, our study was aimed to determine the Median Lethal Concentration (LC_{50}) of the hallucinogen 25C-NBOMe and to evaluate the possible effects caused by exposure to this substance using two *in vivo* animal models. This is the first research reporting LC_{50} values for one member of the NBOMe family. Finally, it represents an advance in the study of the substance 25C-NBOMe in *in vivo* models that contribute to the knowledge of the effects on behavior, morphology, and development. Thus, it can be used to extrapolate its effects on humans and generate public health alerts regarding the consumption of this substance, as well as provide protocols to medical personnel for proper management in cases of overdose.

2. Materials and methods

2.1. Chemical substances

25C-NBOMe HCl was purchased from LIPOMED (Lot 1564.1B1.1). Dimethyl sulfoxide (DMSO 99 % Alpha Aesar) was used as the solvent for the stock solutions of the hallucinogen.

2.2. Animals

The use of the animals was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Universidad de los Andes (C. FUA 17.004) and it complies with the ARRIVE Guidelines [15]. Parental fish housing was done according to the Guide for the Care and Use of Laboratory Animals [16]. Briefly, they had a light/dark cycle 14/10 h, oxygen supplement of 8 ppm, conductivity between 600 and 800 μS/cm, pH in a range of 7.3–7.6, temperature between 26 and 29 °C in a commercial water recirculation system Aquaneering Inc. Their diet consisted of *Artemia salina*, copepods, and Tetramin Tropical Flakes, twice a day. Procedures for obtaining embryos (POE_15-006), feeding (POE_15-007) and euthanasia (POE_15-004) have been approved by the CICUAL.

2.3. Animal models

2.3.1. Artemia salina

Artemia salina cysts obtained from Brine Shrimp Direct (USA) were subjected to decapsulation in a plastic container with 15 mL of a 5 % sodium hypochlorite solution (NaClO). Rinsing with tap water was carried out for eliminating the excess of NaClO. For the *Artemia salina* culture, 25 g of Instant Ocean Sea Salt® were weighed and dissolved in 1 L of Reverse Osmosis (RO) water. This solution together with the decapsulated cysts was transferred to a container with illumination, constant aeration, and temperature control employing a thermostat at 28.5 °C for its hatching [17,18]. For the toxicological tests, individuals were used between 24–36 h post-eclosion (hpe).

2.3.2. Zebrafish

Pairs of wild-line adult zebrafish (AB, TAB-5, and TL) were mated, and their embryos were collected in egg water solution at 28.5 °C. Embryos were dechorionated by 10 h post-fertilization (hpf) using physical means (stainless steel forceps and needles) to be subsequently and homogeneously exposed to different concentrations of 25C-NBOMe.

2.4. Preparation of substance 25C-NBOMe

The substance was dissolved in a 15 % DMSO aqueous solution. The amount of solvent was considered in order to obtain substance concentrations of 0.75, 1, 1.5, 2, 3, 6, 9, 12, 15, 18, 24 and 30 μg/mL for the *Artemia salina* model and 0.75, 1, 1.5, 2, 3, 6, 9, 12, 15 and 18 μg/mL for the zebrafish model. For all solutions, the maximum final concentration of DMSO was less than 1 % v/v (0.028 – 0.86 %) in saline solution (*Artemia salina*) or egg water (zebrafish). It is important to note that we use egg water and DMSO as negative control and vehicle control, respectively. We do not use any other members of the NBOMe family in our experiments. To determine the 25C-NBOMe concentrations, we pretested a wide range of concentrations using different orders of magnitude. Based on this, we evaluate concentrations spaced by a constant factor no greater than 2 in *Artemia salina*, to obtain a more limited range of concentrations to evaluate in zebrafish and thus find the LC_{50} value.

2.5. Lethality test with animal models

2.5.1. Artemia salina

The lethality test with *Artemia salina* was done according to the method described by Meyer et al. (1982) with minor modifications [19].

Briefly, a total of 144 nauplii of 24 hpe were used per experiment. Six nauplii were put into each well in a 24-multiwell plate (a total of three wells for each concentration). For the tests, there were two control groups, one with saline solution (2.5 % w/v) and the other with 1 % DMSO vehicle control. Each solution was added to a final volume of 0.5 mL per well. Subsequently, the plate was incubated at 28.5 °C. For each concentration analyzed, five tests were carried out with three replicas. Monitoring was done every 24 h (24 and 48 h post-immersion -hpi-) to determine the number of live nauplii per well. The estimation of LC_{50} was determined for the *Artemia salina* at 48 hpi, using Probit analysis performed by the STATGRAPHICS Centurion XVI software. The values were determined with a confidence limit of 95 %.

2.5.2. Zebrafish

For the determination of the LC_{50} in zebrafish we used the Test Guidelines for the Chemicals - Fish Embryo Toxicity Test (FET) for zebrafish (*Danio rerio*) proposed by Organisation for Economic Co-operation and Development (OECD - July 26, 2013) and the previous study of Kawahara et al. [4,20].

