Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio rerio) embryos

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HIGHLIGHTS

- Ketamine induce antioxidant defences alterations in zebrafish embryos.
- Changes in glutathione cycle were perceived following exposure to ketamine.
- Ketamine prompted a significant upregulation of antioxidant-related genes expression.
- Apoptotic-related genes were upregulated to some extent.
- Ketamine induced oxidative stress and apoptosis through the involvement of p53-dependent pathways in zebrafish embryos.

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ABSTRACT

Ketamine is a widely used pharmaceutical that has been detected in water sources worldwide. Zebrafish embryos were used in this study to investigate the oxidative stress and apoptotic signals following a 24h exposure to different ketamine concentrations (0, 50, 70 and 90 mg L⁻¹). Early blastula embryos (~2 h post fertilisation-hpf) were exposed for 24 h and analysed at 8 and 26 hpf. Reactive oxygen species and apoptotic cells were identified in vivo, at 26 hpf. Enzymatic activities (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lactate dehydrogenase (LDH) and acetylcholinesterase (AChE)), glutathione levels (oxidised (GSSG) and reduced (GSH)), oxidative damage (lipid peroxidation (LPO) and protein carbonyls (CO)) as well as oxidative stress (gclc, gstp1, sod1 and cat), apoptosis (casp3a, casp6, casp8, casp9, aifm1 and tp53) and cell proliferation (pcna) related-genes were evaluated at 8 and 26 hpf. Caspase (3 and 9) activity was also determined at both time-points by colorimetric methods. Superoxide dismutase (SOD), catalase (CAT), glutathione levels (GSSG), caspase-9 and reactive oxygen species (ROS) were shown to be affected by ketamine exposure while in vivo analysis showed no...
1. Introduction

Over the last few years, numerous studies have reported a rise in water contamination by pharmaceuticals (Fent et al., 2006). These compounds have been detected in wastewaters and surface waters around Europe (Zuccato and Castiglioni, 2009; Petrie et al., 2015). As a result, they have been considered emerging aquatic pollutants (Zuccato and Castiglioni, 2009; Rodayan et al., 2016). Although being present at low concentration levels, they constitute a major risk to aquatic life and to human health (Pomati et al., 2006; Petrie et al., 2015; Rodayan et al., 2016), due to continuous discharges and their resistance to degradation (Santos et al., 2010; Sui et al., 2015). Moreover, most of these compounds are currently not subject to control under European Legislation (Mounteney et al., 2016).

Ketamine, a dissociative glutamatergic hallucinogen widely used in developing countries for medical and non-medical purposes (Jansen, 2000; Lin et al., 2014), has been classified as an emergent contaminant in some countries, as it has been detected in both domestic (recreational use) and hospital waste effluents' sewerage systems (Lin et al., 2010, 2014; Jiang et al., 2014; Castiglioni et al., 2015). The persistency of ketamine in the environment and the formation of more toxic by-products (Lin et al., 2014) could represent an ecological risk that can result in long-term adverse effects, as observed after exposure to different chemicals due to the dysregulation of developmental processes, among others (Lupien et al., 2009; Santos et al., 2010; Perera and Herbsman, 2011; Grandjean et al., 2015).

Zebrafish (Danio rerio) is a well-established alternative laboratory model organism that has been widely used in the ecotoxicology field due to several advantageous characteristics over other animal models (Seok et al., 2008; Dai et al., 2014). Particularly, due to its high sensitivity to environmental stress (Zhu et al., 2015), the early life stages of zebrafish have been extensively used for environmental risk assessment (Scholz et al., 2008). Some toxicity studies that focused on the influence of ketamine short exposure (20 min) during the early organogenesis development of zebrafish include lethality, oxidative stress, apoptosis and behavioural deficits (Felix et al., 2014, 2016a, 2016b, 2017a, 2017c). Additionally, other embryo-larval developmental toxicity assays have been conducted during vulnerable windows of exposure for longer periods (at least for 24 h) in zebrafish (Felix et al., 2017b; Robinson et al., 2017a). This is also the case in other aquatic models, such as Oryzias latipes and Xenopus laevis embryos (Liao et al., 2015; Guo et al., 2016), showing the involvement of oxidative stress and apoptotic pathways in the teratogenicity induced by ketamine (Liao et al., 2017). In our previous study, a 24 h exposure to ketamine during zebrafish embryogenesis showed an increase in mortality, embryo malformations, abnormal growth rates, craniofacial anomalies and behavioural alterations (Felix et al., 2017b). These structural malformations and neurobehavioral deficits need to be better addressed to clarify the underlying mechanism of ketamine in zebrafish early morphogenesis alterations, in which redox-sensitive pathways can be perturbed by chemical exposure (Hansen et al., 2018). Therefore, the novelty of this work was to evaluate the impact of a 24 h exposure to ketamine concentrations high enough to disturb early zebrafish developmental processes, focusing on oxidative stress and apoptosis biomarkers.

