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Short-term exposure to dexamethasone at environmentally relevant concentrations impairs embryonic development in *Cyprinus carpio*: Bioconcentration and alteration of oxidative stress-related gene expression patterns

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ABSTRACT

In recent years and as a result of the Covid-19 pandemic, the consumption of dexamethasone (DXE) has increased. This favors that this corticosteroid is highly released in aquatic environments, generating deleterious effects in aquatic organisms. The information on the toxic effects of DXE in the environment is still limited. Thus, the objective of this work was to determine whether DXE at short-term exposure can cause alterations to embryonic development and alteration of oxidative stress-related gene expression patterns in *Cyprinus carpio*. For this purpose, common carp embryos (2 hpf) were exposed to realistic concentrations of DXE until 96 hpf. Alterations to embryonic development were evaluated at 12, 24, 48, 72 and 96 hpf. In addition, oxidative stress in carp embryos at 72 and 96 hpf was evaluated by cellular oxidation biomarkers (lipoperoxidation level, hydroperoxide and carbonyl protein content) and antioxidant enzymes activities (superoxide dismutase and catalase). Oxidative stress-related gene expression (*sod, cat* and *gpx1*) was also evaluated. Our results showed that DXE concentrations above 35 ng/L are capable of producing alterations to embryonic development in 50 % of the embryo population. Furthermore, DXE was able to induce alterations such as scoliosis, hypopigmentation, craniofacial malformations, pericardial edema and growth retardation, leading to the death of half of the population at 50 ng/L of DXE. Concerning oxidative stress, the results demonstrated that DXE induce oxidative damage on the embryos of *C. carpio*. In conclusion, DXE is capable of altering embryonic development and generating oxidative stress in common carp *C. carpio*.

Abbreviations: API, active pharmaceutical ingredients; ATP, adenosine triphosphate; ANOVA, analysis of variance; BCF, bioconcentration factor; BB, bleeding in the body; BH, body hypopigmentation; Orai/STIM1, calcium release-activated calcium modulator 1/sensor stromal interaction molecule 1; CAT, catalase; *cat, catalase gen*; CS, cyclops or squint; CM, craniofacial malformation; DXE, dexamethasone; DMSO, dimethyl sulfoxide; EC_{50m}, effective concentration 50 of malformations; ESI, electrospray ionization; FM, fin malformation; *gpx*, *glutathione peroxidase gen*; GR, growth retardation; HR, hatching retardation; HH, head hypopigmetation; HPC, hydroperoxide content; K, kyphosis; LC_{50}, lethal concentration 50; LOD, limits of detection; LOQ, limits of quantification; LPX, lipid peroxidation level; LC-MS/MS, liquid chromatography-mass spectrometer; L, lordosis; TOM, membrane translocase; PE, pericardial edema; PCA, principal components analysis; PCC, protein carbonyl content; R, rachischisis; ROS, reactive oxygen species; S, scoliosis; SGK1, serum and glucocorticoid inducible kinase 1; SFE, solid phase extraction; SM, somites malformation; SOCE, store operated calcium entry; SOD, superoxide dismutase; *sod*, *superoxide dismutase gen*; SARS-CoV-2, syndrome due to Coronavirus-2; TCA cycle, tricarboxylic acid cycle; YSM, yolk sac malformation.

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1. Introduction

Dexamethasone (DXE) is a drug belonging to the group of synthetic fluorinated corticosteroids, being one of the most potent drugs of this family (Benedek, 2011; Herráiz, 2017; Mehta et al., 2016; Orduña-Valls et al., 2016; Samuel et al., 2017). This compound has become the prototype of glucocorticoids due to its high tissue penetration (Buttgereit et al., 2005; Stahn and Buttgereit, 2008). Also, DXE is widely employed in the treatment of acute and chronic inflammatory and immune disorders (Coutinho and Chapman, 2011; Lammers et al., 2020), especially in acute congenital adrenal insufficiency syndrome, rheumatoid arthritis, ankylosing spondylitis, eczema and psoriasis. DXE is also used, in certain types of leukemias and lymphomas (Rekers et al., 2016; Kapugi and Cunningham, 2019; McCann-Crosby et al., 2008; Cabrera-Rayo et al., 2020). In the recent covid-19 pandemic, DXE became highly relevant as a drug that has proven to be effective in the management of critically ill patients. As a consequence, concern has arisen about their presence in aquatic ecosystems, as well as the deleterious effects on the organisms present in these environmental matrices (Musee et al., 2021; Morales-Paredes et al., 2022).

The consumption of corticosteroids in the world has increased in recent years. Reports refer that by 2019 it was 4.2 USD billion. In 2020 it increased to 4.47. However, in 2021 consumption increased to 4.56, representing a compound annual growth rate of approximately 2 %. This report covers North America, South America, Asia & Pacific, Europe, and MEA (Middle East and Africa). Projections indicate that by 2025 the consumption of these drugs is expected to increase to 5.33 USD billion (Corticosteroids Global Market Report 2019, 2020 and 2021).

Due to its high use, consumption, and production, DXE reaches variant environment matrices. This compound has been identified in hospital effluents, industrial effluents, wastewater treatment plants (WWTP) influents and effluents, groundwater, seawater, sediments, soil, surface water, and drinking water across the globe at concentrations of ng/L (Kitaichi et al., 2010; Schriks et al., 2010; Tölgyesi et al., 2010; Fan et al., 2011; Kovalova et al., 2012; Herrero et al., 2012; Liu et al., 2012; Oliveira et al., 2012; Zhong-Quan et al., 2012; Arsand et al., 2013; Schriks et al., 2013; Creusot et al., 2014; Macikova et al., 2014; Castro-Pastrana et al., 2015; Jia et al., 2016; Zhou et al., 2016; Praveena et al., 2018; Weizel et al., 2018; Shen et al., 2020).

The constant incorporation of small concentrations of DXE in various aquatic matrices, as well as in domestic wastewater, hospital discharges and industrial effluents, most of which end up in WWTPs, contribute to the presence of DXE in aquatic matrices. In addition, due to the low octanol-water partition coefficient (log Kow ≈ 1.9) of DXE and the fluoro-molecular structure, this compound is resistant to removal in conventional methods using WWTPs. This results in DXE being found in trace amounts in aquatic environments, posing a risk to aquatic organisms (Bain et al., 2014; Jia et al., 2016; Aubakirova et al., 2017; Teodosiu et al., 2018).

The information of the toxic effects of DXE in aquatic organisms is scarce. This corticosteroid has been shown to alter glucose and protein metabolism, generate oxidative stress, endocrine disruption, neurotoxicity, histological alterations at concentrations of 500–19, 700 ng/L in organisms such as *Danio rerio, Gambusia affinis, Hoplias malabaricus* and *C. carpio* (Nakayama et al., 2014; Chen et al., 2016; Zhong et al., 2021).

Several studies have pointed out that reactive oxygen species responsible for oxidative stress act as messengers and as a signaling pathway to induce cell growth or cell death. Therefore, oxidative stress can alter embryo proliferation, development and growth (Hansen, 2006; Dennery, 2007).

In light of the above information and the fact that the toxic effects of DXE have not been evaluated at realistic concentrations and in a species of economic interest such as the common carp, we decided to determine whether this corticosteroid at short-term exposure can cause alterations to embryonic development and oxidative stress in *C. carpio*. Our hypothesis is that DXE will increase reactive oxygen species (ROS) production in common carp embryos, triggering oxidative stress that will generate embryonic alterations in them.