Briefly, a total of 216 fish embryos of 10 hpf were used per experiment. Six fish embryos were put into one well (a total of three wells for each concentration) and were treated with 25C-NBOMe. All experiments were repeated three times, for a total of 54 embryos per concentration treatment. For the tests, there were two control groups, one with egg water and the other with 1% DMSO. Each solution was added to a final volume of 0.5 mL per well. Subsequently, the plate was incubated at 28.5 °C. Monitoring was done every 24 h (24, 48, 72, and 96 h post-immersion -hpi-) to determine the number of live fish embryos per well. Similarly, an embryo viability group ($n = 10$) in egg water was used. The estimation of LC_{50} was determined for zebrafish at 96 hpi, employing Probit analysis performed with STATGRAPHICS Centurion XVI software. The values were determined with a confidence limit of 95 %.

2.6. Behavioral analysis in Artemia salina

To evaluate the effect of hallucinogen 25C-NBOMe on the behavior of the animal model, the following qualitative parameters were considered: swimming pattern, reduction in swimming speed, movement of two antennas without displacement, and movement of one antenna without displacement. These parameters were monitored every 24 h for each concentration of 25C-NBOMe. For the behavioral analysis of the *Artemia salina* model, a descriptive analysis was carried out to determine the percentage of affected individuals in each category (swimming pattern, reduction in swimming speed, movement of two antennas without displacement, and movement of one antenna without displacement) for the different concentrations of 25C-NBOMe at 24 and 48 hpi.

2.7. Analysis of motor response and morphology of zebrafish embryos

Each zebrafish embryo exposed to 25C-NBOMe was monitored every 24 h for four days. To evaluate substance effects on motion pattern and general morphology, we use the following parameters: response to touch, pigmentation, growth and development, the formation of pericardial edema, changes in circulation and heart rate patterns, alterations in ocular development, body curvature, and yolk deformation. Response to touch was done using thick hair fiber on the caudal region of the embryo. An average of 10 larvae without morphological defects was tested per concentration of 25C-NBOMe. Photographic records of the embryos were obtained using a Leica MZ10 F Modular Stereomicroscope, with a magnification of 1.6 ×. Representative images were processed with Adobe Illustrator 2017® using background removal, adjustment of position, and multi-image assembly. These parameters were qualitative evaluations compared to not treated fish, which were observed through light stereomicroscopy.

2.8. Morphometric analysis

To determine the effect of 25C-NBOMe on the growth and development of previously exposed zebrafish embryos, the length of the trunk and the area of the pericardial edema were measured, obtaining average values. For the length of the trunk, nine larvae treated were randomly selected from each one of the previously described concentrations, measuring a total of 108 zebrafish larvae. This measurement was carried out from the first segment of the sarcomere (previous part) to the end of the last sarcomere segment. The pericardial area was calculated by circling the pericardial space. Both parameters were recorded at 72 hpi (82 hpf) using the ImageJ program (NCBI). The values were obtained in pixels and converted to millimeters (mm) for the trunk length and squares millimeters (mm^2) for the pericardial area.

2.9. Statistical analysis for zebrafish assays

The percentage of affected fish for each parameter was determined in the endpoint at 96 hpi (106 hpf) in each concentration. Morphological changes were determined using binary choice (presence/absent) based on Fraysse et al. (2006), using logistic regression [21]. To determine if there was any effect of 25C-NBOMe on trunk length and morphological parameters a Multivariate Analysis of Variance (MANOVA) was performed and significant differences between concentrations were determined using LSD tests for multiple comparisons. The statistical analyses were performed employing the STATGRAPHICS Centurion XVI program with a 95 % confidence limit.

3. Results

3.1. Determination of the LC50 in the Artemia salina model

By exposing the *Artemia salina* nauplii of 24 hpe at different concentrations of the hallucinogen 25C-NBOMe, an LC_{50} value of 20.14 μg/mL was determined at 48 hpi. We observed an increase in mortality with higher concentrations of 25C-NBOMe. At the highest concentration tested (30 μg/mL), a mortality rate of 82 % was observed ($p < 0.05$).

3.2. Determination of the LC50 in the zebrafish model

Zebrafish embryos dechorionated by 10 hpf were exposed to different concentrations of the 25C-NBOMe, resulted in an LC_{50} value of 10.76 μg/mL determined at 96 hpi. For the highest concentration tested (18 μg/mL), the maximum mortality of 67 % was observed in the animal model ($p < 0.05$).

3.3. Behavioral analysis in Artemia salina

Exposure to 25C-NBOMe induces swimming behavior anomalies. At the highest range of 25C-NBOMe concentrations (18−30 μg/mL), the most frequently observed swimming behavior at 48 hpi was two antennas without displacement, affecting between 50–100 % of the population.