2. Material and methods

2.1. Ethical 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 for animal experimentation and welfare. The experiments performed in this work were under project license approval by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária (DGAV, Lisboa, Portugal). All efforts were made to minimize suffering of adult zebrafish.

2.2. 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 sterilised) prepared from City of Vila Real filtered-tap water. Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebourg, France). Caspase substrates were purchased from Enzo Life Sciences International Inc. (Farmingdale, New York, USA) and p-nitroaniline (pNA) was acquired from Santa Cruz Biotechnology (Santa Cruz, California, USA), whereas oligo primers were purchased from Stabvida Lda. (St. Antonio de Oeiras, Portugal). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany).

2.3. Selection of exposure concentrations

Ketamine concentrations of 50, 70 and 90 mg L\(^{-1}\) (0.21, 0.29 and 0.38 mM, respectively) were chosen based on the previous determined mean lethal concentration for a 24 h exposure (24 h-LC50 = 94.4 mg L\(^{-1}\)) (Felix et al., 2017b) established according to OECD standard protocol (OECD 236) with minor modifications.

2.4. Experimental design

Adult zebrafish (Danio rerio) were maintained as previously described (Felix et al., 2014). Briefly, 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-sterilised City of Vila Real tap water (pH 7.3–7.5) at 28 ± 0.5 °C in a 14:10 h light:dark cycle. Feeding was performed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with Artemia sp. nauplii to promote the spawning activity. The spawning of adult zebrafish grouped in breeding tanks, containing a ratio of 2 males:1 female, was induced by the onset of lights in the morning.
Embrions were rinsed in embryo water, bleached according to established protocols (Westerfield, 2007; Varga, 2011) and rinsed again to remove debris. Fertilised embryos with normal morphology were staged under a SMZ 445 stereomicroscope (Nikon, Japan) according to standard methods (Kimmel et al., 1995) and early blastula embryos (~2.0 h post-fertilisation — hpf) were statically exposed in 50 mL beakers, for 24 h, to selected ketamine concentrations as well as to the water system (control group). Fig. 1 shows a schematic diagram of the experimental design used for embryo testing. Throughout all procedures, the temperature was kept at 28 ± 0.5°C.

2.5. In vivo intracellular ROS and apoptosis

The generation of reactive oxygen species (ROS) and cell death were analysed at 26 hpf in dechorionated zebrafish embryos (2 mg mL−1 pronase (Roche Diagnostics, Mannheim, Germany) (Westerfield, 2007) using the oxidation-sensitive fluorescent probe dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA 20 μg mL−1) and the acridine orange (5 μg mL−1) staining, respectively for ROS and apoptosis evaluation, for 30 min at room temperature, according to previously described methodologies (Felix et al., 2014, 2016b, 2017c). Fluorescent images were obtained from twelve animals of each of the five replicates under an inverted microscope (IX 51, Olympus, Antwerp, Belgium) equipped with an Olympus U-RFL-T fluorescent light source (Olympus, Antwerp, Belgium) and FITC filter, using a 4X Olympus UIS-2 objective lens (Olympus Co., Ltd., Tokyo, Japan). Data was then acquired using Cell R software (Olympus, Antwerp, Belgium) and fluorescent images were processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, USA). Furthermore, whole-embryo fluorescence of individual embryos was quantified using the ImageJ2 program (version 2.0.0, U.S. National Institutes of Health, Bethesda, USA) (Schneider et al., 2012) after correcting for the yolk auto-fluorescence.

