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Integrative approach to elucidate the embryological effects of caffeine in *Cyprinus carpio*: Bioconcentration and alteration of oxidative stress-related gene expression patterns

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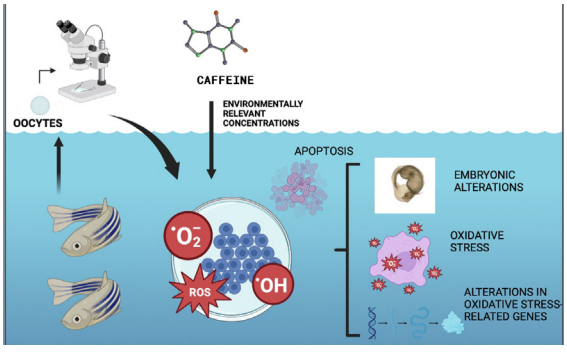
HIGHLIGHTS

- CAF generated embryological alterations in *C. carpio*.
- CAF induced cardiac malformations, somite alterations, pericardial edema and chorda malformations.
- CAF induced oxidative damage to *C. carpio* embryos.
- CAF modified the gene expression of *sod*, *cat* and *gpx* in embryos.
- CAF was highly uptaken in *C. carpio* embryos.

GRAPHICAL ABSTRACT



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ABSTRACT

Caffeine (CAF) is an alkaloid, which acts as a central nervous system (CNS) stimulant drug. In recent years, CAF has been recurrently detected in water bodies, generating deleterious effects in aquatic organisms. The information on the toxic effects of CAF in the environment is still limited. Thus, the objective of this work was to determine whether CAF at environmentally relevant concentrations (CAF concentrations were selected based on studies on the worldwide occurrence of this compound and on the toxicity of CAF in aquatic species) is capable of inducing 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 CAF until 96 hpf. Alterations to embryonic development and teratogenic effects were evaluated at 12, 24, 48, 72 and 96 hpf. In addition, oxidative

Abbreviations: AP-1, activating protein factor 1; AC, adenylyl cyclase; AR, adenosine receptors; A_1AR, adenosine A1 receptors; A_2AR, adenosine 2 receptors; B, bleeding; BCF, bioconcentration factor; CAF, caffeine; CAT, catalase; CFM, caudal fin malformation; CNS, central nervous system; CM, chorda malformation; CrM, craniofacial malformation; cAMP, cyclic adenosine monophosphate; EH, early hatching; FDA, Food and Drug Administration; GRAS, generally recognized as safe; GR, growth retardation; HM, heart malformation; HPC, hydroperoxide content; H, hypopigmentation; HIF-1, hypoxia-inducing factor; JNK, c-Jun N-terminal kinase; LH, late hatching; LPX, lipoperoxidation level; LOD, limits of detection; LOQ, limits of quantification; MAPK/ERK, mitogen-activated protein kinases; REML, mixed-effects model; NF-kB, nuclear Factor κB; NF, nuclear factor; Nrf-1, E2-related Factor 1; OH, oral hyperplasia; OM, ocular malformation; PFM, pectoral fin malformation; PE, pericardial edema; PCC, protein carbonyl content; ATK, protein kinase; PAK2, p21-activated protein kinase 2; Ref-1, redox effector factor; SM, somites malformation; SOD, superoxide dismutase; TP, total protein content; YSM, yolk sac malformation; WF, without fin.

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stress in carp embryos at 72 and 96 hpf was evaluated by cellular oxidation biomarkers (lipoperoxidation level, hydroperoxide content 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 CAF concentrations above 500 ng/L are capable of producing teratogenic effects. Furthermore, CAF was able to induce alterations such cardiac malformations, somite alterations, pericardial edema and chorda malformations. Concerning oxidative stress, the results demonstrated that CAF induce oxidative damage on the embryos of *C. carpio*. Our outcomes also showed up-regulations in genes related to antioxidant activity *sod*, *cat* and *gpx* by CAF exposure. In conclusion CAF at environmentally relevant concentrations is able to alter the embryonic development of common carp by the oxidative stress pathway. Based on the above evidence, it can be inferred that acute exposure to CAF can lead to a toxic response that significantly harms fish's health, adversely affecting their essential organs' functioning.

1. Introduction

Caffeine [CAF] (1,3,7-trimethylxanthine) is an alkaloid, which acts as a central nervous system (CNS) stimulant drug (De Carvalho et al., 2019; Rah et al., 2017). Currently, approximately 80 % of the world's population consumes products containing caffeine on a daily basis, making it the most widely consumed stimulant substance and the most socially accepted worldwide (Rah et al., 2017; Espinosa Jovel and Sobrino Mejía, 2017; Depaula and Farah, 2019).

People can ingest an average of 50 mg of CAF per day, and in developed countries, the consumption can reach up to 400 mg per day (De Carvalho et al., 2019). Of the CAF-containing products, tea and soft drinks are the main products consumed in Africa and Asia, while coffee and soft drinks are preferred in Europe, North America, Latin America and the Caribbean (Reyes and Cornelis, 2018). The Food and Drug Administration (FDA) classifies CAF as a GRAS substance (Generally Recognized As Safe), this means that under normal conditions of use it is safe. However, the European Union does establish a maximum limit for CAF in soft drinks, which is 150 mg/L (Reyes et al., 2015).

In recent years, CAF has been recurrently detected in water bodies. Some relevant occurrence data are listed below: 1) In a study of an effluent from a wastewater treatment plant in Spain up to 66 mg/L of CAF was found (Mijangos et al., 2018); 2) In some rivers in China concentrations of up to 1280 ng/L were found (Dai et al., 2015); In Korea concentrations of 51.6 ng/L were found in drinking water (Nam et al., 2014); in streams in India concentrations of 44 ng/L were detected (Bernot et al., 2013); in municipal sewage in a city in Spain 11,600 ng/L were detected (Del Río et al., 2013); in treated water in the US, 51300 ng/L were detected (Li et al., 2013); while in Mexico in irrigation canals 18,500 ng/L were detected (Lesser et al., 2018).