It was observed that at 24 hpi in the lower concentrations up to 6 μg/mL of 25C-NBOMe characteristic swimming prevailed, comparable to DMSO 1% vehicle control. For the concentrations of 9–18 μg/mL, there was a reduction in the swim speed in more than 50 % of the treated *Artemia salina*. In concentrations of 24 and 30 μg/mL, the movement behavior of two antennas without displacement for 16 % of the biomodel was presented. At 48 hpi, the characteristic swimming was maintained until the concentration of 1.5 μg/mL of the hallucinogen. At concentrations of 2–6 μg/mL, the swim speed was reduced in 50 % of the population. In concentrations of 9–30 μg/mL, an increase in the movement behavior of two antennas without displacement was observed until reaching the total immobilization of the individuals evaluated (Fig. 1).

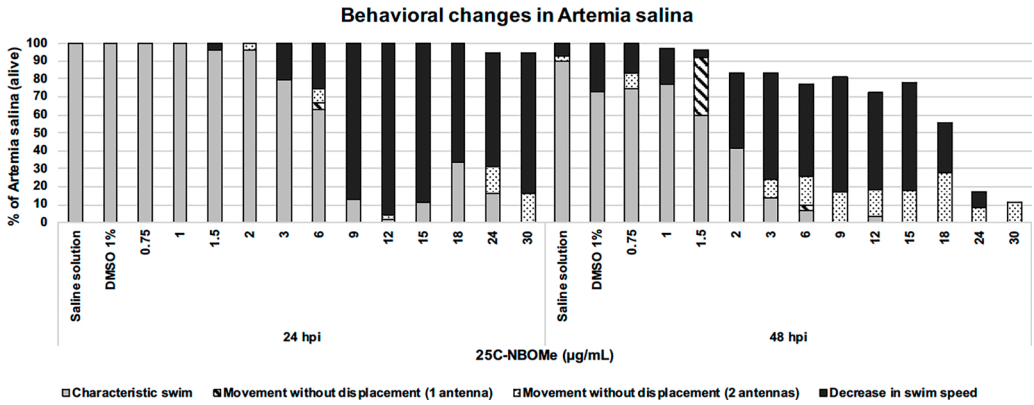

Fig. 1. The hallucinogen 25C-NBOMe affects the motor function of Artemia salina. Effects on the behavior of the Artemia salina as a function of 25C-NBOMe concentrations, after 24 and 48 hpi. The hallucinogen 25C-NBOMe effects on motor function are discriminated in 4 categories in grayscale representing the proportion of individuals displaying a particular effect. Exposure to 25C-NBOMe causes a decrease in swimming speed and, in some cases, the movement of antennas without displacement.

3.4. Analysis of motor response in zebrafish

Zebrafish embryos exposed to different concentrations of 25C-NBOMe show response to touch alterations. No response to touch was observed starting at 6 µg/mL being the predominant effect at 72 hpi ($p < 0.05$) (Fig. 2). The analysis of this effect showed that with increasing concentrations of 25C-NBOMe, there is a decrease in the motor response.

3.5. General effects 25C-NBOMe on zebrafish development

Developmental alterations were observed such as changes in pigmentation patterns, growth and development rates, heart rhythm and peripheral circulation, and ocular development. In addition to these alterations, we observed, the formation of pericardial and perivitelline edemas, deformation in the yolk and its elongation, and changes in the curvature of the body (Fig. 3).

3.6. Specific effects on the zebrafish growth and development rates

The effects on the growth and development rates of zebrafish were established by morphometric analysis. We determined that the average length of the larvae of the control group was 3.4 mm, while for the larvae exposed to concentrations of 9 and 18 µg/mL of 25C-NBOMe the lengths were 3.19 and 2.92 mm, respectively ($p < 0.05$). From the above, a decrease in the length of the trunk was evident.

Regarding pigmentation patterns, a reduction of these in the body cavity, yolk, and yolk extension was observed. This effect was significant mainly in concentrations of 2, 12, and 18 µg/mL ($p = 0.0300$, 0.0201 and 0.0209, respectively), related to the vehicle DMSO 1% (Fig. 4).

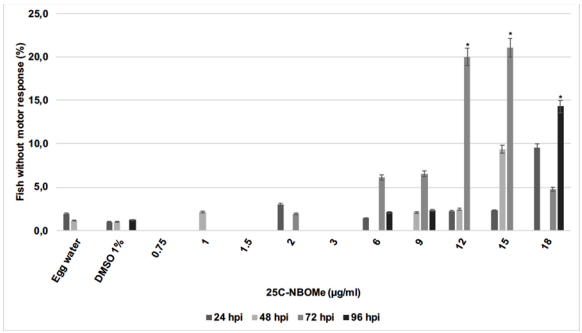

Fig. 2. The hallucinogen 25C-NBOMe affects the motor response in zebrafish. Percentage of fish that do not present a motor response to a stimulus after exposure of 25C-NBOMe as a function of time (hpi). The column color indicates evaluation time (hpi). An average of 10 larvae without morphological defects were tested per concentration of 25C-NBOMe. The error bars represent the sample standard deviation. Asterisks mark significant differences relative to controls in concentrations of 12, 15 and 18 µg/mL of 25C-NBOMe ($p < 0.05$).