2. Methods

2.1. Ethics statement

The procedures performed in this work adhered to the norms of the Ethics and Research Committee of the Autonomous University of the State of Mexico (approval ID: RP.UAEM.ERC.075.2021).

2.2. Chemical and reagents

Dexamethasone (9-fluoro-11β, 17, 21-trihydroxy-16α-methylpregna-1, 4-diene-3, 20 dione) with CAS number: 50-02-2 and all other reagents used in this work were purchased from Sigma-Aldrich (St. Louis, MO). The stock solution of DXE was prepared by dissolving DXE powder in DMSO 0.01 %.

2.3. Common carp housing and egg production

The carp used for this study were provided from the Centro Carpicola Tiacaque located in the municipality of Jocotitlán, State of Mexico. This center is the main producer of common and grass carp in the country. The organisms used for the study were of reproductive age with a size of 50 to 62 cm and a weight between 6 and 8 kg.

The test organisms were housed in breeding ponds for 20 days prior to the experiment. In these ponds, males were separated from females. Water quality for fish maintenance and exposure was measured daily (Table 1). Also, we ensured that the temperature (23 ± 1 °C) and light-dark cycles (12:12 h) were constant during the fish housing process. Fish were fed daily with protein supplemented food (35 %), enriched with carbohydrates and other nutrients to ensure that the carps improve the desired husbandry index.

One day prior to the embryotoxicity experiment, two males and four females in reproductive age were placed in the spawning ponds to obtain the eggs. To promote fertilization, the females were administered a dose of gonadotropin by IV 500 IU/kg. The bottom of the spawning pond was covered with casuarina branches to favor egg deposition. For embryotoxicity tests, only fertilized eggs that were in the blastula stage at 2 hpf were used.

2.4. Embryotoxicity test

Embryonic toxicity of all nine DXE realistic concentrations (20, 25, 30, 35, 40, 45, 50, 55 and 60 ng/L) was established following the OECD guidelines (OECD, Test No. 236: fish embryo acute toxicity (FET) test) modified by Luja-Mondragón et al., 2019. Briefly, eggs in the blastula stage at 2 hpf were selected and randomly distributed into 20-well plates (one embryo per well). For test systems, DXE realistic concentrations used were selected based on the occurrence studies (Kitaichi et al., 2010; Schriks et al., 2010; Tölgyesi et al., 2010; Fan et al., 2011; Kovalova et al., 2012; Herrero et al., 2012; Liu et al., 2012; Oliveira et al., 2012; Zhong-Quan et al., 2012; Arsand et al. 2013; Schriks et al., 2013; Creusot et al., 2014; Macikova et al., 2014; Castro-Pastrana et al., 2015; Jia et al.,

Table 1
Measured water parameters in breeding and spawning ponds.

Water parameter	Measured value
Dissolved oxygen	95 % saturation
Nitrite	0.031 ± 0.011 mg/L
Nitrate	3.0 ± 0.42 mg/L
pH	7.17 ± 0.12
Un-ionized ammonia	0.012 ± 0.005 mg/L

Values are expressed as mean ± standard deviation.

2016; Zhou et al., 2016; Praveena et al., 2018; Weizel et al., 2018; Shen et al., 2020). Simultaneously a DXE free control and a control with a 0.01 % Dimethyl sulfoxide (DMSO) solution were used. The experiments were replicated in quadruplicate. Plates were incubated in a *Prendo bioclimatic chamber (CB-14)* for five days at 24 ± 1 °C and light-dark photoperiods (13:11). The systems were refreshed with medium every 24 h. The temperature and humidity parameters of the bioclimatic chamber were controlled until the end of the test and there were no alterations. The number of live, dead and malformed embryos at 12, 24, 48, 72 and 96 h were counted. The data obtained were used to calculate the lethal concentration 50 (LC_{50}), effective concentration 50 of malformations (EC_{50m}) and their respective 95 % confidence intervals ($p < 0.05$), using the USEPA Toxicity Estimation Software Tool (TEST) ver. 5.1.2. The teratogenic index (TI) was calculated using the ratio LC_{50}/EC_{50m}. If the TI was >1, DXE was considered as teratogenic and if it was <1 as embryolethal, according to criteria of (Weigt et al., 2011).

2.5. Evaluation of morphology scoring and embryonic developmental alterations

The systems referred to in Section 2.4 were used for this purpose. DXE-induced alterations to embryonic development were calculated using the score established by Kimmel et al. (1995) and Hermsen et al. (2011) and modified by Luja-Mondragón et al., 2019. The entire mortality and alterations to embryonic development of *C. carpio* were monitored and recorded at 24, 48, 72 and 96 h post-fertilization (hpf). Coagulation, lack of somites, non-detachment of the tail and no heartbeat were considered as the lethal endpoint. In addition, somites malformation, bleeding in the body, rachischisis, kyphosis, lordosis, scoliosis, fin malformation, cyclops or squint, craniofacial malformation, hatching retardation, body hypopigmentation, head hypopigmetation, pericardial edema, yolk sac malformation, growth retardation (although within these we were able to identify morphological alterations and teratogenic effects, we categorized them as alterations to embryonic development to be consistent with the previously mentioned criteria), were evaluated as alterations to embryonic development endpoint. The percentage of mortality was also estimated. The alterations considered as lethal endpoints were:

(1) Embryonic coagulation - when it occurs within hours of the onset of exposure and indicates a generic acute toxic effect and is related to embryo death.
(2) Lack of somite formation: somites should be visible at 12 hpf. In case of lack of somites, this may alter the development of the embryo and may lead to its death;
(3) No tail detachment: tail detachment from the yolk can be observed 24 h after fertilization, indicating normal embryo growth. If it does not occur, the embryo will die.
(4) Absence of heartbeat: the heartbeat is easily detectable 30 h after fertilization; its absence indicates embryo death. Coagulation of the embryo and absence of heartbeat were considered criteria for mortality.

The criteria for embryo lethality and embryonic alterations were considered according to the methodology established by Kimmel et al. (1995) and Hermsen et al. (2011).

2.6. Quantification of DXE and determination of bioconcentration factors

For the determination of DXE in water and *C. carpio* embryos exposed to realistic concentrations of DXE the following methodology was used:

Briefly, the determination of DXE in water samples was performed by a two-step filtration (first an 8 μm membrane was used followed by a 2.5 μm membrane). Phenomenex, Strata XL (Torrance, CA) solid phase extraction (SFE) cartridges were used, these were conditioned with 10 mL of methanol followed by 10 mL of milli Q® water. The water samples with DXE internal standard were added to the SFE cartridges at a flow rate of 5 mL/min and then eluted with 6 mL of methanol. The solvent was evaporated to dryness using N-Evap 112 nitrogen (Organomation, Haverhill, MA). Samples were reconstituted with 1 mL of methanol: water (50:50) and passed through a 0.2 μm syringe filter prior to injection.