The presence of CAF in water bodies is attributed to increased population density, the consumption of CAF-added products and the inefficiency or absence of wastewater treatment systems (Chen et al., 2008; Lozano et al., 2007; Ramirez and Rivera, 2017; Marasco Júnior et al., 2019). Currently, CAF is considered an emerging contaminant, due to its occurrence and toxicological effects (Rah et al., 2017).

Some of the embryological effects of CAF have been documented especially in *D. rerio*. For example, Chen et al. (2008) documented at concentrations of 17.5, 35, 50, 50, 100, 150 mg/L CAF, alterations in body length, muscle fiber misalignment and motor neuron defects, especially axonal growth defects of secondary motor neurons in *D. rerio*. Rah et al. (2017) identified at 25 μM, 125 μM, 250 μM, 500 μM body and heart malformations in zebrafish. In agreement, Basnet et al. (2017) observed that at concentrations of 0.25, 0.5, 0.5, 0.75, 1.5, 2.5 ng/L structural alteration of the heart, transient increase in heart rate occurred in the same species. Félix et al. (2021) showed that 0.5 mM of CAF induced an increase of mortality up to 3, and these findings were associated with high incidence of edema and malformations of tail, body length, head, eye, and yolk area.

The toxicological effects associated with CAF in aquatic organisms are related to down-regulation of adenosine receptors, induction of catecholamine release, inhibition of phosphodiesterase, and release of calcium in intracellular depot cells that are sensitive to ryanodine (Rana et al., 2010; Bode and Dong, 2007). Non-selective antagonism of adenosine receptors is believed to be the main pharmacological mechanism of action of CAF (Fisone et al., 2004; Menezes et al., 2018; De Carvalho et al., 2019).

The species *C. carpio* is frequently used as a bioindicator, since cyprinids are quantitatively the most important group of teleost fishes cultured worldwide for commercial purposes and, in addition, they are very sensitive and easy to maintain organisms (Yalsuyi et al., 2021; Banaee et al., 2022; Vali et al., 2022).

Based on the previously identified findings, this study aims to evaluate the embryological effects induced by CAF in a commercially important species, *Cyprinus carpio*, and examine alterations in gene expression patterns related to oxidative stress.

This is done through an integrative approach that relates morphological aspects, biochemical biomarkers and gene expression. We hypothesize that the embryological damage produced by CAF may be due to an oxidative stress mechanism.

2. Material and methods

2.1. Caffeine

Caffeine powder, ReagentPlus® (1,3,7-trimethylxanthine), CAS number: 58-08-2, purity >599 % was purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was prepared at a concentration of 10 mg/L using water as a solvent and kept at −20 °C.

2.2. Test organisms and egg collection

Cyprinus carpio eggs were obtained from the Tiacaque aquaculture center, located in Jocotitlan, State of Mexico. The eggs were obtained from adult of *C. carpio* of reproductive age, with an average length of 45 ± 5 cm and an approximate weight of 3.8 ± 0.8 kg. Four females and eight males were placed in breeding ponds for spawning. The carp were placed in breeding ponds with water at two-thirds capacity, at a temperature of 23 ± 1 °C, conductivity of 740 ± 100 μS/cm, pH of 7.0 ± 1.0 and dissolved oxygen saturation ≥ 95 %. The natural photoperiods used were 14 h of light and 10 h of darkness. The carp were supplemented daily with protein, carbohydrates and other nutrients to ensure better egg production. The breeding pond was covered with plastic mesh on the sides and casuarina branches at the bottom to facilitate egg collection. Fertilized eggs were collected the following morning from the plastic nets and casuarina branches and placed in containers with proper temperature and oxygenation. The eggs were washed with saline solution and selected for normal morphology using a stereoscopic microscope (Nikon, Japan), the selection was made, including only fertilized eggs with normal morphology (Kimmel et al., 1995). This research was conducted in accordance with the official Mexican standard on the breeding, care and use of laboratory animals (NOM-062-ZOO-1999). In order to guarantee the welfare of the organisms.

2.3. 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.082.2021).

2.4. Embryotoxicity test

The embryotoxicity test was performed according to the guidelines established by the OECD [Test No. 236: Fish Embryo Acute Toxicity (FET), 2013] modified by Luja-Mondragón et al., 2019. The CAF concentrations used in this study were selected based on studies on the worldwide occurrence of this compound and on the toxicity of CAF in aquatic species (Fisone et al., 2004; Bode and Dong, 2007; Rana et al., 2010; Bernot et al., 2013; Del Río et al., 2013; Li et al., 2013; Nam et al., 2014; Dai et al., 2015; Lesser et al., 2018; Mijangos et al., 2018; Menezes et al., 2018; De Carvalho et al., 2019). The concentrations used were: 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, and 2500 ng/L, and a control (CAF-free). In 24-well plates, 2.5 mL of each concentration of CAF tested was placed in 2.5 mL of each concentration of CAF tested and an egg was placed in the wells at mid-blastula stage (2 h post fertilization *-hpf-*). Plates were placed in bioclimatic chambers, with light and temperature control (12:12 h and 23 ± 1°) and observed in the stereomicroscope at 12, 24, 48, 72 and 96 hpf. The experiment was replicated in quadruplicate. 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, CAF was considered as teratogenic and if it was <1 as embryolethal, according to criteria of (Weigt et al., 2011). Embryological evaluation and mortality at the different concentrations of CAF tested was performed considering the OECD FET test and assigning the score according to Kimmel et al. (1995) and Hermsen et al. (2011), modified by Luja-Mondragón et al. (2019) for *C. carpio*. Morphological scoring was assigned considering the contrast of exposure-free (control) and CAF-exposed organisms considering body shape, pigmentation, somites, heart, eyes, craniofacial structures, yolk, tail, corda & fins. The frequency of malformations was analyzed for the 10 concentrations used.