2.6. Biochemical determinations

For biochemical determinations, at least four replicates of 100 embryos per concentration were collected at 8 and 26 hpf as previously reported (Felix et al., 2016b). Zebrafish embryos were homogenised in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of MgCl2 and 0.5 mM of phenylmethyl sulphonylfluoride (PMSF, prepared in ethanol to prevent protein degradation, pH 7.4) (Deng et al., 2009), using a pellet mixer and a cordless motor (VWR International, Carnaxide, Portugal). Homogenates were centrifuged at 15 000 × g at 4°C for 20 min (Sigma model 3K30, Osterode, Germany) and supernatants (post-mitochondrial supernatant (PMS) collected for biochemical analysis and stored at −80°C for no more than 2 months until analysis. The activity of superoxide dismutase (Cu/Zn-SOD) was determined by the nitroblue tetrazolium (NBT) reduction generated by the xanthine/xanthine oxidase system at 560 nm (Durak et al., 1993). SOD from bovine erythrocytes was used to construct a standard curve (0–3.75 U mL−1). The activity of catalase (CAT) was determined at 240 nm in accordance to the method previously described (Aebi, 1984) and was calculated using bovine catalase as a standard (0–5 U mL−1). Glutathione peroxidase (GPx) activity was determined at 340 nm (Paglia and Valentine, 1967) using the extinction coefficient of 6.22 mm−1 cm−1. For acetylcholinesterase (AChE) activity, a method described for microplates (Rodriguez-Fuentes et al., 2015) and based on Ellman’s method (405 nm) (Ellman et al., 1961) was applied using the 5-thio-2-nitrobenzoic acid (TNB) extinction coefficient of 13.6 M−1 cm−1. For lactate dehydrogenase (LDH) activity, the method described by Domingues (Domingues et al., 2010) at 340 nm was used using the extinction coefficient of 6.22 mm−1 cm−1. The glutathione levels were determined by measuring both the reduced (GSH) and the oxidised states (GSSG) using the fluorochrome ortho-phtalaldehyde (OPA) at 320 nm and 420 nm for excitation and emission wavelengths, respectively (Gartaganis et al., 2007). Concentrations were estimated based on GSH and GSSG standard curves (0–10 μM), whereas the ratio between GSH and GSSG was calculated as the oxidative-stress index (OSI). Malondialdehyde (MDA) content, an indicator of lipid peroxidation (LPO), was measured at 535 nm (excitation) and 550 nm (emission) wavelengths, by chromogenic assay via a thiobarbituric (TBA) acid-based method described elsewhere (Gartaganis et al., 2007). MDA was estimated based on a standard curve (0–4 nM) of malonaldehyde bis(dimethyl acetal). The carbonylated proteins (CO) were measured as dinitrophenylhydrazine (DNPH) derivatised protein at 370 nm as described by Reznick and Packer (1994) (Reznick and Packer, 1994). Determination of total ROS was performed at 485 nm and 530 nm for excitation and emission wavelengths, respectively, using the fluorescent probe DCFH-DA, as previously described (Deng et al., 2009) and ROS accumulation was estimated based on a DCF standard curve (0–6.25 nM). Caspase-3 (Ac-DEVD-pNA) and caspase-9 (Ac-LEHD-pNA) activities were measured at 405 nm through release of the chromophore p-nitroaniline (pNA) (Deng et al., 2009). Activities were calculated from a pNA calibration curve (0–100 μM) and data were normalised by the protein content of each sample and the results expressed as fold change over control. Protein quantification in samples was performed at 595 nm according to the Bradford method (Bradford, 1976) with bovine serum albumin as a standard (0–1.4 mg mL−1). All samples were performed in duplicate and measured against a reagent blank in the corresponding microplate. All enzymatic assays were carried out with 20 μL of sample at 30°C using a Pow-erWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Varian Cary Eclipse (Varian, USA)

![Diagram showing the timing of ketamine exposure in zebrafish early development](image-url)
spectrofluorometer, equipped with a microplate reader.

2.7. Analysis of gene expression

To determine gene expression patterns of oxidative-stress (sod1, cat, gstp1, gelc), apoptosis (casp3a, casp6, casp8, casp9, aifm1 and tp53) and cell proliferation (pca) related genes, three independent replicates of 300 viable embryos were used and 50 animals/replica were collected at 8 and 26 hpf for qRT-PCR analysis. Total RNA was isolated from whole zebrafish embryos stored in RNAlater (Sigma, Steinheim, Germany) using the Illustri RNAspin kit (GE Healthcare, Munich, Germany) according to previously described methodologies (Luzio et al., 2013; Felix et al., 2017c). Total RNA concentration and quality (260/280 nm ratio = 2) was quantified by spectrophotometric readings at 260 and 280 nm in a Take3 micro-volume plate (Powerwave XS2, BioTek Instruments, Inc. USA). RNA integrity and DNA contamination were verified by gel electrophoresis, using green safe premium staining (NZYTech, Ltd., Lisbon, Portugal) and detection under UV light using BioCapt software (v99.02, Vilber Lourmat, France). The ratio between 28S/18S bands intensity was quantified using the ImageJ2 program (version 2.0.0, U.S. National Institutes of Health, Bethesda, USA) (Schneider et al., 2012) and used as a measurement of quality. Only samples presenting a ratio equal or higher than 1.0 were used for further analysis. The cDNA was synthesised from 500 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, California, USA) following the manufacturer’s instructions. The qRT-PCR reactions were performed in triplicate using 1 μL of cDNA as template in a 20 μL reaction mixture containing 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) and 200 nM of each specific primer (Table 1). Gene-specific primers for proliferating cell nuclear antigen (pca) were designed using the online Primer3 primer design tool (http://primer3.ut.ee/) and analysed by Primer-BLAST to ensure specificity for the intended target gene within the zebrafish genome. The following thermal cycling conditions were used: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, primer annealing for 40 s (annealing temperatures in Table 1) and extension at 72°C for 20 s followed by a final extension at 72°C for 5 min. The melt curve analysis was carried out to confirm the specificity of the amplification reaction. Subsequent analysis of the results were carried out in duplicated reads using the software supplied with the Stratagene Mx3005P Real-Time PCR system (Agilent Technologies, Santa Clara, USA), whereas the expression was calculated by normalisation to the β-actin gene, used as housekeeping. Values were then normalised to the control average value calculated using the ΔΔCt method with efficiency correction (Pfaffl, 2001) based on standard curves of a 5-fold dilution series prepared from a mix of the used samples.