In the case of DXE determination in carp embryos, these were washed with water and dried. 0.50 g of carp embryos were weighed and added DXE internal standard. The DXE-enriched sample was shaken in a vortexer (Jeho Tech VM-96B, Korea) for 30 s and stored at −20 °C for 30 min. Briefly, 20 mL of methanol: milli Q® water (1:1) was added and extracted for 10 min using an ultrasonic cleaner (Nexul NXP 1002, Japan). Subsequently, the sample was centrifuged at 24,000 rpm for 10 min to achieve a good separation of the organic phase. SPE cleanup was performed by conditioning the cartridge with 5 mL of methanol, 5 mL of ultrapure water (UPW) and 5 mL of methanol: milli Q® water (1:1). After sample loading, 5 mL of milli Q® water was used to rinse the cartridge, which was dried under vacuum for 15 min. For sample elution, 10 mL of MeOH followed by 5 mL of acetone:methanol (1:1) was used and the cartridge was dried under vacuum for 3 min. The sample eluent was first concentrated using a rotary evaporator before being concentrated again under a gentle flow of nitrogen (N_2) to near dryness. The eluent was reconstituted to 1 mL using acetonitrile: milli Q® water (3:7) and filtered using a 0.2 μm PTFE membrane filter.

LC-MS/MS (liquid chromatography-mass spectrometer) by Waters (Mildford, MA, US) was used for quantification and identification of DXE in water and embryos. An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) was used. The mobile phases were 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in methanol (B). The column was maintained at 40 °C. The injection volume was 10 μL and the flow rate was 0.3 mL/min. Total run time of 10 min with 90 % A at 0.1 min, 10 % A at 8 min and 90 % A at 10 min. Quattro Premier XE triple quadrupole mass spectrometer fitted with electrospray ionization (ESI) was used in positive mode.

The limits of quantification (LOQ) and detection (LOD) were calculated according to Brubaker (1999):

$LOD = t_{0.99} \times S$ and $LOQ = 3 \times LOD$. Where, $t_{0.99}$ = the one-tailed statistic at the 99 % confidence level for n replicates, S = the standard deviation of recovery results from n samples fortified at the estimated LOQ.

The mass spectrometer parameters used in the quantification of DXE were: ionization mode positive, parent ion (m/z) = 363.2, product ion (m/z) = 147.1, cone voltage (V) = 20, Detection limit (LOD) = 1.05 ng/L, quantification limit (LOQ) = 3.15 ng/L for water. Detection limit (LOD) = 0.13 ng/L, quantification limit (LOQ) = 0.39 ng/L for carp embryos.

Employing the DXE concentration in both the embryos of *C. carpio* and the surrounding environment, the bioconcentration factor (BCF) was calculated using the following formula: BCF = [concentration in embryos] / [concentration in surrounding environment] (García-Medina et al., 2022).

To evaluate the matrix effect, the slopes obtained in the matrix-fitted calibration curves were contrasted with those obtained with the solvent standards. Matrix effects (ME%) were calculated by subtracting 1 from the ratio of the slope of the matrix-matched calibration curve (B) to the slope of the DXE standard solution curve (C), and then multiplying by 100:

$$ME(\%) = \left(\frac{CB}{C} - 1\right) 100\%.$$

The signal is enhanced if the value is positive, whereas it is suppressed if the value is negative. A signal enhancement or suppression effect is considered as acceptable if the matrix effect values range from 20 % to +20 % (Lopes et al., 2012).

2.7. Assessment of oxidative stress in zebrafish embryos

Eleven systems, each with 1000 *Cyprinus carpio* embryos at the gastrula stage, were formed in aquariums of 4 L capacity. Each system was spiked with the realistic concentrations of DXE previously noted. All along the exposure period, temperature was kept constant (24 ± 1 °C). At 72 hpf and 96 hpf, 500 embryos were randomly selected and homogenized in 1 mL of phosphate buffer solution (pH 7.4) employing an Ultra-turrax T25 (IKA, Germany). These endpoint times were selected because at that time the *C. carpio* embryos had already hatched and their enzymatic system was already working.

In order to minimize the biases in this research, we considered the following points: 1) the main researcher of this research would know the treatments at which embryos were exposed; 2) one lab technician would be in charge of preparing the test solutions and the maintenance of the systems; and 3) another lab technician would be in charge of the randomization process. This way, lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization.

Samples were separated into two Eppendorf tubes. For tube 1, 300 μL of the homogenate was taken and 300 μL of a 20 % trichloroacetic acid solution was added, centrifuged at 11,495 rpm at 4 °C for 15 min, the precipitate was used for the determination of protein carbonyl content (PCC), and the supernatant for the degree of lipoperoxidation level (LPX) and hydroperoxide content (HPC). For tube 2, 700 μL of the homogenate was taken, centrifuged at 12,500 rpm at 4 °C for 15 min for the determination of superoxide dismutase (SOD) & catalase (CAT) activity and total protein (TP) content. Samples were analyzed in a spectrophotometer (Shimadzu, UV-2600). The experiments were replicated in quadruplicate. For each replicate, 4 readings were performed. Reproducibility of results was assessed calculating the precision of them. Precision was calculated using the following formula: C.V. = ($^{\mu s}$) 100 %.

The details of the methods used are summarized in Table 2.

2.8. Gene expression

For the evaluation of *gene expression* of oxidative stress-related genes, the technique established by Collins et al. (2022) was used. The analysis was carried out in the molecular biology room, wish has a laminar flow hood. Briefly, 100 common carp embryos exposed to realistic concentrations of DXE at different exposure times (12, 24, 48, 72 and 96 hpf) were homogenized and RNA was isolated with Qiagen RNeasy® protect mini kit following the manufacturer's procedure. Concentration was determined by 260/280 ratio using a microvolume spectrophotometer and RNA purity was assessed by agarose gel electrophoresis. Reverse transcription was performed using at least one microgram of total RNA and the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF 205313), considering the following conditions: 42 °C for 15 min and 95 °C for 3 min. The quantitative polymerase chain reaction (qPCR) was performed with a Rotor-Gene Q (Qiagen), under the following conditions: 94 °C for 15 s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Each reaction was carried out in a 50 μL solution containing 0.3 μmol of primers, 25 μL of SYBER Green QuantiTec® (QIAGEN, Hilden, Germany) and 500 ng of cDNA template. *β-actin* was used as a housekeeping gene to normalize all samples. The housekeeping gene was assessed for each organism in both control and expressed organisms. Experiments were replicated in quadruplicate. The genes used for qRT-PCR are related to the response to oxidative stress (Table 3).

Once the qPCR cycles were completed, a dissociation curve was performed to distinguish between specific and non-specific products, using the following conditions: 10 s at 95 °C, 10 s at 65 °C and 10 s at 95 °C with 0.2 °C increments. Data were collected at each increment of the melting curve.

The change of mRNA expression in the studied genes was calculated using the $2^{-\Delta\ \Delta Ct}$ method (Livak and Schmittgen, 2001). The Ct of all study genes were normalized to the actin reference gene. In all cases each qPCR reaction was repeated four times to minimize experimental error.

2.9. Statistical data analysis

Once all endpoints were evaluated, results were analyzed by a researcher who did not know the treatment at which embryos were exposed. Results, from all four replicates of each experiment were

Table 3
Genes used for qRT-PCR.

Gene	Sequence (5′ -3′)	Reference
β-actin	Forward: AGCTAGGCCTTGAGCTAT Reverse: CCTGCTTGCTAATCCACA	Sinha et al., 2012
sod	Forward: CTGGACAAATCTGTCACCTA Reverse: TGCAGCAATCCTCAGTCT	Karaca et al., 2014
cat	Forward: AGCCAAAGTGTTCGAGCATGT Reverse: TCACCAGCCACAGTGGAAAA	Zheng et al., 2014
gpx	Forward: TGCAACCAGTTCGGACATCA Reverse: GAAGCCATTTCCAGGACGGA	Agus et al., 2015

sod = superoxide dismutase gen; cat = catalase gen; gpx = glutathione peroxidase gen.