The validity and reliability of the test was considering the following criteria: fertilization rate was ≥90 % and not showing a mortality rate and lethal teratogenic effects >10 % at the end of exposure in the control group.

2.5. Determination of oxidative damage

One liter of water previously aerated at 23 ± 1 °C was placed in a glass container, 1 g of carp eggs (2 hpf) belonging to the same broodstock group (Section 2.2) were added to the system. They were randomly selected and exposed to 10 concentrations of caffeine previously described in Section 2.3 (500–2500 ng/L), in addition to the control system. The experiment was replicated in quadruplicate. The exposure times used were 72 and 96 hpf (this was due to the fact that at 72 hpf the eggs had hatched and the embryos already showed enzymatic activity). After the exposure time, the systems were filtered, the embryos were placed in Eppendorf tubes and 1.5 mL of phosphate buffer solution (PBS, pH 7.4) was added, homogenized (rotor-stator IKA T 10 basic ULTRA-TURRAX) and frozen at −20 °C for the determination of biomarkers of cellular oxidation and antioxidant enzyme activity.

The homogenate was 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 11495 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.

The evaluation of cellular oxidation biomarkers was performed at 72 and 96 h of exposure. HPC content was determined by the technique of Jiang et al. (1992), LPX content by the method of Buege and Aust (1978), protein carbonyl content PCC by Levine et al. (1994). SOD enzyme activity by the method of Misra and Fridovich (1972), CAT enzyme activity by the technique of Radi et al. (1991) and PT content was related to the other biomarkers for analysis using the method of Bradford (1976).

2.6. Gene expression determination

Ten embryos were placed in Petri dishes using the caffeine concentrations tested in section 2.4 (500–2500 ng/L and control), as well as the control (CAF free), these systems were placed in bioclimatic chambers under the previously mentioned conditions. The exposure period was 96 h. After the exposure period, the embryos were placed in 10 μL of RNA later solution (Qiagen) at −20 °C for subsequent analysis. Using a QIAGEN RNeasy kit and following manufacturer's instructions, RNA was isolated, the analysis were carried under controlled condition in a special room to avoid contamination or interference, and the RNA concentration was determined with a spectrophotometer (Thermo Scientific NanoDrop 2000/2000c) with absorbances of 260 nm for RNA and 280 nm for proteins, and RNA purity was assessed by agarose gel electrophoresis. Reverse transcription reaction was performed using the QuantiTect® (QIAGEN, Hilden, Germany, REF 205313) reverse transcription kit and 10 μg of total RNA. The conditions for the reaction were 42 °C for 15 min and 95 °C for 3 min. The q-PCR used a Rotor-Gene Q (Qiagen) the conditions were: 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 (Table 1), 25 μL of SYBER Green QuantiTec® (QIAGEN, Hilden, Germany) and 500 ng of cDNA template. ß-actin was used as a housekeeping gene to normalize all samples. The housekeeping gene was assessed for each organism in both control and expressed organism. The genes used for qRT-PCR are related to the response to oxidative stress (Table 1).

Once the q-PCR cycles were completed, a dissociation curve was performed to distinguish between specific and non-specific products, using the following conditions: 10s at 95 °C, 10s at 65 °C and 10s 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.

2.7. Determination of the bioconcentration factor (BCF)

The quantification of CAF in water and eggs of *C. carpio* was carried out in accordance with Fick et al. (2009). For CAF extraction, 5 mL of previously filtered water (Filtropur S of 0.45 μm) and 0.1 g of eggs were added 1.5 mL of acetonitrile along with 10 zirconium beads.. Samples were homogenized at 42,000 oscillations per minute (Mini Beadbeater, BioSpec Bartlesville, USA) and centrifuged at 17,500 rpm for 10 min (Beckman Coulter Microfuge 22R Centrifuge). To the supernatant was added 1.5 mL of acetonitrile and the process was repeated. Supernatants were evaporated to near dryness (TurboVap Classic LV, Biotage AB, Suecia), the eluent was reconstituted with 150 μL of methanol. The final extracts were frozen for a minimum of 24 h to ensure protein precipitation and centrifuged again directly before analysis. A solid-phase system coupled to liquid chromatography-tandem mass spectrometry (SPE LC-MS/MS) was used for the extraction. A triple-stage quadrupole mass spectrometer (TSQ Quantiva, Thermo Scientific, San Jose, CA) equipped with a heated electronebulization ionization ion source (HESI), coupled to an Accela LC pump (Thermo Fisher Scientific, San Jose, CA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used. CAF separation was

Table 1
Genes used for qRT-PCR.