2.8. Statistical analysis

For this study, a sample size calculation was performed with the G’Power 3 (University of Düsseldorf, Germany) based on the standard deviations from previous studies from our group (Oliveira et al., 2013; Felix et al., 2014; Santos et al., 2014). In order to detect significant changes, assuming an α error probability of 0.05 and a power of 90%, it was determined that at least three replicates per group for gene expression and four replicates for biochemical analysis and in vivo staining procedures would be necessary. The normal distribution and the homogeneity of the data variances were tested by Kolmogorov–Smirnov and Levene’s tests, respectively. When the data did failed to accomplish the previous assumptions, groups were compared by a non-parametric independent samples test, Kruskal–Wallis, followed by Dunn’s pairwise comparison tests and data expressed as median and interquartile range (25th; 75th percentiles). Data normally distributed was analysed by a one-way analysis of variance (ANOVA) followed by Tukey’s pairwise comparison tests and data expressed as mean ± standard deviation. In the figures, data following both normal and non-normal distributions, both are expressed as median and interquartile range, as it is a more conservative approach. All tests performed were two-tailed and statistical analyses were carried out using SPSS for Windows (Version 22.0; Chicago, IL, USA) and differences were considered significant at p < 0.05.

3. Results

3.1. In vivo embryonic apoptosis and ROS

In vivo assays for apoptosis and ROS patterns were performed at 26 hpf. Acidine orange staining photographs of the zebrafish

### Table 1

Information of specific primers used for amplification in real-time PCR with GenBank accession numbers shown in parentheses.

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Fragment size (bp)</th>
<th>Annealing Temp. (°C)</th>
<th>R²</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping</td>
<td>β-actin (NM_181 601.4)</td>
<td>ACT GTA TGT TCT GGT GGT AC</td>
<td>TAC TCC TGC TTG GTA ATC C</td>
<td>197</td>
<td>60</td>
<td>0.993</td>
<td>94.0</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>gelc (NM_199 277.2)</td>
<td>CTA TCT GGA GAA CAT GGA GG</td>
<td>CAT TTT CTC TGG ACC GG</td>
<td>264</td>
<td>60</td>
<td>0.992</td>
<td>104.6</td>
</tr>
<tr>
<td></td>
<td>gsp1 (NM_131 734.3)</td>
<td>TCC AGT CCA ACC CCA TCG</td>
<td>ATG AGA TCT GAT CAC CAA AC</td>
<td>255</td>
<td>60</td>
<td>0.992</td>
<td>102.0</td>
</tr>
<tr>
<td></td>
<td>sod1 (Y12236.1)</td>
<td>AAG AAG CCA TGC AAG AGC GTC ACT</td>
<td>ACA TTA CCC AGC TCC CCC AC</td>
<td>165</td>
<td>60</td>
<td>0.989</td>
<td>113.5</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>cat (AF170069.1)</td>
<td>AGA TAC AAC TGT GGA AGG AGG GTC</td>
<td>AAA CAC TTT GGC TTT GGA CAT GGG</td>
<td>269</td>
<td>60</td>
<td>0.990</td>
<td>110.2</td>
</tr>
<tr>
<td></td>
<td>casp3a (NM_131 877.3)</td>
<td>CCG TGT CCC ACT ACT A</td>
<td>ATC TCT TCA CGA CCA TCT</td>
<td>129</td>
<td>60</td>
<td>0.987</td>
<td>102.5</td>
</tr>
<tr>
<td></td>
<td>casp6 (NM_001020497.1)</td>
<td>AGG ACA GCC CTT CAG CAC A</td>
<td>TGA GAC CCA TCC CCC TCT TGT</td>
<td>132</td>
<td>60</td>
<td>0.982</td>
<td>99.8</td>
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<td></td>
<td>casp8 (NM_131 510.2)</td>
<td>GGG CAA AGC TGG CAA CAT C</td>
<td>CTT CTA CTA GAG CAA GTC TGC</td>
<td>200</td>
<td>58</td>
<td>0.995</td>
<td>106.7</td>
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<tr>
<td></td>
<td>casp9 (NM_001067402.4)</td>
<td>CTC GCG CAA GCC ATC G</td>
<td>ACA AGA CAT CCG AAT AGC CT</td>
<td>248</td>
<td>62</td>
<td>0.987</td>
<td>102.6</td>
</tr>
<tr>
<td></td>
<td>aifm1 (AY423007.1)</td>
<td>AAA GTC CCG AAA CAG GGT</td>
<td>GCC TGG ACG TCA TCA TAC AC</td>
<td>224</td>
<td>60</td>
<td>0.998</td>
<td>106.8</td>
</tr>
<tr>
<td></td>
<td>tp53 (NM_131 327.2)</td>
<td>ACC ACT GCG ACC AAA CAG ATG</td>
<td>CAG AGT CCG TTC TTC CTC G</td>
<td>310</td>
<td>60</td>
<td>0.983</td>
<td>105.2</td>
</tr>
<tr>
<td>Cell Proliferation</td>
<td>pca (NM_131 404.2)</td>
<td>GCT CTT CCG GCA TTT CT</td>
<td>CAG AGT CTT CCA GGC</td>
<td>572</td>
<td>58</td>
<td>0.996</td>
<td>109.1</td>
</tr>
</tbody>
</table>