Table 2
Methods used for oxidative stress biomarkers determination.

	Biomarker	Sample amount	Reagents	Wavelengths	Method used
Tube 1	LPX	50 μL supernatant	450 μL Tris-HCl 150 nM 1 mL TCA-TBA	535 nm	Buege and Aust, 1978
	HPX	100 μL supernatant	900 μL mixture ($FeSO_4$, H_2SO_4, dehydroxytoluene butylate, and xylenol orange).	560 nm	Jiang et al., 1992
	POX	Precipitate	150 μL DNPH/HCl 10 nM 500 μL TCA 1 mL guanidine 6 M	366 nm	Levine et al., 1994
Tube 2	SOD	40 μL supernatant	260 μL CO_3 buffer (50 mM Na_2CO_3 and 0.1 mM EDTA) 200 μL adrenaline 30 mM	480 nm	Misra and Fridovich, 1972
	GPX	100 μL supernatant	290 μL reaction buffer (3.5 mM GSH, 1 mM NaN_3, and 0.12 mM NADPH) 100 μL H_2O_2 20 mM 12 μL GR	340 nm	Gunzler and Flohe-Clairborne, 1985
	CAT	30 μL supernatant	420 μL isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, and 5 mM KH_2PO_4) 300 μL H_2O_2 20 mM	240 nm	Radi et al., 1991
	TP	13 μL supernatant	300 μL distilled water 1.25 mL Bradford reagent (Coomassie blue, Et-OH 96 %, H_3PO_4).	595 nm	Bradford, 1976

TCA: trichloroacetic acid. EDTA: Ethylenediaminetetraacetic acid. NADPH: Nicotinamide adenine dinucleotide phosphate. TCA-HCl: Tris hydrochloride. TCA-TBA: thiobarbituric-trichloroacetic acid. DNPH: 2,4-Dinitrophenylhydrazine.

pooled and evaluated as follow: oxidative stress biomarkers data was examined using a one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test or Kruskal-Wallis followed by Tukey's multiple-comparison test depending on the data (parametric or non-parametric distribution). Embryotoxic and teratogenic effects data were evaluated by one-way ANOVA followed by Kruskal-Wallis followed by Student-Newman-Keuls multiple comparison. The gene expression data were subjected to statistical analysis using a one-way ANOVA followed by the Student-Newman-Keuls post hoc test, while normality was evaluated through the Shapiro-Wilk test. The results were expressed as mean ± standard deviation (SD) for parametric data distribution or median ± interquartile range (25th–75th) for non-parametric data and analyzed in SigmaPlot 12.3 software. LC_{50} and EC_{50m} were determined by a probit analysis (USEPA Toxicity Estimation Software Tool (TEST) ver. 5.1.2). With the relationship LC_{50} and EC_{50m}, the TI was calculated. We carried out a principal component analysis to correlate all variables measured (R software; $p < 0.001$).

3. Results

3.1. Bioconcentration factor

Table 4 shows that although the bioconcentration factor (BCF) values of DXE for the nine concentrations tested in the study were low (between 0.051 and 0.543), DXE could be absorbed in *C. carpio* embryos as early as 24 hpf, although in a very low percentage.

3.2. Lethality study

The cumulative mortality rate of *C. carpio* embryos exposed to each concentration of DXE is shown in Fig. 1. This shows that with respect to the control (T) and DMSO (C) groups, DXE significantly increased the mortality rate of the embryos exposed, time and concentration dependent. It was observed that at 60 ng/L the highest mortality rate and the lowest survival of embryos occurred.

Considering the data of dead and malformed embryos at the different concentrations of DXE, the LC_{50} and EC_{50m} with their 95 % confidence intervals were calculated, obtaining values of 50 ng/L (46.75–54.74) and 28.8 ng/L (24.51–35.20), respectively. Furthermore, the teratogenic index of DXE in *C. carpio* was 1.7. According to the criteria of Weigt et al. (2011), DXE should be classified as teratogenic.

3.3. Alterations to embryonic development induced by DXE in C. carpio

During the exposure period, the embryos of the control group showed normal development as shown in Fig. 2. However, DXE caused developmental alterations at all exposure times and at all concentrations tested. The most observed alterations to embryonic development were pericardial edema, body hypopigmentation, craniofacial malformation, flipper malformation, scoliosis, somite malformation, yolk sac malformation, among others. The severity (lethality) of the alterations was more evident with increasing DXE concentration, for example at 60 ng/L a higher incidence of chordal alterations such as kyphosis, lordosis and scoliosis were observed, as well as pericardial edema and yolk sac deformation that in many cases led to the death of the embryos.

As shown in Fig. 3, the alterations in the development of *C. carpio* embryos due to exposure to DXE were evident. The higher the concentration and the longer the exposure time, a deterioration in embryo development was observed. Many of the alterations presented in the development of the common carp embryo were very severe and led to its death; the main alterations were related to the formation of somites, pericardial alterations, edema and hemorrhages, and alterations of the chorda.

Table 4
BCF of DXE in carp embryos at 24, 48.72 and 96 hpf.

DXE nominal concentrations ng/L	12	24	48	72	96
	BCF	BCF	BCF	BCF	BCF
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
DMSO	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
20	<LoQ	0.055	0.188	0.336	0.508
25	<LoQ	0.051	0.197	0.312	0.425
30	<LoQ	0.065	0.168	0.343	0.513
35	<LoQ	0.067	0.164	0.35	0.68
40	<LoQ	0.073	0.234	0.322	0.543
45	<LoQ	0.069	0.183	0.288	0.61
50	<LoQ	0.07	0.196	0.257	0.524
55	<LoQ	0.066	0.142	0.231	0.391
60	<LoQ	0.073	0.166	0.228	0.414

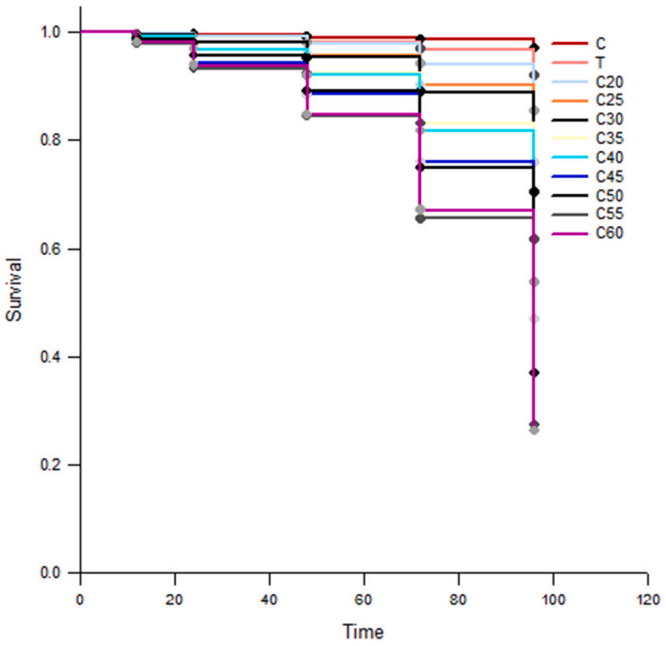

Fig. 1. The cumulative mortality rate of *C. carpio* embryos exposed to each DXE concentration at 12, 24, 48, 72 and 96 hpf.