When exposing the zebrafish embryos at 25C-NBOMe concentrations between 1 and 18 µg/mL, pericardial and/or perivitelline edema were observed. These effects were more frequent at concentrations of 12 and 18 µg/mL ($p = 0.0311$ and 0.0438, respectively) compared to the 1% DMSO control (Fig. 5a). Associated with this, the formation of a small and elongated heart with reduced heart rate and alterations in blood circulation was evident (Fig. 5b).

Another observed effect was the optic fissure closure alterations in zebrafish embryos, generating a "Pacman" effect, where ocular depigmentation was usually present (Fig. 6a and b).

Additionally, it was determined that 25C-NBOMe causes lordosis in a percentage higher than 50 % of the fish exposed to concentrations between 1 and 18 µg/mL. This structural defect was significant in the concentrations of 1, 1.5, 2, 6, 12, 15 and 18 µg/mL ($p = 0.0383$, 0.0423, 0.0260, 0.0258, 0.0294, 0.0417 and 0.0383, respectively) when compared with the vehicle of DMSO 1% (Fig. 7a). It is important to note that this alteration corresponds to the most predominant morphological effect of the study (Fig. 7b).

Finally, the 25C-NBOMe substance causes a significant decrease in the yolk extension in the exposed embryos at concentrations of 6, 12, and 18 µg/mL at 24 hpi ($p = 0.0037$, 0.0121, 0.0048, respectively) related to the control DMSO 1 % (Fig. 8).

It is important to highlight that our results show a concentration-dependent lethality correlation. However, for morphological and behavioral effects, this concentration-dependency is not observed. All described effects are commonly known as toxicity indicators and have also been found in other investigations with other chemical compounds. Hermsen et al. (2011) developed a quantitative scoring system based on the evaluation of the different characteristics during the development of the zebrafish, as well as its teratogenicity [22].

4. Discussion

The animal models of *Artemia salina* and zebrafish allow us to evaluate morphological, physiological, and behavioral changes, due to the ease of studying the brain and nervous system in its early development. This makes them excellent models to investigate chemical exposure and neurotoxicity [23]. *Artemia salina* is a marine crustacean that has a great capacity for osmoregulation, which contributes to a great resistance against the toxicological effects of different compounds [24]. On the other hand, the nervous system in the *Artemia salina* is characterized by the presence of monoaminergic neurons in the outer medulla and different areas of the brain [25]. To limit the concentrations to be evaluated in the zebrafish animal model, the *Artemia salina* model was used. Although the 5-HT_{2A} receptor gene has been duplicated in a teleost, it retains its functions as observed in mammals. It shows similar expression patterns in smooth muscle fibers, skeletal muscle, endothelial cells, frontal cortex, basal ganglia, and the synaptic cleft [4], [26].

Fig. 3. The hallucinogen 25C-NBOMe induces morphological changes in zebrafish. Images of most common defects observed in zebrafish embryos exposed to concentrations of 1.5, 3, 12, 15 and 18 µg/mL at 24 hpi (34 hpf), 48 hpi (58 hpf), 72 hpi (82 hpf) and 96 hpi (106 hpf). Asterisk indicates that for 15 µg/mL of 25C-NBOMe at 96 hpi, no photographic record was obtained. Scale bar =1.0 mm.

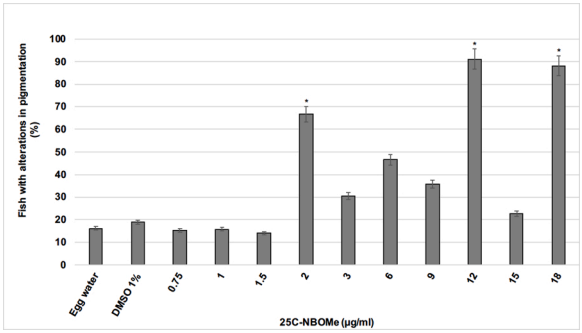

Fig. 4. The hallucinogen 25C-NBOMe delays pigmentation pattern in zebrafish. Quantification of the effect of 25C-NBOMe on pigmentation patterns in affected larvae. The error bars represent the data standard deviation. Asterisks mark significant differences in concentrations of 2, 12 and 18 µg/ mL of 25C-NBOMe ($p = 0.0300$, 0.0201 and 0.0209) relative to controls.