Gene	Sequence (5′-3′)	Reference
sod	5′: CTGGACAAATCTGTCACCTA 3′: TGCAGCAATCCTCAGTCT	Karaca et al., 2014
cat	5′: AGCCAAAGTGTTCGAGCATGT 3′: TCACCAGCCACAGTGGAAAA	Zheng et al., 2014
gpx	5′: TGCAACCAGTTCGGACATCA 3′: GAAGCCATTTCCAGGACGGA	Agus et al., 2015

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

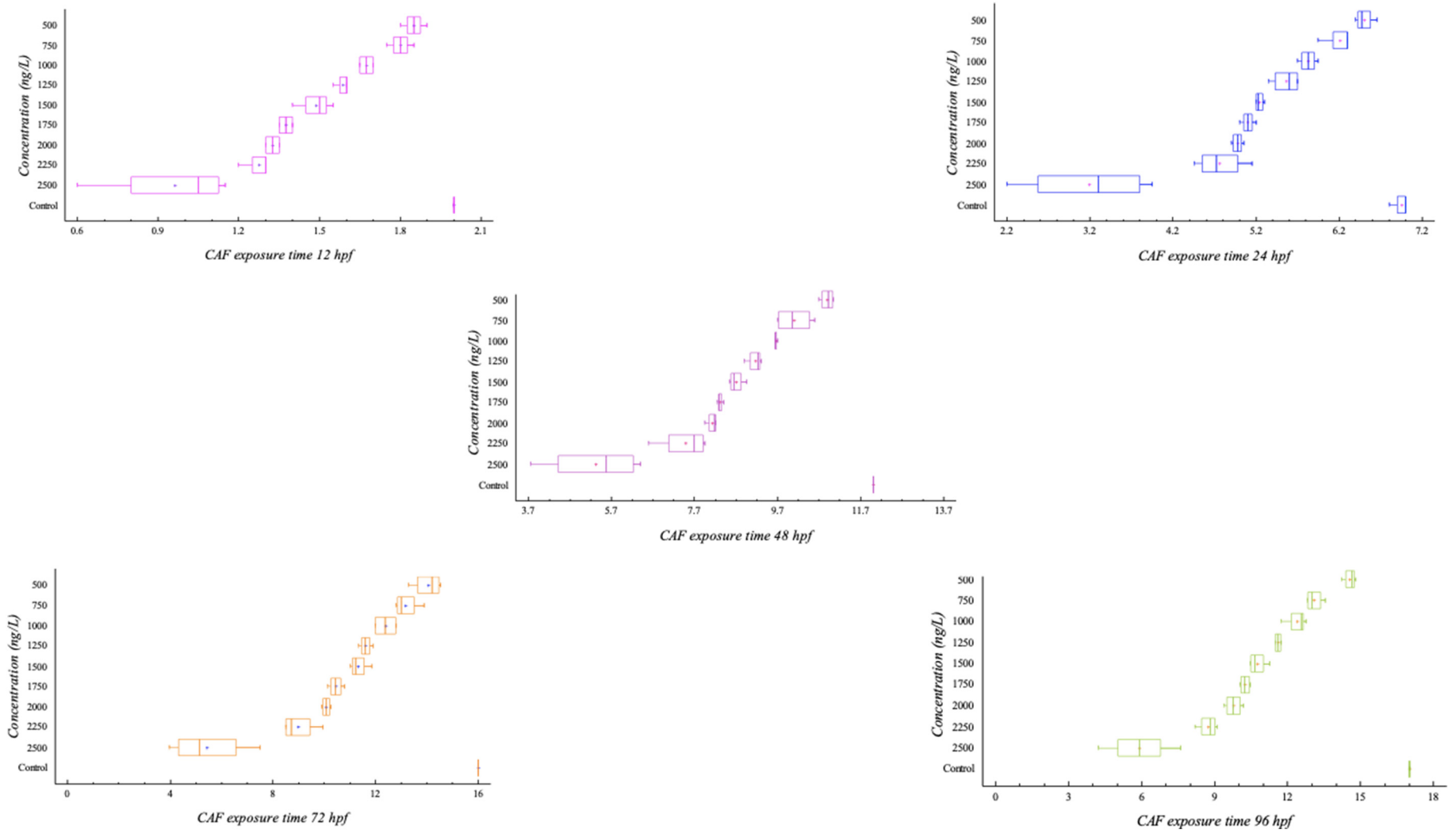


Fig. 1. Dose-response of morphological evaluation of *C. carpio* embryos. Score of the morphological characteristics of the normal progressive development of *C. carpio* (control), compared with embryos exposed to different concentrations of caffeine, during 12 (a), 24 (b), 48 (c), 72 (d) and 96 (e) hpf. Statistical analysis was performed using test Kruskal-Wallis followed by Student-Newman-Keuls multiple-comparison test. All show significant differences between to the control group ($p < 0.05$).

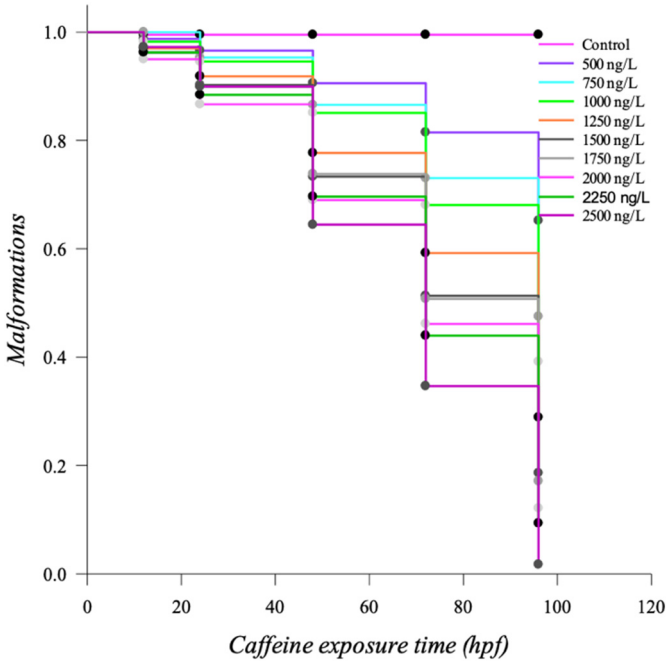


Fig. 2. Kaplan Meier graph. Represents the non-parametric probability distribution of malformations with respect to CAF exposure time in *C. carpio* embryos. Statistical analysis was performed using log rank statistic for the malformation followed by All Pairwise Multiple Comparison Procedures (Holm-Sidak method) $p < 0.05$.

performed using a C18-phase Hypersil gold column (50 mm × 2.1 mm ID × 3 μm particles, Thermo Fisher Scientific, San Jose, CA, USA).