b. Usenko et al. (2012).
c. Liu et al. (2013).
d. Deng et al. (2009).
e. Sanden et al. (2012).
g. Luzio et al. (2013).
h. Chakraborty et al. (2009).
embryos (Fig. 2A) showed that embryos exposed to ketamine exhibited a dose-dependent delay in development as well as a dose-dependent increase \((X^2 (3) = 13.15, p = 0.004)\) in overall apoptosis patterns. In this regard, a higher fluorescence intensity was observed in the embryos exposed to 70 \((p = 0.028)\) and 90 mg L\(^{-1}\) \((p = 0.001)\) ketamine comparatively to the control group. Differences in fluorescence intensity were also observed between embryos exposed to the lowest and the highest ketamine concentration \((p = 0.011)\). Overall, apoptosis in ketamine exposed embryos was mainly observed in the tail, fins, pericardium zone and hindbrain and in the midbrain. The ROS distribution in control and ketamine exposed embryos showed a similar distribution \((F (3,13) = 1.19, p = 0.362)\), as seen in DCFH-DA stained embryos (Fig. 2B).

### 3.2. Oxidative stress and apoptosis related biomarkers

The ROS production, enzymatic determinations, glutathione levels and oxidative damage biomarkers were measured in embryos at 8 and 26 hpf and the results are presented in Fig. 3. No significant differences between groups are presented in Table S1. In comparison to the control group, the activities of SOD \((26.5 \pm 3.91 \text{ U mg protein}^{-1})\) and CAT \((47.6 [46.4–60.1] \text{ U mg protein}^{-1})\) at 8 hpf were dose-dependently increased \((X^2 (3) = 12.16, p = 0.007)\) and \(F (3,18) = 10.40, p < 0.001\) comparatively to the control group \((41.1 [27.6–52.2] \text{ nmol pNA mg protein}^{-1})\). At 26 hpf, all these differences disappeared. Notwithstanding, a dose-dependent increase in ROS production \((F (3,21) = 6.29, p = 0.003)\) was observed at 26 hpf with 70 and 90 mg L\(^{-1}\) groups showing significantly differences from the control group \((310 [265–415] \text{ nmol DCF mg protein}^{-1})\) \((p = 0.004)\). An increase of caspase-3 activity \((F (3,18) = 6.72, p = 0.004)\) in the groups 50 and 90 mg L\(^{-1}\) \((p = 0.014)\) comparatively to the control group \((41.1 [27.6–52.2] \text{ nmol pNA mg protein}^{-1})\).

Likewise, at the same time-point, GSSG levels were increased \((X^2 (3) = 9.30, p = 0.026)\) in ketamine groups relative to the control group \((94.5 [77.0–124] \text{ nmol mg protein}^{-1})\) \((p = 0.018)\) and \(p = 0.004\) comparatively to the control group \((41.1 [27.6–52.2] \text{ nmol pNA mg protein}^{-1})\). At 26 hpf, all these differences disappeared. Notwithstanding, a dose-dependent increase in ROS production \((F (3,21) = 6.29, p = 0.003)\) was observed at 26 hpf with 70 and 90 mg L\(^{-1}\) groups showing significantly differences from the control group \((310 [265–415] \text{ nmol DCF mg protein}^{-1})\) \((p = 0.004)\). An increase of caspase-3 activity \((F (3,18) = 6.72, p = 0.004)\) in the groups 50 and 90 mg L\(^{-1}\) \((p = 0.014)\) comparatively to the control group \((41.1 [27.6–52.2] \text{ nmol pNA mg protein}^{-1})\). Although slightly variations in the remaining parameters were observed, no statistically significant differences were observed.