4.1. *Effect of 25C-NBOMe on the Artemia salina model*

Our data shows that 25C-NBOMe induces a decrease in the survival of *Artemia salina* as the concentration and time of exposure increases. We determined that for 25C-NBOMe, the LC_{50} value was 20.14 µg/mL considered as highly toxic (Level II: LC_{50} 10−100 µg/mL), according to previous toxicological criteria [27,28]. The above indicates a high lethality. We also show that 25C-NBOMe produces alterations in the swimming speed of the *Artemia salina* model. This agrees with that published by Manfra et al. (2016), who determined that alterations on swimming speed can be used as an expression of acute toxicity and toxicological stress [29]. Thus, it is the most frequently used behavioral measurement to assess the physiological state of an aquatic organism.

Similarly, regarding the swimming patterns and antennae movement, our data showed aberrant and discontinuous swimming as well as immobility under treatment with 25C-NBOMe. Some of these alterations have been described previously when the *Artemia salina*, has been exposed to different concentrations of copper sulfate pentahydrate ($CuSO_4 \bullet 5\ H_2O$) and sodium dodecyl sulfate (SDS) [29]. Therefore, our data suggest high toxicity not only by LC_{50} value but also by the behavioral tests.

4.2. *Effect of 25C-NBOMe on the zebrafish animal model*

Our data in zebrafish embryos exposed to 25C-NBOMe show an LC_{50} value of 10.76 µg/mL. Studies with other NBOMe family members have shown a reduction in the survival rate of approximately 40 %, for the concentration of 0.5 µg/mL for 25B-NBOMe [4]. However, this exposure was only done starting at 4 dpf by Kawahara et al. (2017). Furthermore, using the exposure protocol described previously with 25C-NBOMe, we found no mortality (Data not shown). This difference between NBOMe family members could be explained according to the affinities (Ki) of these molecules for the 5-HT_{2A} receptor. It also could be related to the halogens present in each structure (bromine and chlorine for 25B-NBOMe and 25C-NBOMe, respectively). The above was described by Rickli et al. (2015) who determined that the substance 25B-NBOMe has a higher affinity value (0.0005 ± 0.0000 µM), compared with the hallucinogen 25C-NBOMe (0.0007 ± 0.0002 µM) [30].

Regarding the effects on the nervous system, our data show that 25C-NBOMe, an agonist of the serotonergic receptors 5-HT_{2A}, produces

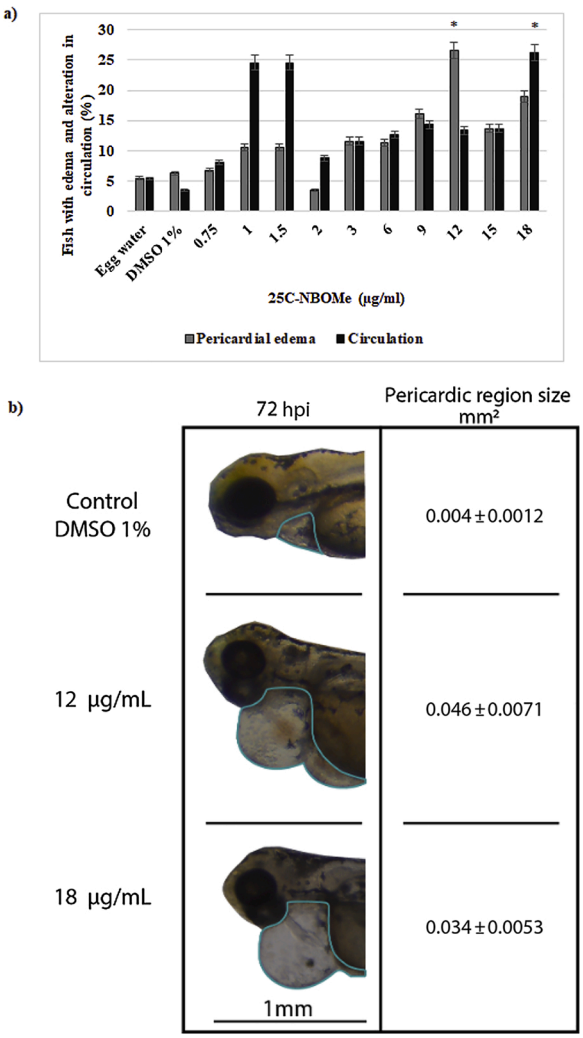

Fig. 5. 25C-NBOMe causes heart defects and circulatory problems in zebrafish. a Effect of 25C-NBOMe on the appearance of pericardial and perivitelline edemas, and alteration in circulation in zebrafish. The error bars represent the data standard deviation. Asterisks show significant differences in the concentrations of 12 and 18 µg/mL of 25C-NBOMe ($p = 0.0311$ and 0.0438) relative to controls. **b** Pericardial and perivitelline edemas (blue line) observed in zebrafish exposed to concentrations of 12 and 18 µg/mL of 25C-NBOMe at 72 hpi ($p = 0{,}0041$ and 0.0254). Scale bar =1.0 mm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