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

LOD = $t_{0.99}$ × S and LOQ = 3 × 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. Detection limit (LOD) and quantification limit (LOD) were 15 and 25 ng/L, respectively.

Employing the CAF 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).

2.8. Statistical analysis

For the morphological dose-response evaluation of CAF in *C. carpio*, we used the Kruskal-Wallis test followed by a Student-Newman-Keuls multiple comparison test, with a confidence level of 95 % using the Software Sigma Plot 12.3.

For the determination of biomarkers of cellular oxidation and antioxidant activity, a linear mixed model was performed to determine the effects of caffeine concentrations and exposure time. Subsequently, a Dunnett's multiple comparison test was performed with a 95 % confidence level, using the Software *Graphpad* Prism 8.

3. Results

3.1. Caffeine-induced morphological evaluation of C. carpio

Fig. 1, describes the morphological score of normal progressive development of *C. carpio* (control), compared to the 9 different CAF concentrations (500, 750, 1000, 1250, 1500, 1750, 2000, 2250 and 2500 ng/L) and exposure time (12, 24, 48, 72 and 96 hpf). All concentrations tested were significantly different from the control group at 12 hpf [H (9) = 38.97], 24 hpf [H (9) = 38.01], 48 hpf [H (9) = 38.58], 72 hpf [H (9) = 38.32] and 96 hpf [H (9) = 38.52] ($p \leq 0.05$). This was tested using a Kruskal-Wallis nonparametric test, followed by a Student-Newman-Keuls multiple comparison test.

Alterations in morphological scoring of *C. carpio* embryos exposed to caffeine was concentration dependent. A progressive effect of dysmorphogenesis was observed as CAF concentration increased ($p < 0.05$).

3.2. Embryolethality test

The LC_{50} and EC_{50} values and their respective 95 % confidence intervals were 2089.29 ng/L (0.55 ng/L–3.063 ng/L) and 1096.48 ng/L (0.83 ng/L–2.56 ng/L), respectively. The teratogenic index of CAF was 1.90, taking into account the criteria of Weigt et al. (2011), caffeine is considered a teratogenic substance.

With the malformation data obtained at the different CAF concentrations, a Kaplan Meier plot was constructed to represent the probability of malformations with respect to time (Fig. 2). As can be seen, all CAF concentrations presented significant differences with respect to the control group, i.e., in the different concentrations there is a probability of malformations due to CAF action with respect to exposure time in *C. carpio*.

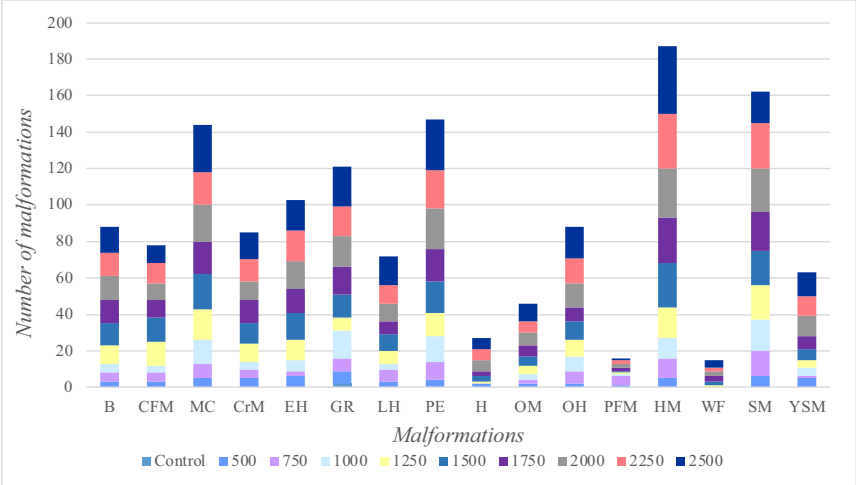


Fig. 3. Frequency of malformations in *C. carpio* embryos: B: bleeding; CFM: caudal fin malformation; CM: chorda malformation; CrM: craniofacial malformation; EH: early hatching; GR: growth retardation; LH: late hatching; HM: heart malformation; H: hypopigmentation; OM: ocular malformation; OH: oral hyperplasia; PFM: pectoral fin malformation; PE: pericardial edema; WF: without fin; YSM: yolk sac malformation; SM: somites malformation.

3.3. Embryological alterations of CAF in C. carpio

The total frequency of embryological alterations in *C. carpio* is described in Fig. 3. We distinguish heart malformations (12.97 %); somite malformations (11.23 %); pericardial edema (10.19 %) and chorda malformation (9.99 %). The 2500 ng/L concentration showed the highest percentage of malformations. As previously mentioned, the embryological alterations induced by CAF were concentration-dependent. The percentages of malformations presented at each concentration of CAF are shown below: 2500 ng/L (17. 55 %), 2250 ng/L (14.84 %), 2000 ng/L (14.49 %), 1750 ng/L (12.62 %), 1500 ng/L (12.41 %), 1250 ng/L (10.06 %), 1000 ng/L (7.56), 750 ng/L (6.24 %) & 500 (4.09 %).

Table 2 shows that the most severe malformations were identified at the 2250 and 2500 ng/L concentrations. At these concentrations, the optic primordia had little or no development, with little or no somite formation, and the caudal bud did not develop. In some cases the bud was so large that it occupied most of the cell space, with no optic primordia and no caudal bud. There was no development of the craniofacial structure, or it was delayed, with little or no movement. Some organisms had early hatching, with hypopigmentation or no pigmentation, delayed eye, yolk sac, and pectoral fin development, with larger and deformed hearts.