3.4. Oxidative stress evaluation

Fig. 4 shows the oxidative stress biomarkers data at 72 and 96 hpf in *C. carpio* embryos exposed to DXE. In the case of HPC, PCC, SOD and CAT, significant time and concentration dependent increases were observed with respect to the control and DMSO groups ($p \leq 0.001$). In the case of LPX, the largest increases were observed at the 40 ng/L DXE concentration.

3.5. Oxidative stress-related gene expression patterns

All nine tested concentrations of DXE induced an up-regulation of oxidative stress-related genes (Fig. 5). These increases were statistically significant up to 3–5-fold over the control and DMSO groups ($p \leq 0.001$) and at the highest DXE concentrations.

3.6. Principal component analysis (PCA)

The principal components analysis (PCA) showed that the most representative contributing variables in Dim. 1 were the activity of the antioxidant enzymes SOD and CAT, the content of carbonyl proteins, the gene expression of *sod* and *gpx*, and the concentration of DXE in *C. carpio* embryos. For Dim. 2 the parameter that contributed the most was BCF, while for Dim. 3, the alterations to embryonic development. Dim. 4 showed that there was a greater contribution of HPX, LPX and the gene

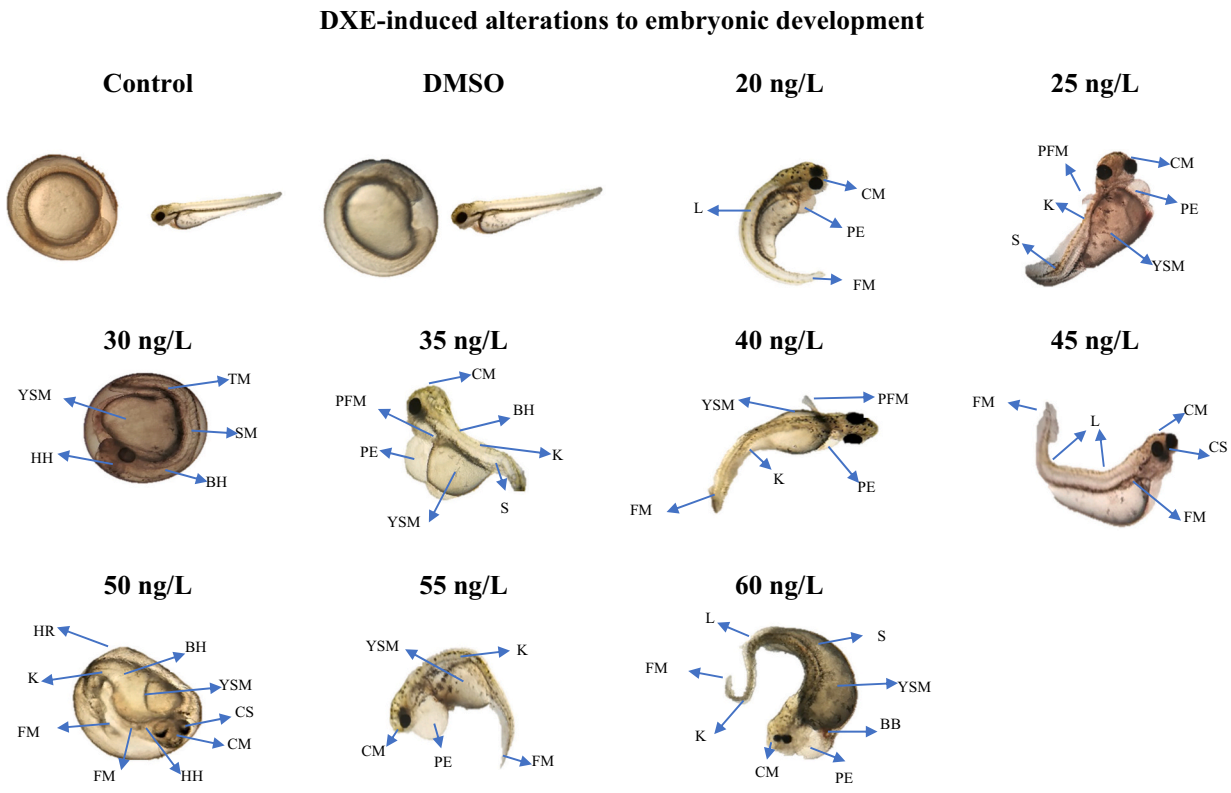

Fig. 2. Main alterations to embryonic development induced by each nominal concentration of DXE in *D. rerio* embryos.
Somites malformation (SM); Bleeding in the body (BB); Rachischisis (R); Kyphosis (K); Lordosis (L); Scoliosis (S); Fin malformation (FM); Cyclops or Squint (CS); Craniofacial Malformation (CM); Hatching retardation (HR); Body hypopigmentation (BH); Head hypopigmetation (HH); Pericardial edema (PE); Yolk sac malformation (YSM); Growth retardation (GR).

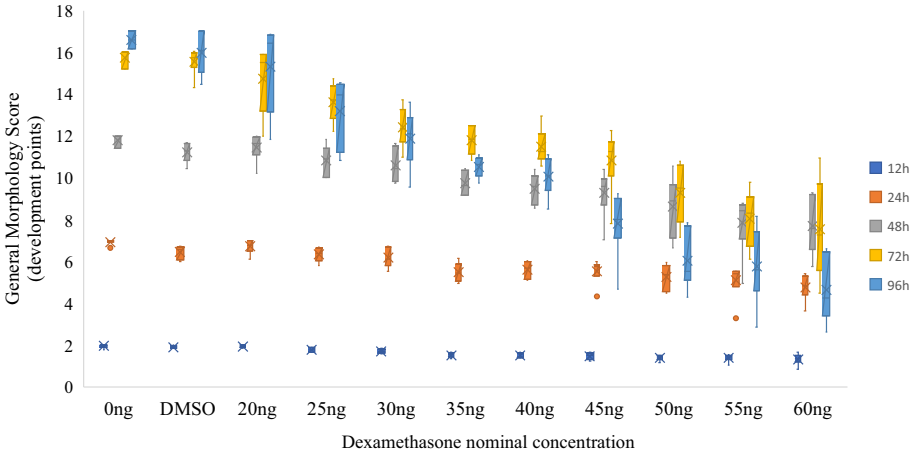

Fig. 3. Exposure time-response of the general morphology score of DXE on embryos of *C. carpio*. Data represents median ± interquartile range (25th–75th) for non- parametric data of four exposures. Statistical analysis was performed using one-way ANOVA followed by Kruskal-Wallis followed by Student-Newman-Keuls multiple comparison procedure: 12hpf, $X^2(10) = 173.62$; 24hpf, $X^2(10) = 264.26$; 48hpf, $X^2(10) = 179.26$; 72hpf, $X^2(10) = 233.37$; 96hpf $X^2(10) = 446.78$; $p < 0.001$.

expression of *cat* (Fig. 6).