motor alterations. Zebrafish embryos exposed to the substance did not respond to touch but frequently showed a spiral swimming pattern (parameter not quantified). This behavior was explained by Neelkantan et al. (2013) who highlighted the zebrafish as an ideal model for the study of motor and behavioral alterations, which are possibly caused by the affectation of the monoaminergic neurotransmission system [31]. Similarly, Kyzar et al. (2012) conducted studies using mescaline, a phenylethylamine that acts as an agonist of the serotonergic receptors 5-$HT_{1A/2A/B/C}$, showing that this substance produces anxiolytic effects in the treated fish which subsequently reacted with erratic movements [32]. Additionally, studies by Xu et al. (2019) showed that 25C-NBOMe affects the viability of SH-SY5Y, PC12, and SN4741 cell lines, which are models used to assess neurotoxicity. On the other hand, they made a comparison with methamphetamines showing that 25C-NBOMe is 50 times more potent in the SH-SY5Y line [9]. This could suggest that the behavior observed by us may be due to an effect of 25C-NBOMe on cells of the central nervous system.

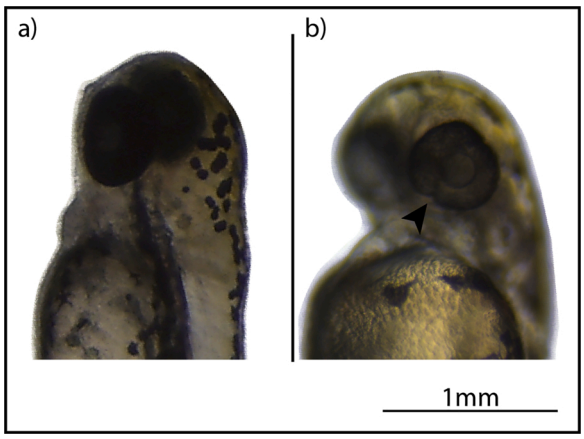

Fig. 6. 25C-NBOMe causes eye morphogenesis defects. a Normal ocular development in control fish at 60 hpf. **b** Fish with alteration in the closure of the optic fissure (arrowhead) at 60 hpf exposed to 9 µg/mL of 25C-NBOMe. Scale bar =1.0 mm.

Our results also showed that 25C-NBOMe causes a decrease or absence of pigmentation in the zebrafish. The melanocytes begin to produce melanin and become visible by 24 hpf, establishing the main pattern of pigmentation by 48 hpf [33]. However, in the present study, we observed that in certain concentrations this pattern was only completed by 84 hpf (72 hpi). These changes may be caused by a disruption in the melanocytes development, impairment in the migration of the neural crest cells, blockage in the synthesis of melanin, or global developmental delay [34]. Lee et al. (2011) studied the effect of some agonists on different serotonin receptors, including the 5-HT_{2A} receptor, and they determined that serotonin induces the process of melanogenesis, morphological changes, and migration in cells *via* the 5-HT_{2A} receptor [35]. This could explain the effects on the pigmentation of zebrafish embryos due to the substance 25C-NBOMe affects this receptor. Even more, Stewart et al. (2013) showed that the serotonin signaling pathway is associated with the dispersion and aggregation of melanophores in several vertebrate species [36].

On the other hand, all these effects on the zebrafish may reflect serotonergic toxicity and conditions associated with the serotonin syndrome. This syndrome besides the alteration in pigmentation includes structural changes in skeletal muscle consistent with rhabdomyolysis and autonomic hyperactivity. In humans, it also includes mental status changes. In agreement with Kawahara et al. (2017) the NBOMe compounds, which are serotonergic drugs, could induce effects associated with this syndrome caused mainly by excessive activation of serotonin receptors [4].

Although no tests were performed related to alterations in muscle structures, we observed a difference in trunk length, body curvature, and yolk formation in embryos exposed to 25C-NBOMe. According to Nagel R., (2002), the reduction in trunk length by exposure to various substances indicate toxicity and teratogenicity [37]. Therefore, our results are consistent with the effects previously described, also related to developmental lags due to the exposure to toxics. Similarly, B. Fraysse et al. (2006) evidenced a reduction in the length of the embryo when the fish were subjected to concentrations of propranolol (32 mg/L), malathion (2 mg/L), and cadmium (1.5 mg/L) [21]. Regarding the alterations in the body curvature, these are consistent with irregularities of the myosepta and rhabdomyolysis, which are part of the neuromuscular abnormalities of the serotonin syndrome, caused by the excessive reuptake of serotonin and activation of 5-HT_{2A} receptors [4,38]. Likewise, Sfakianakis et al. (2015) mentioned that the majority of reported deformities in fish occur in the vertebral column or the notochord, on which many tissues depend. Based on this, they stated that toxic substances that alter the development and differentiation of the notochord