3.4. Oxidative damage in C. carpio embryos exposed to CAF

Fig. 4 shows the response of cellular oxidation biomarkers LPX, HPC and PCC and antioxidant activity of SOD and CAT in *C. carpio* embryos exposed to different concentrations of CAF (500–2500 ng/L) compared to a control group (CAF-free), at 72 and 96 hpf. At all CAF concentrations, an increase in biomarkers was observed with respect to the control group, both at 72 and 96 hpf.

In LPX (a), the increase with respect to the control group was significant from concentration 500 ng/L (both at 72 h and 96 h) [$F(9,220) = 150.7$, $p < 0.05$]. For HPX (b), a significant increase was presented starting at 750 ng/L during 72 and 96 h of exposure [$F(9,110) = 21.07$, $p < 0.05$]. While for POX (c), SOD (d) and CAT (e); the increase was significant from 1000 ng/L (72 h of exposure), and 750 ng/L (96 h of exposure). [$F(9,110) = 7.46$, $p < 0.05$], [$F(9,110) = 7.46$, $p < 0.05$] and [$F(9,110) = 10.45$, $p < 0.05$] respective.

3.5. Oxidative stress-related gene expression patterns

Gene expression of genes related to antioxidant activity (*sod*, *cat* and *gpx*) induced by CAF exposure in *C. carpio* embryos is described in Fig. 5. A concentration-dependent increase in the up-regulation of the three genes *sod*, *cat* and *gpx* was observed. In *sod* the increases in gene expression were most pronounced at 1750 ng/L, in *cat* at 2250 ng/L and in *gpx* at 2000 ng/L of CAF.

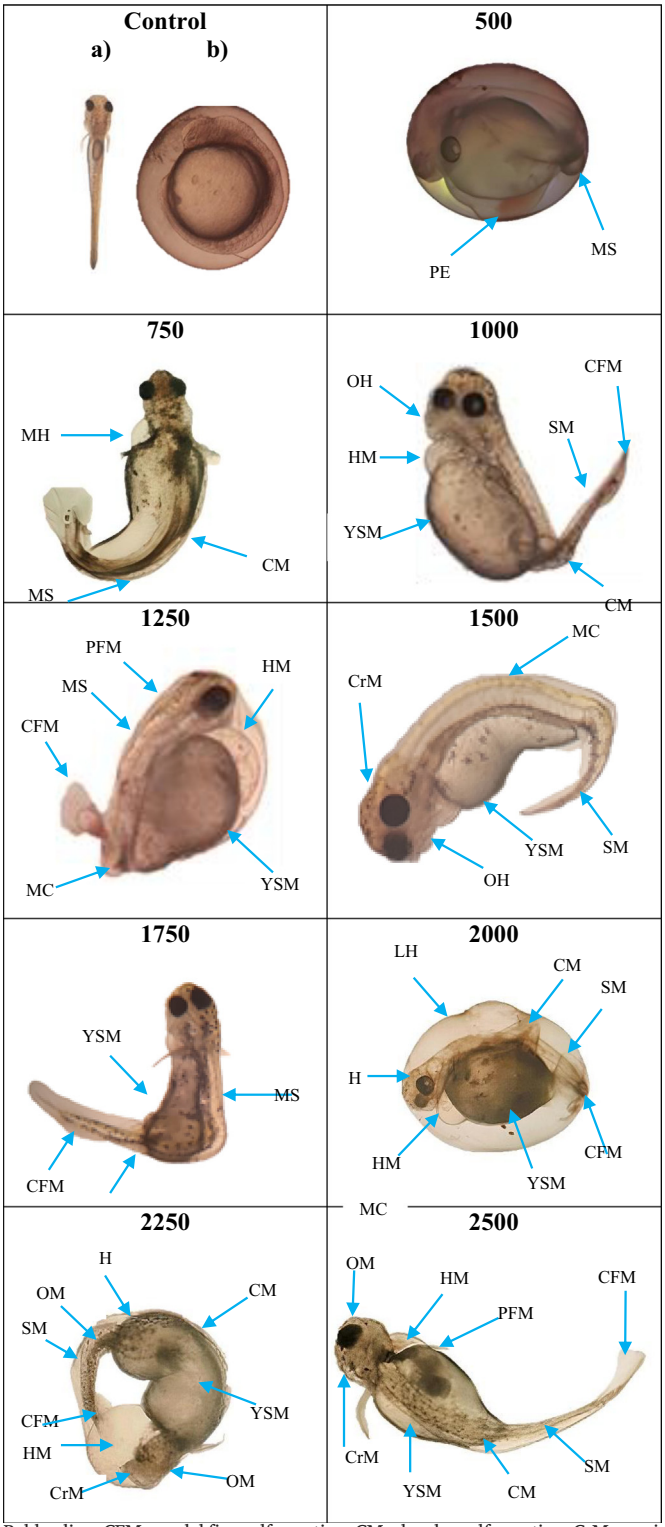
3.6. Bioconcentration factor

Table 3 shows the CAF concentrations in the exposure water, embryos and their respective partial bioconcentration factor (BCF) at 12, 24, 48, 72, and 96 hpf. As can be seen at 12 hpf no uptake of CAF is observed in the carp embryos. However, at 24 hpf the embryos are already capturing CAF from the medium. At 96 hpf, BCF values close to one or slightly above 1 were already observed in several concentrations. These results indicate that CAF has a good capacity to pass through the *C. carpio* chorion.