4. Discussion

DXE is released in various aquatic matrices, as well as domestic sewage, hospital discharges, and industrial effluents in concentrations mainly ranging from ng/L to μg /L (Kitaichi et al., 2010; Schriks et al., 2010; Tölgyesi et al., 2010; Fan et al., 2011; Kovalova et al., 2012; Herrero et al., 2012; Liu et al., 2012; Oliveira et al., 2012; Zhong-Quan et al., 2012; Arsand et al. 2013; Schriks et al., 2013; Creusot et al., 2014; Macikova et al., 2014; Castro-Pastrana et al., 2015; Jia et al., 2016; Zhou et al., 2016; Praveena et al., 2018; Weizel et al., 2018; Shen et al., 2020). The presence of DXE in aquatic matrices is indicative evidence of its persistence and the inability of conventional wastewater treatment systems to remove it from wastewater (Chang et al., 2007; Fan et al., 2011; Herrero et al., 2012). Due to its chemical structure, DXE can be resistant to removal using conventional treatment techniques and, in turn, persists in different environmental matrices. Additionally, owing to the continuous release of small quantities from hospitals, households, and livestock, DXE can be termed pseudo-persistent active pharmaceutical ingredients (API) (Deblonde et al., 2011; Weizel et al., 2018). Overall, despite the very low or negligible reported DXE concentrations, continuous introduction of the parent drug and its associated metabolites may eventually result in pseudo-persistent behavior. This, in turn,

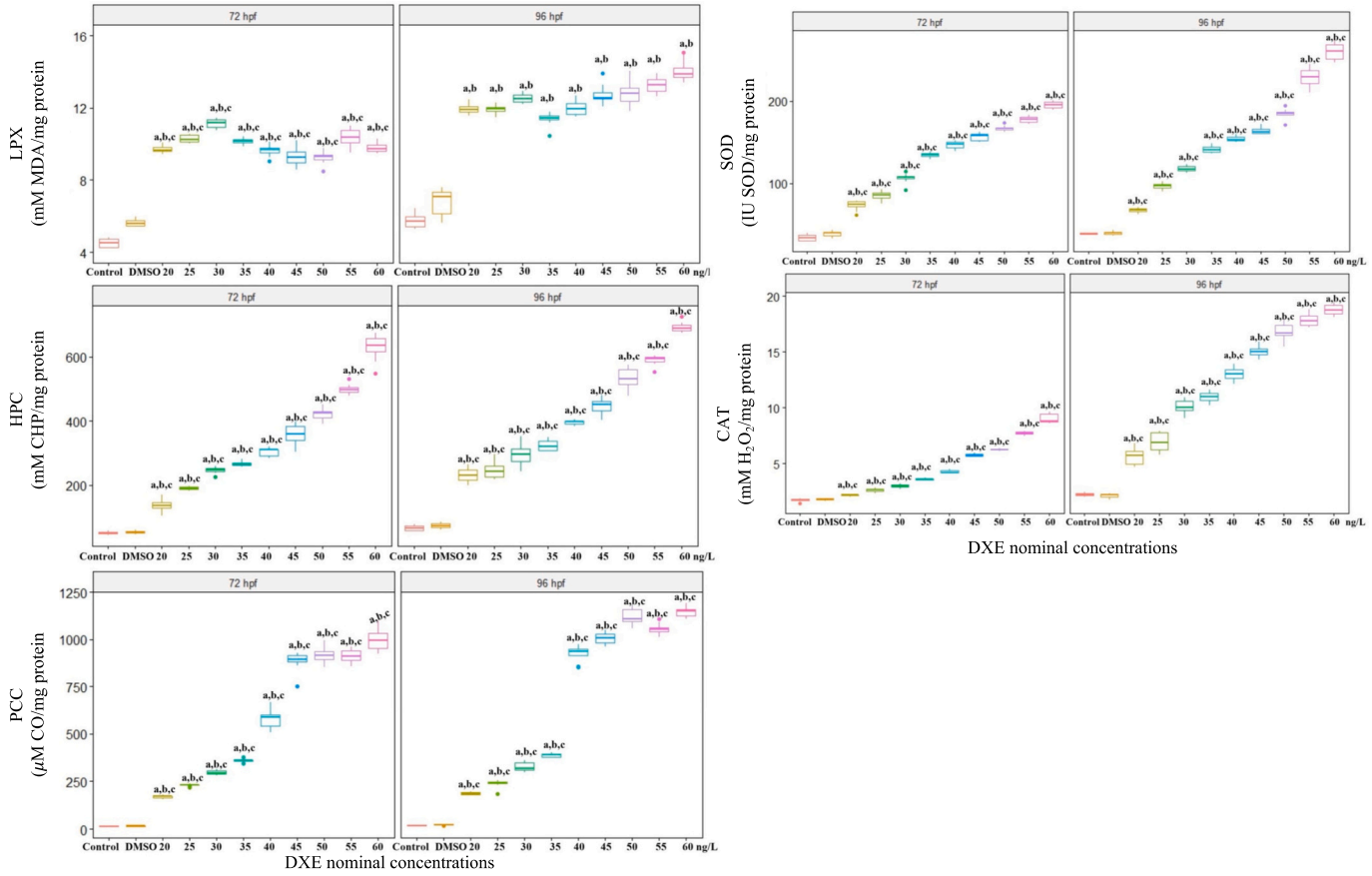

Fig. 4. Oxidative Stress Biomarkers in *C. carpio* embryos at 72 and 96 h after exposure to dexamethasone.
Data represents median ± interquartile range (25th–75th). Statistical analysis was performed using one-way ANOVA followed by Kruskal-Wallis followed by Tukey's multiple-comparison test depending on the data.
Different lowercase letters indicate significant differences between to the control group (a); DMSO (b); and (c) between 72 and 96hpf. $p \leq 0.001$.

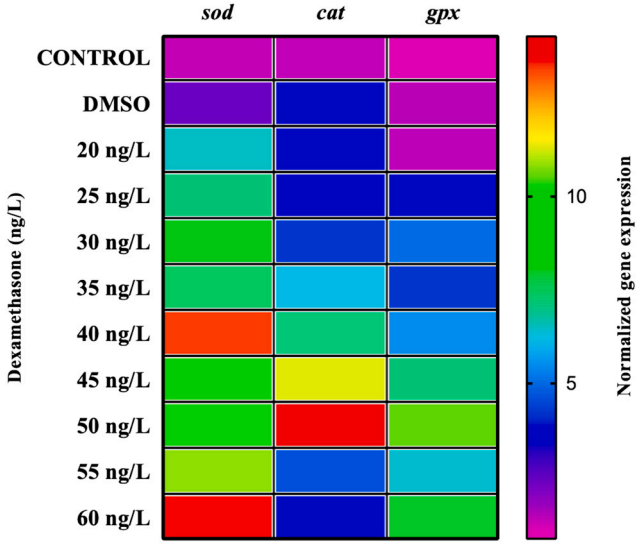

Fig. 5. Short-time exposure to DXE alters different genes related to oxidative stress (*sod, cat* and *gpx*) in *C. carpio* embryos. Values were normalized compared to β-actin (used as a housekeeping gene) and represent the mean mRNA expression value ± SEM. Meaningful increases over the control group were observed for *sod* ($F(10,33) = 905.27$; $p \leq 0.001$), *cat* ($F(10,33) = 1857.99$; $p \leq 0.001$) and *gpx* ($F(10, 33) = 176.48$; $p \leq 0.001$).

can affect drinking water quality, with probable risk to human health, livestock, and wildlife over extended periods (Herrero et al., 2012) This motivated the development of this work, where we evaluated whether DXE at environmentally relevant concentrations in short-term exposure can generate alterations in embryonic development and alterations in gene expression patterns related to oxidative stress in the common carp *C. carpio.*

The ecotoxicological effects of DXE in the literature are highly variable in various folds, e.g., intraspecies (from ng/L to mg/L) and between different species (fish > daphnids > algae). This therefore reflects a high sensitivity to DEX as a function of species biology, age, growth stage, exposure methods used and endpoints measured (Burkina et al., 2015).