**Fig. 2.** Quantification and distribution of apoptotic cells (A) and reactive oxygen species (B) in 26 hpf zebrafish embryos after exposure to ketamine for 24 h. Data represented as median and interquartile range from at least four independent samples (12 embryos/each) and expressed as fold-change of control. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple-comparison test or using the Kruskal–Wallis test followed by Dunn’s test. Different lowercase letters indicate significant differences between groups \((p < 0.05)\). An increasing number of apoptotic cells stained with acridine orange was observed in the tail (t), fins (f), pericardial zone (pc), hindbrain (hb) and midbrain (mb) with increasing ketamine concentrations. The pericardial (pc), yolk sac (ys) and yolk extension (ye) were the zones were an increase in the ROS distribution (DCFH-DA staining) was detected. Scale bar represents 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
exposure. To our knowledge, this is the first study to examine the oxidative stress and apoptosis were examined following a 24 h exposure. No other statistically significant differences were observed between groups (p < 0.05).

3.3. Gene expression profile

The statistically significant differences in expression levels of the studied genes are summarised in Fig. 4 while remaining results are presented in Table S2. Changes in the expression patterns of sod1 at 8 (F (3,10) = 4.81, p = 0.025) and 26 hpf (F (3,10) = 7.82, p = 0.003) as well as in the cat levels at 8 (F (3,10) = 7.36, p = 0.007) and 26 hpf (F (3,10) = 8.73, p = 0.002) were observed. The highest dose of ketamine induced an increase of sod1 gene expression at 8 hpf (p = 0.030) and an inhibition at 26 hpf (p = 0.013) compared to the control group (relative expression of 1.35 ± 0.02 and 1.38 ± 0.05 at 8 and 26 hpf, respectively). A significant increase in cat mRNA levels was observed at 8 hpf in the groups exposed to 50 mg L⁻¹ (p = 0.027) compared to the control group (relative expression of 1.35 ± 0.02). At 26 hpf, a significant down-regulation of cat gene was observed for the 70 and 90 mg L⁻¹ groups (p = 0.004 and p = 0.008, respectively) compared to the control group (relative expression of 1.38 ± 0.05). The expression of apoptosis-related genes was also affected by ketamine exposure. Almost a 2-fold increase regarding control group (relative expression of 1.31 ± 0.03) was observed in the levels of casp6 mRNA at 8 hpf (F (3,8) = 5.65, p = 0.022) in the highest ketamine group (p = 0.029). Relative to the levels of casp8 (F (3,8) = 4.48, p = 0.040), changes were observed between the lowest and the highest ketamine group (p = 0.042). Changes in the levels of casp9 gene were also affected by ketamine exposure at 8 and 26 hpf (X² (3) = 8.231, p = 0.041 in both cases). In this regard, the expression of casp9 gene was increased at 8 hpf and at 26 hpf (p = 0.009) by the highest ketamine dose compared to the control group (relative expression of 1.52 ± 0.02 and 1.58 ± 0.03, respectively). At 8 hpf, the levels of tp53 mRNA were increased (X² (3) = 9.667, p = 0.022) after exposure to 70 (p = 0.024) and 90 mg L⁻¹ (p = 0.005) of ketamine compared to the control group (relative expression of 1.21 ± 0.02).

No other statistically significant differences were observed for the remaining genes.

4. Discussion

In the present study, the mechanisms of ketamine-induced oxidative stress and apoptosis were examined following a 24 h exposure. To our knowledge, this is the first study to examine the oxidative stress and cellular death effects of a 24 h acute exposure to ketamine in zebrafish early development stages. The results of this work show that ketamine exposure during a 24 h period during early organogenesis of zebrafish induced, besides the already described abnormal development (Felix et al., 2017b), changes on enzymatic biomarkers in addition to a substantial impact on the expression of oxidative stress- and apoptotic-related genes.