3.7. Pearson correlation between biomarkers of oxidative stress, embryolethality and gene expression

Fig. 6 shows the correlation between the biomarkers evaluated in this study. The colors show the strength of the correlation of the variables with each other. As the intensity of the color decreases, the correlation between the variables is stronger. As can be seen there is a positive correlation between the biomarkers of oxidative stress, gene expression and embryolethality. This would help us to prove our initial hypothesis that the embryological damage produced by CAF is related to oxidative damage produced by this methylxanthine.

Table 2
Main malformations induced by different concentrations of CAF (ng/L) in *C. carpio* embryos.



B: bleeding; CFM: caudal fin malformation; CM: chorda malformation; CrM: craniofacial malformation; EH: early hatching; GR: growth retardation; LH: late hatching; HM: heart malformation; H: hypopigmentation; OM: ocular malformation; OH: oral hyperplasia; PFM: pectoral fin malformation; PE: pericardial edema; WF: without fin; YSM: yolk sac malformation; SM: somites malformation.

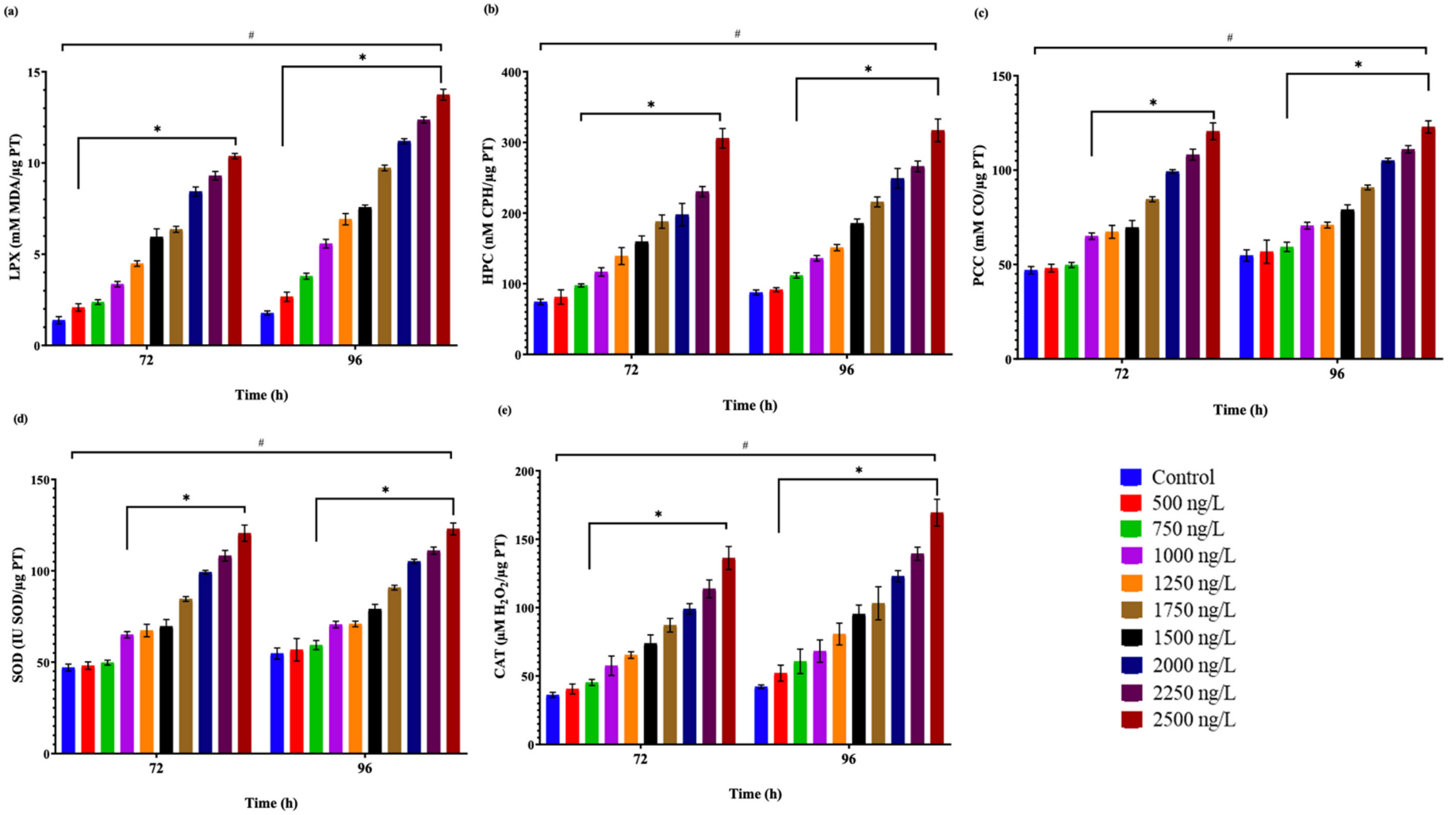


Fig. 4. Determination of lipoperoxidation level (a); hydroperoxides (b); carbonylated proteins (c). Enzymatic activity SOD (d) and CAT (e) induced by different concentration of caffeine during 72 y 96 h. Data represents the mean ± SD, a Mixed-effects model (REML) was applied establishing as fixed effects the groups with different concentrations of caffeine and time. Subsequently, a Dunnett's multiple comparisons test was applied, with statistical significance set to $p < 0.05$. Significant differences between to the control group (*) and significant differences between the exposure time 72 and 96 h (#) $p < 0.05$.