In our study we found that the LC_{50} and EC_{50m} in *C. carpio* embryos were 50 and 28.8 ng/L, respectively. These results are in contrast with other studies, for example Cuzziol Boccioni et al. (2020) found that for toad embryos of *Rhinella arenarum* the LC_{50} of DXE at 96 h were 10.7 mg/L. In the study of DellaGreca et al. (2004), they found LC_{50} values for beaver-tail fairy shrimp (*Thamnocephalus platyurus*) and planktonic rotifer (*Brachionus calyciflorus*) >10 mg/L. In contrast, Bal et al. (2017) found an LC_{50} value for water flea (*Ceriodaphnia dubia*) LC_{50} of 0.75 mg/L. However, fish have been shown to be more sensitive species to this compound, for example, LaLone et al. (2012) showed that the LC_{50} for Fathead Minnow Fish (*Pimephales promelas*) was 254 μg/L. But zebrafish proved to be more sensitive to DXE as Chen et al. (2016), determined an LC_{50} for *Danio rerio* larvae of 652 ng/L. These studies show that the sensitivity to this compound in different aquatic organisms is highly variable. Our results show that *C. carpio* is a highly sensitive species to

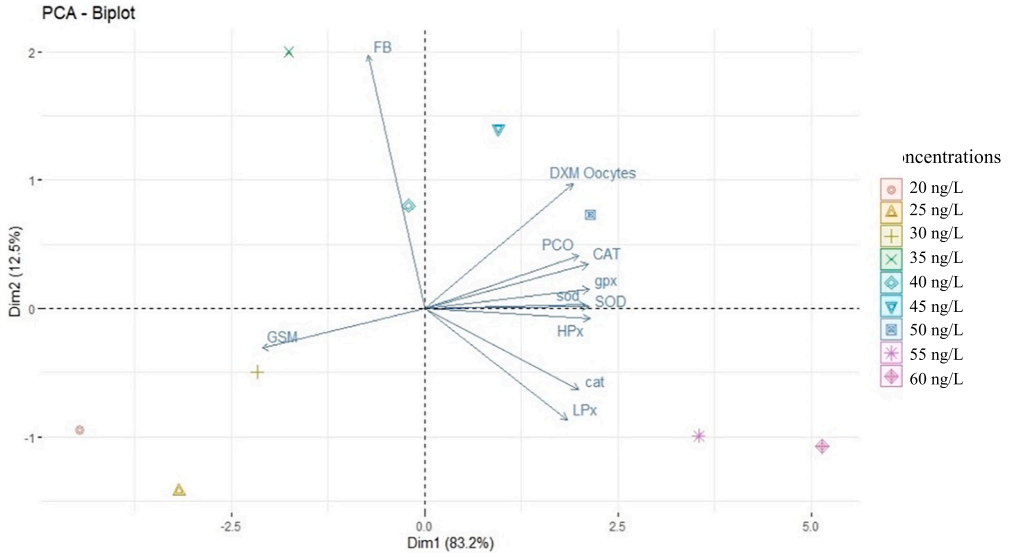

Fig. 6. Principal Component Analysis of biomarkers evaluated in *C. carpio* embryos exposed to DXE.

DXE, especially in the larval stage. A possible explanation for this result could be the hormetic behavior that DXE has shown to generate toxic effects at very low concentrations of this glucocorticoid (Calabrese and Calabrese, 2022; Orzechowski et al., 2002).

Some studies showing that DXE is toxic at environmentally relevant concentrations (ng/L) are shown below: Burkina et al., 2015 conducted an in vivo experiment to identify the toxic effects of DEX on rainbow trout. In this study, *Oncorhynchus mykiss* were exposed to 3, 30, 300 and 3000 ng/L. Their results showed that DEX at concentrations ranging from 3 to 300 ng/L can modify CYP450 activity when expressed as amount of product formed per min per nmol total CYP450, and it can affect total CYP450 content. However, at 3000 ng/L, DEX did not affect the parameters investigated, suggesting that DEX is regulated by CYP450 in a biphasic manner, a response to xenobiotics that is not uncommon (Heinrichs et al., 1994). These data may also suggest that fish were adapted to DEX exposure. In addition, this study demonstrates the hormetic behavior that dexamethasone can have.

In addition, Collins et al., 2022, identified that 40 ng/L concentrations of DXE at 96 h of exposure, up-expressed *acsl5, scd, odc1* and *gadd45ga* genes, and down-expressed *c27a6* and *dgat2* genes in adult male fathead minnows (*Pimephales promelas*). These genes were evaluated to better understand the sensitivity of in vivo hepatic gene expression responses to glucocorticoid receptor (GR) agonist exposure.

In order to corroborate the penetration of DXE into common carp embryos, in this study, the concentration of glucocorticoid in the exposure medium and within the egg was determined, and also its bioconcentration factor was determined. The results showed that as early as 24 hpf DXE was present in common carp embryos. These results showed that DXE has a high penetration of the chorion through the inner vitelline membrane, the perivitelline fluid space and the outer layer that protects the embryo during its development (Kutub Ali et al., 2017). Although it is true that the chorion is soft, as soon as oviposition occurs, a hardening process begins and ends shortly before fertilization, when an increase in the concentration of Ca^{+2} ions is generated in the peripheral zone (Mekkawy and Osman, 2006; Jaramillo et al., 2012). Likewise, the transport of substances through the chorion is influenced by changes in hardness and permeability, as greater penetration force is needed during the blastula and gastrula stage and decreases prior to hatching (Kais et al., 2013; Pelka et al., 2016).

The results of our study are in agreement with those of Steenbergen et al. (2017), who demonstrated the dynamics of DXE uptake in *D. rerio* oocytes. In this study, zebrafish oocytes were exposed to the tritiated variant 1,2,4,6,7-^{3}H-dexamethasone and it was observed that 85 % of the compound was taken up by the fish egg from 24 hpf.

The toxic effects of DXE presented in this study are directly related to the mechanism of action of this glucocorticoid. To date, four mechanisms of action have been proposed, these include: 1) Glucocorticoids intercalate into the lipid bilayer and induce changes in the biophysical properties of the plasma membrane, leading to the activation of intracellular signaling pathways; 2) Through the activation of a novel transmembrane protein belonging to the G protein-coupled receptor family, mediating the activation of second messengers including cyclic adenosine monophosphate (cAMP) and Ca^{+2} (Wang et al., 2014; Gong et al., 2016); as well as pathways involving protein kinase A, B, C (PKA, B and C) phosphorylation; 3) Through modulation of the glucocorticoid receptor by coupled glycoproteins or translocation of these receptors to the plasma membrane leading to activation of rapid signaling; and 4) By increasing calcium levels, which acts as a second messenger (Das et al., 2018; Johnstone III et al., 2019). In summary, DXE has the ability to intercalate into membranes and change the functionality of associated proteins, inducing lipoperoxidation and modifying membrane permeability. These findings would explain the exposure time- and concentration-dependent increases observed in the degree of LPX, PCC and HPC in this study. It is known that glucocorticoids such as DXE are capable of generating metabolic changes and can generate alterations in lipid composition (Costantini et al., 2011; Guiloski et al., 2015). High metabolic intensity may facilitate the progression of oxidative stress as observed in this study. The increase in SOD and CAT enzyme activity observed in our work has been previously reported in other investigations, which state that DXE is directly involved in cell death mechanisms (Yi et al., 2016).