It is worth noting that the concentrations of ketamine used in this study are not routinely detected in aquatic systems, as the maximum environmental concentration of ketamine has been reported to be around 10 μg L⁻¹ (Lin et al., 2014) with peak concentrations up to about 140 μg L⁻¹ (Jiang et al., 2015). In this regard, the highest concentration used was 600× above the environmental concentration reported. Still, there is an uncertainty about the pharmacological impact of ketamine in aquatic environments with studies using different aquatic models showing different responses with either relevant environmental conditions (Liao et al., 2015) or even higher concentrations (up to 500 mg L⁻¹) (Guo et al., 2016; Li et al., 2017a). The potential risk assessment of ketamine based on the calculated risk quotients (RQ) for aquatic environments, suggests that there is no ecotoxicological risk (RQ < 1) (Escher et al., 2011). RQ is a widely standardised measure of risk (Escher et al., 2011) based on the comparison of the estimated environmental exposure with a toxicity end-point value, such as the LC50 (Peterson, 2006). Notwithstanding, ketamine has been shown to remain chemically and biologically active in the aquatic environment following natural and conventional purification processes (Lin et al., 2014; Yadav et al., 2017). Moreover, ketamine loads have shown an overall increasing trend (Castiglioni et al., 2015; Du et al., 2015) that will result in increasing environmental contamination with possible low-level cumulative ecological impacts (Wang and Lin, 2014). Although the approach used is based on a concentration-effect relationship, which may overestimate the toxicological impact of low exposure concentrations (Zurlo, 2012), it allows further understanding of the biological effects, providing an estimation of ketamine’s ecotoxicological risk through the establishment of the minimum concentration with detectable toxic effects. Indeed, our previous study showed that 50 mg L⁻¹ and higher ketamine concentrations induced mortality, and alterations on morphology and behaviour (Felix et al., 2017b). The present study better clarifies these toxicological effects and the
mechanisms involved, which are imperative for assessing the risk in non-target species.

The early zebrafish development is a complex process driven by a highly coordinated cascade of events (Bonneau et al., 2011). The redox balance is essential to the embryonic development by playing defined functions in signalling pathways such as proliferation, differentiation, cell death and migration (Dennery, 2007, 2010; Covarrubias et al., 2008). It is well established that SOD and CAT are important enzymes acting together to reduce ROS (Slaninova et al., 2009) while GPx operates at low hydrogen peroxide concentrations (HalliwelI and Gutteridge, 2015). According to this principle and knowing that in the current work GPx had an unaltered activity, the increase in SOD and CAT activity observed after 6 h of ketamine exposure, suggests a high level of hydrogen peroxide resulting from superoxide dismutase that is manageable by catalase, thus, causing a delay in ROS accumulation. These results suggest an adaptive response to ketamine, promoted through the increased antioxidant enzymes activity as an attempt to eliminate surplus reactive radicals at 8 hpf, which is no longer manageable at 26 hpf, when ROS production increases and probably contribute to the effects observed later in development. Previous data has also shown that ketamine induces oxidative stress responses in medaka (Oryzias latipes) embryos following 7 days of exposure to environmentally relevant concentrations (Liao et al., 2015). Furthermore, an up-regulation of sod1 and cat genes was observed at 8 hpf whereas a down-regulation of mRNA levels of both genes was observed at 26 hpf. This may represent a dual response of the embryo to ketamine exposure by augmenting transcript levels until a threshold value above which the transcript levels underlies the augmented levels of ROS.

Notwithstanding, a decrease in protein carbonyls was observed in embryos exposed to the lowest ketamine doses, thereby, suggesting a protective mechanism to lower the negative effects of ROS induced by ketamine. Additionally, changes in glutathione homeostasis (increase in GSSG levels at 8 but not at 26 hpf and slight variations in GSH levels) were observed during ketamine exposure, but alterations in the oxidative stress ratio (GSH/GSSG ratio) and in the expression of the glutathione biosynthetic gene (gclc) were not perceived. The glutathione redox system is tightly regulated during embryonic development (Hansen and Harris, 2015) and is important for maintaining the biological state of a cell. The balance between GSH and GSSG is maintained intracellularly through the action of several additional enzymes and compounds that, when disrupted, affect several redox-signalling mechanisms (Schafer and Buettner, 2001, Jones, 2002). Despite the relation between the cellular redox state and an abnormal embryo development, the underlying mechanism is not entirely understood (Hansen and Harris, 2015). In the last few years, the excessive ROS production during development and the resulting phenotypes induced by substances have been linked to mitochondrial pathway (Pinho et al., 2013; Zhao et al., 2016). Indeed, ketamine has been shown to adversely affect zebrafish mitochondria membrane (Robinson et al., 2017b), supporting an increase in ROS production. Based on the results of the present work, a mechanism is proposed in which ketamine stimulates hydrogen peroxide production. Then the levels of GSSG increase, as observed, as an attempt to maintain the glutathione cycle balance. Supporting this hypothesis, previous studies have also reported ketamine to induce hydrogen peroxide generation by mitochondrial dysfunction in other organisms (Venancio et al., 2015), but more extensive examination is needed to further establish this association.