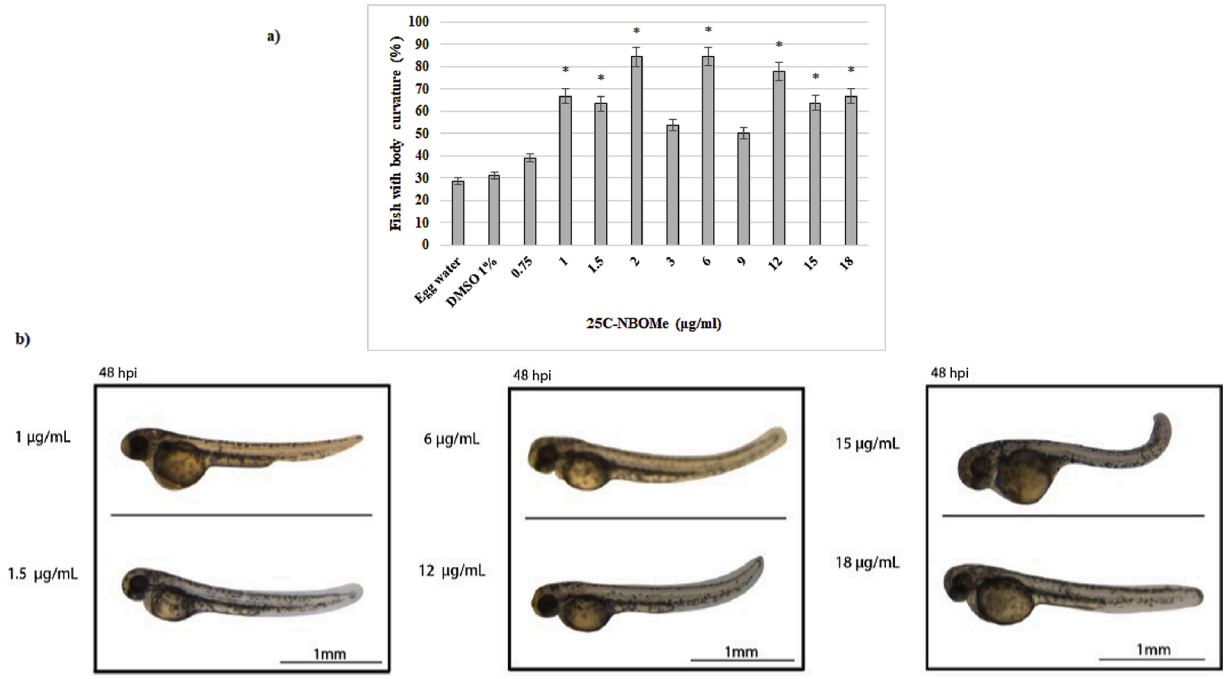

Fig. 7. The hallucinogen 25C-NBOMe induces lordosis in zebrafish. **a** Percentage of fish that show lordosis when exposed to 25C-NBOMe. The error bars represent the data standard deviation. Asterisks show significant differences in the concentrations of 1, 1.5, 2, 6, 12, 15 and 18 µg/mL ($p = 0.0383, 0.0423, 0.0260, 0.0258, 0.0294, 0.0417$ and 0.0383) relative to controls. **b** Lordosis observed in zebrafish exposed to concentrations of 1, 1.5, 6, 12, 15 and 18 µg/mL of hallucinogen 25C-NBOMe. Scale bar =1.0 mm.

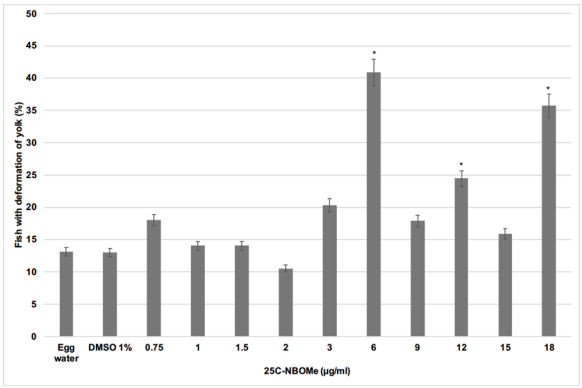

Fig. 8. The hallucinogen 25C-NBOMe induces yolk deformation in zebrafish. Percentage of fish displaying yolk deformation at 24 hpi per concentration. The error bars represent the data standard deviation. The asterisk shows the significant difference between the vehicle and the concentrations of 6, 12, and 18 µg/mL ($p = 0.0037, 0.0121, 0.0048$), relative to controls.

can generate muscle abnormalities, skeletal deformities, and uncontrolled muscle spasms [39]. Furthermore, changes in body curvature are also associated with yolk deformation. Nagel R., (2002) suggests that the deformation of yolk in embryos is an effect previous to the appearance of lordosis being indicative of a teratogenic effect [37]. Fraysse et al. (2006) also associated yolk deformation with an alteration of blood flow, which is related to the decrease in heart rate [21]. Similarly, Kabir et al. (2020) determined the incidence of arsenic on the embryonic development of zebrafish. At high levels of this substance, an increase in mortality was evidenced, as well as physiological abnormalities in early development, prolonged hatching time, abnormalities in the cardiovascular systems, and decreased growth [40]. In the present work, we demonstrated that a high proportion of embryos exposed to the substance had effects on the yolk extension triggering lordosis and circulation alterations.

