

Morphological and behavioral responses of zebrafish after 24 h of ketamine embryonic exposure

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ABSTRACT

Ketamine, one anesthetic used as an illicit drug, has been detected both in freshwater and marine ecosystems. However, knowledge of its impact on aquatic life is still limited. This study aimed to test its effects in zebrafish embryos by analyzing its time- and dose-dependent developmental toxicity and long-term behavioral changes. The 24 h-LC₅₀ was calculated from percent survival using probit analysis. Based on the 24 h-LC₅₀ (94.4 mg L⁻¹), embryos (2 hour post-fertilization - hpf) were divided into four groups, including control, and exposed for 24 h to ketamine concentrations of 50, 70 or 90 mg L⁻¹. Developmental parameters were evaluated on the course of the experimental period, and anatomical abnormalities and locomotor deficits were analyzed at 144 hpf. Although the portion of ketamine transferred into the embryo was higher in the lowest exposed group (about 0.056 ± 0.020 pmol per embryo), the results showed that endpoints such as increased mortality, edema, heart rate alterations, malformation and abnormal growth rates were significantly affected. At 144 hpf, the developmental abnormalities included thoracic and trunk abnormalities in the groups exposed to 70 and 90 mg L⁻¹. Defects in cartilage (alcian blue) and bone (calcein) elements also corroborated the craniofacial anomalies observed. A significant up-regulation of the development-related gene *nog3* was detected by qRT-PCR at 8 hpf. Early exposure to ketamine also resulted in long-term behavioral changes, such as an increase in thigmotaxis and disruption of avoidance behavior at 144 hpf. Altogether, this study provides new evidence on the ketamine teratogenic potential, indicating a possible pharmacological impact of ketamine in aquatic environments.

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1. Introduction

Novel psychoactive substances (NPS) have been emerging worldwide since 2008, leading to a total of over 450 NPS only in Europe (Mounteney et al., 2016). Ketamine, has been a widely used anesthetic in human and veterinary medicine for >40 years (Jansen, 2000), being one of the oldest NPS. According to the United Nations Office on Drugs and Crime 2014 World Drug Report (UNODC, 2014), and due to its hallucinogenic effects, ketamine unauthorized non-medical use and PCP-type substances recreational abuse corresponds to about 4% of NPS worldwide (Domino et al., 1965). This has raised concerns about the

physical and psychological harmful consequences of this drug abuse (Morgan et al., 2012).

Ketamine is not completely metabolized by humans and other organisms (Lin et al., 2014) with about 10% being found partially unchanged in faeces and urine (Quibell et al., 2011). Ketamine detection in wastewater effluents (Castiglioni et al., 2015), from medical and non-medical practices (Lin et al., 2014), has led to its classification as an emergent contaminant in some countries. Nevertheless, there are limited data on the efficiency of the removal of illicit drugs during wastewater treatment and some compounds remain chemically and biologically active in the aquatic environment (Petrie et al., 2015). In this sense, and given the relatively long half-life of ketamine in the environment (Wang and Lin, 2014), the risk of chronic exposure is high. In fact, ketamine has been reported to persist through conventional wastewater treatment plants (WWTPs), as it still is detected in both surface and sea waters in concentrations up to 10 µg L⁻¹ (Lin et al., 2010; Jiang et

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al., 2014; Lin et al., 2014). The environmental contamination by wastewater discharges is an ecotoxicological concern (Prasse et al., 2015), however the evidence of the toxic consequences and ecotoxicity of ketamine to aquatic organisms is still limited despite physiological, biochemical and behavioral consequences have been associated with ketamine exposure at early stages (Felix et al., 2014; Liao et al., 2015; Felix et al., 2016a; Felix et al., 2016b; Guo et al., 2016).

In the past few years, zebrafish has proved to be a general sensitive model for ecotoxicological and toxicological studies (Dai et al., 2014). Therefore, the aim of this study was to establish the 24-hour lethal concentration (LC_{50}) of ketamine to zebrafish (*Danio rerio*) embryos and to evaluate ketamine exposure impact on zebrafish development. To this end, morphological and behavioral changes were evaluated. Data from this study should contribute to a better understanding of the pharmacological action and environmental cumulative risk of ketamine to fish species at early life stages of development.

2. Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations for care and use of laboratory animals of the EU directive (2010/63/EU) and National (Decreto-Lei 113/2013) legislation on the protection of animals used for scientific purposes. The experiments performed in this work were under project license approval by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária (DGAV). All efforts were made to minimize suffering.

2.2. Chemicals and other reagents

Ketamine (ketamine hydrochloride, Imalgene1000, 100 mg mL⁻¹) was obtained from Merial Portuguesa-Saúde Animal Lda (Rio de Mouro, Portugal). All solutions were freshly made with embryo water (28 ± 0.5 °C, 200 mg L⁻¹ Instant Ocean Salt and 100 mg L⁻¹ sodium bicarbonate; UV sterilized) prepared from City of Vila Real filtered-tap water. Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebouurg, France). All other chemical reagents were either of analytical or HPLC grade, and obtained from Sigma-Aldrich (Steinheim, Germany). To establish a standard curve for HPLC analysis, ketamine hydrochloride was purchased from Biotrend (Cologne, Germany).