According to the literature (Schafer and Buettner, 2001; Dennery, 2007, 2010), changes in the cellular redox environment can impact embryonic development by shifting the fate of cells in the embryo toward proliferation, differentiation or cellular death. Therefore, proliferation is favoured by a reduced oxidative state...
whereas differentiation and cell death are favoured by mild and high oxidative settings, respectively. The results of the present study suggest a general change to a more oxidised environment induced by ketamine exposure, as observed by the increase of GSSG in ketamine groups. Thus, in agreement, it was expected to observe an increase in apoptosis rather than proliferation and differentiation. Indeed, no changes in proliferation were observed as confirmed by the outcome of pcna gene expression while changes in apoptosis-related genes were detected. Accordingly, the gene expression of casp6, casp9 and tp53 were significantly increased and a trend to increase casp8 transcript levels was observed during ketamine exposure.

Typically, p53 integrates a variety of signals that control lethality and teratogenesis (Choi and Donehower, 1999). Furthermore, p53 activates the apoptotic machinery through stimulation of cytochrome c release from mitochondria (Schuler et al., 2000; Mihrara et al., 2003) which promotes the activation of caspase-9 (Jiang and Wang, 2000). Active caspase-9 then prompts an executioner caspase activation cascade (caspase-3, -6 and -7) (Cullen and Martin, 2009; Mcllwain et al., 2013). Caspase-6 has been established as a major activator of caspase-8 in vivo, which in turn has an important role in the positive feedback loop, linking caspase activation to mitochondrial dysfunction (Cowling and Downward, 2002). Furthermore, caspase-6 can regulate its own activity through unique intramolecular mechanism of self-activation (Klaiman et al., 2009; Edington et al., 2012). Considering the above, and the outcomes of this study concerning alterations in the caspases activity and expression, it is hypothesised that ketamine-induced apoptosis not only involves the mitochondrial pathway, as described in vitro (Braun et al., 2010), but it also occurs via p53-dependent pathway, as previously reported in developing rats (Yan et al., 2014). This is also supported by the observed transient increase in GSSG levels, which precede cytochrome c release and apoptosis activation (Pias and Aw, 2002). Moreover, the AO staining assay was consistent with gene expression patterns observed, thus, confirming ketamine-induced apoptosis. Still, variations in caspase-9 catalytic activity were observed, and further investigation is needed as it is known that caspase catalytic activity is regulated by many cellular processes (Shi, 2002, 2004; Li et al., 2017b).

Moreover, severe developmental defects, similar to the ones observed when zebrafish embryos were exposed to ketamine (Felix et al., 2014, 2017b), have been associated with abnormal glycolysis (Xu et al., 2014, 2016), the predominant source of energy during these early stages of zebrafish (Stackley et al., 2011). As such, prior to the mid-blastula transition, there is a potential for oxidative damage to occur as embryos switch from anaerobic to aerobic metabolism (Stackley et al., 2011). Still, little is known about the interaction between toxic compounds and this pathway in zebrafish development. Notwithstanding, evidence in the literature supports a critical function of p53 in the development of the nervous system at later matured stages by regulating the proliferation and differentiation of neural progenitor cells (Jacobs et al., 2006; Tedeschi and Di Giovanni, 2009). Indeed, an up-regulation of p53-related apoptotic genes in neural crest progenitors has been associated with abnormal zebrafish development (Rinon et al., 2011). Furthermore, ketamine has been shown to alter the neurogenesis in zebrafish through possible interactions with notch1a or gli2b (Cuevas et al., 2013; Kanungo et al., 2013). Notch signalling is involved in a variety of cell-fate decisions for nervous system development through a p53-dependent mechanism (Yang et al., 2004). Again, this apparent interconnection seems to corroborate a model in which p53 signalling plays an important role in ketamine toxicity. In accordance, a recent study has shown that ketamine induces the disruption of p53-regulated apoptosis pathways in medaka larvae (Liao et al., 2017). Nevertheless, interactions with other signalling pathways could not be excluded. For instance, calcium signalling plays an essential role in the development of the nervous system (West et al., 2001; Toth et al., 2016) and mediates the activation of p53 (Liu et al., 2007). Also, previous published data highlighted the teratogenic potential of ketamine by showing dose-dependent and stage-specific malformed phenotypes, accompanied by changes in molecular markers associated with distinct signals related to oxidative stress, osmoregulation and apoptosis processes (Felix et al., 2014, 2016a, 2016b, 2017a, 2017b, 2017c). Thus, further research using different technical approaches is needed to clarify the pathways and molecular mechanisms of p53 signals involved.

In summary, a stage-dependent mechanism by which ketamine triggers the transcripational activation of p53 in response to ketamine-induced oxidative stress is proposed. Although tested concentrations were above the concentrations expected to occur in the environment, the results of this study extend the current knowledge on ketamine toxicological effects that may be useful in risk assessment procedures through the establishment of concentrations associated with no or only minimal toxic effects in this species. Further investigation on the toxic effects to aquatic species and human health should be undertaken, due to the complex interactions of combined illicit drugs in aquatic ecosystems and unexpected pharmacological interactions.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.03.049.

**References**