Similarly, we observed a decrease in the circulation of the caudal blood, the formation of edema, and lower heart rate in most embryos exposed to 25C-NBOMe. Billiard et al. (1999) found that decreased blood flow may be associated with pericardial edema [41]. On the other hand, Chen J., (2013) suggested that these alterations correspond to cardiotoxic responses presented when exposing zebrafish embryos of 72 hpf to toxic substances [42]. Likewise, zebrafish embryos exposed to severe changes in their environment during critical periods of heart development can trigger responses that end up in heart failure, edema, and circulatory collapse. Fraysse et al. (2006) determined that the pericardial area increased as the concentration of the toxicants used in their study increased and that a reduction in the frequency of the heartbeat decreased the size of the heart [21]. Finally, Yoon et al. (2019) showed that 25C-NBOMe and 25D-NBOMe have cardiotoxic effects on *in vitro* and *in vivo* models, especially on heart rate, suggesting cardiotoxicity [10]. The foregoing is in accordance with our results concerning a decrease in circulation and heart rate.

About the alterations in ocular development like the failure in the optic fissure closure upon exposure to 25C-NBOMe, Weiss et al. (2012) suggested that an interruption in the closure of the optic fissure in *lmo2*^{vu270} mutant embryos at 48 hpf, is due to the formation of cephalic edema resulting in altered cell proliferation or cell death in the retina [43]. Thus, it may cause eye abnormal morphogenesis. Another possible cause of this optic defect may be related to an alteration in the neurotransmitters and their receptors present in the vertebrate retina during the early stages of development, which contribute to the signaling, proliferation, and differentiation of progenitor cells. Martins et al. (2008) highlighted in their review several studies in which genetic manipulations were performed to demonstrate that signaling through the 5-HT$_{2B}$ receptors controls cell proliferation and cell death in the development of the Xenopus retina [44]. On the other hand, Weiss et al. (2012) determined that an abnormal increase in blood vessels passing through the optic fissure interfere with her closure and because serotonin controls processes associated with vasoconstriction, a change in its normal concentration may affect the contraction of the blood vessels that pass through the eye, causing an effect on its closing [43,45]. In the

case of the 25C-NBOMe substance, because it is an agonist of serotonergic receptors, it could cause an alteration in the optic fissure closure we have observed.

Finally, the non-dependence between morphological effects and concentrations of 25C-NBOMe in the zebrafish model could be due to the desensitization of the 5-HT_{2A} receptor. It has been shown that this receptor can be internalized and degraded by the prolonged action of agonists and antagonists generating this effect [46,47]. On the other hand, Renieri et al. (2017) found that when treating zebrafish with high concentrations of cadmium, there was no dose-dependent response concerning survival and effects. This was related to the hormesis phenomena, concluding that exposure to high doses inhibits toxic effects, due to a modification in the uptake of the substance [48].

5. Conclusions

In this study, we showed that the *in vivo* models allow the evaluation of the toxicity and effect of hallucinogens, due to their susceptibility to the effect of neurotoxic substances. We determined LC_{50} values for two animal models confirming that 25C-NBOMe is highly toxic and also causes teratogenic effects on the development of zebrafish embryos. Altogether our findings contribute to the understanding of the effects caused by the substance 25C-NBOMe in two different organisms and could be related to results observed in studies with cell lines, in which it was determined that this substance is neurotoxic and cardiotoxic. Thus, it could be the foundation for new studies associated with the assessment of the cellular effects of 25C-NBOMe in humans. Our data would be the starting point to generate public health alerts and prevention policies highlighting the risk of 25C-NBOMe consumption, particularly for reproductively active or pregnant women.

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Compliance with ethical standards

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Procedures for obtaining embryos (POE_15-006), feeding (POE_15-007), and euthanasia (POE_15-004) have been approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL- UNIANDES). The use of animals was approved in April 2017 by the C. FUA 17.004.

CRediT authorship contribution statement

Natalie Álvarez-Alarcón: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Jhon Jairo Osorio-Méndez:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Adis Ayala-Fajardo:** Conceptualization, Methodology, Writing - review & editing, Supervision. **William F. Garzón-Méndez:** Conceptualization, Resources, Writing - review & editing. **Zayra V. Garavito-Aguilar:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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