2.3. Animals

Zebrafish maintenance and embryo collection were performed as previously described (Felix et al., 2014). Wild-type (AB strain) zebrafish embryos, were maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) in an open water system supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5) at 28 ± 0.5 °C in a 14:10 h light:dark cycle. Animals were fed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. nauplii. The onset of light in the morning induced the spawning of fish allocated in breeding tanks, containing a ratio of 2 males:1 female. Embryos were rinsed in embryo water, bleached according to established protocols (Westerfield, 2007; Varga, 2011) and rinsed again to remove debris. Fertilized embryos with normal morphology were staged under a SMZ 445 stereomicroscope (Nikon, Japan) according to standard methods (Kimmel et al., 1995).

2.4. 24 h LC_{50}

The lethal median concentration (LC_{50}) in embryos was estimated by establishing concentration-response curves according to OECD standard protocol (OECD 236) with minor modifications. Briefly, replicates of 100 collected zebrafish embryos (~2.0 hour post-fertilization – hpf)

were randomly exposed in 50 mL beakers to each six ketamine concentrations (25, 50, 70, 90, 100 and 200 mg L⁻¹, respectively 0.11, 0.21, 0.29, 0.38, 0.42 and 0.84 mM) for a static 24-h period, based on preliminary studies, and mortality was recorded. Control sets were simultaneously prepared to correct mortalities. The experiments were repeated three times and averages were calculated. The LC_{50} value (Fig. 1) was derived through probit analysis (Finney, 1971) and the upper and lower confidence intervals were calculated using SPSS for Windows (Version 22.0; Chicago, IL, USA). Based on the data acquired, the median lethal dose for 24-h exposure was calculated at 94.4 (92.0–96.8) mg L⁻¹ or 0.40 (0.39–0.41) mM. These results were used to select concentrations for the remaining experimental conditions.

2.5. Embryo assay

Fig. 2 shows a schematic diagram of the experimental design used for embryo testing. Based on the LC_{50} calculation, early blastula embryos (~2.0 hour post-fertilization – hpf) were statically exposed in 50 mL beakers for 24 h to freshly ketamine concentrations (50, 70 and 90 mg L⁻¹ respectively 0.21, 0.29 and 0.38 mM), along with controls containing no ketamine. Following treatment exposures, embryos were washed three times in embryo water and collected or allowed to develop up to 144 hpf, with daily water renewal. Dead embryos/larvae and debris were daily removed. Throughout all procedures, temperature was kept at 28 ± 0.5 °C.

2.6. HPLC-UV quantification of ketamine

In order to quantify ketamine absorption, for each of the experimental test concentrations, three independent biological replicates of 100 pooled zebrafish embryos were collected immediately after (T0) and 24 h after (T24) ketamine exposure for high performance liquid chromatography (HPLC). Samples were collected as previously described (Bolze and Boulieu, 1998) with slight modifications (Cuevas et al., 2013; Trickler et al., 2014). The HPLC separations (50 µL) were performed with a Hichrom ACE Ultracore 5 Super C18 (150 × 4.6 mm, 5 µm) at 20 °C and a flow rate of 1.5 mL min⁻¹ in a Dionex UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) coupled with a diode array detector (Dionex PDA 100 photodiode array, Dionex, Olten, Switzerland) at 210 nm and controlled by the Dionex Chromeleon Software 6.70 Build 1820. Control samples were used as blanks and ketamine was quantified by measuring the area under the peak, which was drawn from the analysis of seven known ketamine concentrations (from 0.05 to 10 pmol). The amount (pmol) of ketamine in each sample was interpolated from the linear correlation plot between the integrated areas

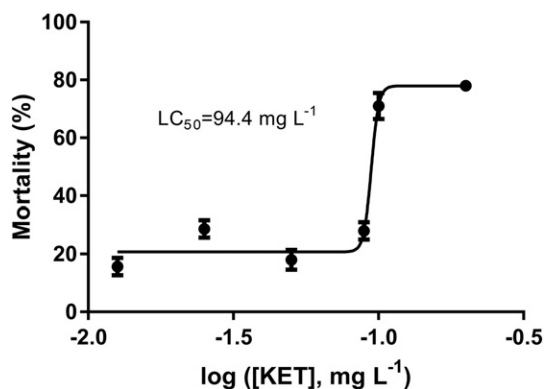


Fig. 1. Dose–response curve of mortality of zebrafish following 24 h exposure to ketamine. Log-transformed ketamine concentrations are plotted on the x axis in function of the mortality, taken into account the correction of control mortality. The 24 h lethal concentration (LC_{50}) value of ketamine was 94.4 mg L⁻¹ (0.40 mM) with the 95% confidence interval ranging from 92.0 to 96.8 mg L⁻¹ (0.39 to 0.41 mM). The values are presented as mean ± SD from three independent replicate exposures.

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