Other effects of glucocorticoids on plasma membranes include the rapid reduction of calcium and sodium exchange across plasma membranes. Glucocorticoids also cause decreased adenosine triphosphate (ATP) production by inhibiting oxidative phosphorylation and increasing mitochondrial proton leakage (Stahn et al., 2007; Stahn and Buttgereit, 2008; Lee et al., 2013).

In teleost's such as *C. carpio* and *D. rerio*, at the onset of oviposition, glucocorticoid receptor transcripts and cortisol from the mother are deposited in the oocyte just before fertilization occurs. This receptor plays a preponderant role in embryogenesis. Cortisol-glucocorticoid receptor binding (C-CGR) is involved in both retrograde calcium communication, activation of related genes in cell cycle management, cell multiplication and mitochondrial activity, as well as in embryogenesis, organogenesis and morphogenesis (Nesan and Vijayan, 2013).

While cortisol is important during embryogenesis in teleosts,

pollutants such as DXE can interfere with the mechanisms that govern their development and growth. DXE at concentrations of ng/L, produces aberrant changes in craniofacial cartilage development during embryogenesis by altering chondrocytes (Hillegass et al., 2008). This glucocorticoid produces early hatching and changes in enzyme activity of embryos, as well as masculinization in hatched larvae after 60 days, this has been reported in species such as *Oryzias latipes* and *Oncorhynchus mykiss* (Mommsen et al., 1999). In addition, it can prevent the normal expression of metalloproteins (MMP), specifically MMP-13 which is essential in normal zebrafish development. Acute exposure to DXE results in craniofacial abnormalities, impaired somitogenesis, yolk sac and pericardial edema, blood pooling (Hillegass et al., 2007). Glucocorticoids can also interfere with retrograde Ca^{+2} communication between the nucleus and mitochondria, which is of great importance in contraction, secretion, as well as in long-term responses such as transcription, growth and cell division (Soboloff et al., 2006; Webb and Miller, 2006; Lee et al., 2013). Thus, the results obtained such as somite malformation, growth retardation, chordal malformations (syphosis, scoliosis, lordosis and rachischisis), craniofacial malformation, hypopigmentation are the result of DXE concentrations in the embryo.

Mitochondrial dysfunction may also be caused by increased intracellular calcium concentration, high concentrations of glucocorticoids may cause a sustained flux of this cation inducing the production of ROS, which would explain the increase in biomarkers of cellular oxidation and the increased activity of the antioxidant enzymes SOD and CAT, found in this study. The pathway involved is mediated by SOCE (store operated calcium entry) through a critical pathway involving calcium release-activated calcium modulator 1/sensor stromal interaction molecule 1 (Orai/STIM1) that supports Ca^{+2} influx. In addition, there is a relationship between calcium homeostasis and mitochondrial dysfunction, which ultimately leads to cell death. Furthermore, the excess of ROS causes Ca^{+2} overload in the cytosol, which results in an excess of mitochondrial incorporation leading to depolarization of the mitochondrial membrane potential (Soboloff et al., 2011; Soboloff et al., 2012; Orrenius et al., 2015). Simultaneously, exposure to DXE leads to an increase in energy expenditure and hypermetabolism (Desquiret et al., 2008), based on an increase in cytosolic Ca^{+2} that results in the stimulation of the Tricarboxylic acid cycle (TCA cycle), which manifests itself in ATP synthesis (Wiederkehr et al., 2011). It should also be taken into consideration that the effects of DXE treatment have both short-term and long-term effects (Desquiret et al., 2008).

According to the results in the determination of gene expression, we obtained that the gene that is expressed from 20 ng/L is the *sod* having maximum expression at 40 ng/L and 60 ng/L. These results are in agreement with those obtained by Hira et al. (2020), who found that dexamethasone, through the induction of superoxide anion, has the ability to increase the mRNA of mitochondrial proteins, such as the mitochondrial outer membrane translocase (TOM) subunits TOM20 and TOM70, whose possible function is to import proteins that have been synthesized in the cytosol, to increase their presence within the mitochondrion itself.. At the same time, an increase in SGK1 (serum and glucocorticoid inducible kinase 1) mRNA and dose-dependent MnSOD (mitochondrial-specific MnSOD) was detected. On the other hand, catalase has the ability to inhibit SGK1 and consequently its effects generated on TOM20, TOM70 and MnSOD.

As mentioned above, glucocorticoids not only have non-genomic effects, but also genomic effects, as shown below. Since gene expression corresponds to molecules of a diffusible nature that generate morphogenetic gradients at the site of action, interact with membrane receptors, triggering intracellular responses at the transcriptional level. Signals mediated by wingless e int (Wnts) ligands are classified into 4 intracellular pathways: 1) The canonical pathway uses β-catenin/tcf as transcription factors to activate important target genes in embryonic development; 2) Jun-kinase (JNK or planar cell polarity) pathway that controls the reorganization of the cytoskeleton.; 3) The Wnt/Ca^{2+} pathway controls protein kinases C (PKC) and Ca^{2+}/calmodulin-dependent kinases (CamKII) and regulates cell adhesion and motility; 4) There is a final pathway, not well characterized, that regulates the axis of orientation and asymmetric cell division (Aguirre-Gutiérrez, 2007). The findings found in this study corroborate that the embryotoxic and teratogenic effects induced by DXE are directly related to the oxidative stress mechanism as evidenced by the biomarkers evaluated and the expression of the *sod, cat* and *gpx* genes. The results also demonstrate that DXE is an unsafe drug for organisms such as *C. carpio*.

5. Conclusions

Unlike previous findings, herein, results demonstrated that exposure to realistic concentrations and acute exposure to DXE (20–60 ng/L) can generate different toxic responses in common carp. These responses included embryonic alterations and oxidative stress in *C. carpio*. In addition, the expression of genes related to oxidative stress was altered. Embryonic alterations may be associated with DXE-induced oxidative damage. However, further investigations are needed to relate oxidative damage to embryonic alterations. It is also suggested to evaluate genes related to apoptosis to complement the molecular information and to be able to establish a possible mechanism of action of DXE. In addition, it is suggested to perform studies with the other synthetic fluorinated corticosteroids, to see if they comprise the mechanism of action.

The results observed in this study are worrying, since DXE, by generating embryonic alterations, can affect the reproduction of common carp, which is a species of economic and ecological interest in many Latin American countries.

CRediT authorship contribution statement

Veronica Margarita Gutiérrez-Noya: Data curation, Writing – original draft. **Leobardo Manuel Gómez-Oliván:** Formal analysis, Writing – review & editing, Supervision, Project administration. **Idalia Casas-Hinojosa:** Methodology. **Sandra García-Medina:** Data curation. **Karina Elisa Rosales-Pérez:** Methodology. **José Manuel Orozco-Hernández:** Methodology. **Gustavo Axel Elizalde-Velázquez:** Methodology. **Marcela Galar-Martínez:** Formal analysis. **Octavio Dublán-García:** Data curation. **Hariz Islas-Flores:** Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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