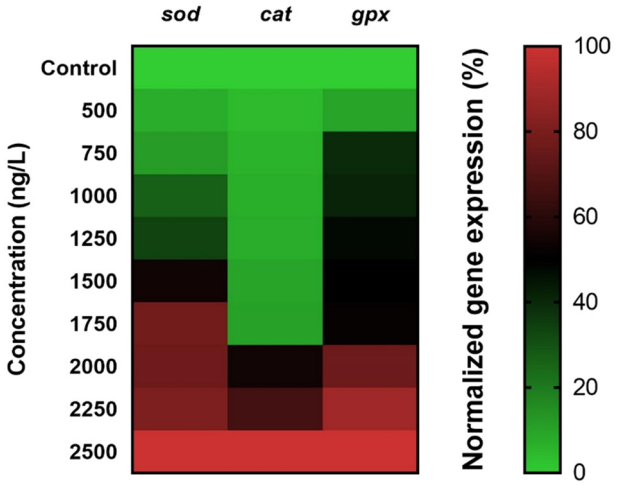


Fig. 5. Gene expression in *C. carpio* embryos induced at different concentrations of caffeine for 96 hpf.

4. Discussion

Caffeine is the most widely consumed psychoactive substance worldwide. In recent years its consumption has increased, due to the existence of new and novel products, population growth and its daily consumption by people of all ages. In addition, wastewater treatment plants are not efficient in removing this compound, and there is no legislation regulating the concentration of this methylxanthine in aquatic environments. This means that CAF is present in several bodies of water around the world, causing deleterious effects on aquatic organisms.

Some of the toxic effects induced by CAF have been documented in various aquatic species. Cruz et al. (2016) identified alterations in energy reserves, metabolic activity and oxidative damage in *Ruditapes philippinarum*. Rah et al. (2017), reported alterations in hair cells during embryogenesis in zebrafish. Chen et al. (2008) also observed movement and neuromuscular disorders in embryos of the same species. Félix et al. (2021) indicate that CAF induces possible teratogenic effects and neurological damage in zebrafish, through apoptotic mechanisms mediated by oxidative stress. These findings led us to conduct this research in which we assessed the embryological effects induced by CAF at environmentally relevant concentrations in a species of economic interest and economic importance in Mexico such as the common carp *C. carpio*. In addition to relate these effects with oxidative damage and the expression of genes related to oxidative stress.

In this study, progressive alterations in embryonic development were observed due to concentration-dependent exposure to CAF. The most frequent alterations were related to heart malformations, altered somite formation, pericardial edema and chordal malformations. These results agree with those observed by Rah et al. (2017), who demonstrated that in *Danio rerio* embryos exposed to CAF at concentrations of 25–500 μM, alterations in somite formation, body malformations, alterations in the notochord, dorsal fin, tail and heart malformations were identified. These researchers also reported that the embryonic development score decreases significantly as occurred in this study, and associate these alterations to an increase in apoptosis and mitochondrial damage. Furthermore, Rana et al. (2010) reported abnormal cardiovascular phenotypes in *D. rerio* embryos with exposure to 1–25 mM CAF.

It is well known that regardless of the species, the cardiovascular system is the first functional component in the embryonic development of vertebrates. The cardiovascular system in fish is functionally complete within 2 days after fertilization (Schwerte, 2009). Adenosine A1 receptors (A1ARs) are the dominant regulators of embryonic heart rate, which plays a key role in protecting the embryo against stress. Exposure to high concentrations of CAF during embryonic development in fish generates a down-regulation of A1ARs; this decrease, generates changes in the

Table 3
SPE LC-MS/MS Analysis of CAF concentrations in water and carp embryos samples, and BCF in carp embryos at 24, 48, 72 and 96 hpf.

Nominal	Exposure time														
Concentration CAF	12 hpf		24 hpf			48hpf			72 hpf			96 hpf			
	CAF in exposure water	CAF in carp embryos	CAF in exposure water	CAF in carp embryos	FBC	CAF in exposure water	CAF in carp embryos	FBC	CAF in exposure water	CAF in carp embryos	FBC	CAF in exposure water	CAF in carp embryos	FBC
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
500 ng/L	489.6 ± 3.1	<LoQ	418.1 ± 2.3	75.3 ± 3.8	0.018	416.6 ± 1.1	83.1 ± 7.1	0.199	403.9 ± 6.1	88.7 ± 5.7	0.219	116.7 ± 13	124.1 ± 7.3	1.063
750 ng/L	734.5 ± 4.6	<LoQ	643.6 ± 2.6	102 ± 3.1	0.158	631.3 ± 6.7	114.6 ± 3.1	0.181	712.6 ± 8.1	123.6 ± 12.1	0.173	443.9 ± 012.6	301.7 ± 22.6	0.679
1000 ng/L	989.4 ± 5.9	<LoQ	798.3 ± 2.6	196.3 ± 4.1	0.245	763.2 ± 6.8	224.3 ± 5.7	0.293	682.6 ± 6.1	307.6 ± 17.1	0.450	533.9 ± 13.3	437.8 ± 4.9	0.820
1250 ng/L	1244.9 ± 3.2	<LoQ	1139.6 ± 7.6	283.1 ± 4.6	0.248	850.6 ± 3.4	394.1 ± 7.7	0.463	781.7 ± 7.3	459.7 ± 18.3	0.588	713.7 ± 7.8	534.6 ± 32.1	0.750
1500 ng/L	1489.6 ± 4.6	<LoQ	1050.3 ± 2.9	421.6 ± 3.8	0.401	1055.3 ± 7.8	432.6 ± 3.9	0.409	1000.6 ± 13.2	496.5 ± 0.12.7	0.496	745.8 ± 11.7	723.6 ± 12.3	0.970
1750 ng/L	1739.7 ± 3.1	<LoQ	1190.9 ± 6.9	552.4 ± 7.9	0.463	1710.8 ± 6.7	731.8 ± 3.6	0.427	923.7 ± 12.4	810.6 ± 21.3	0.877	807.7 ± 17.6	940.3 ± 8.9	1.164
2000 ng/L	1978.6 ± 4.5	<LoQ	1260.8 ± 7.2	722.7 ± 4.6	0.573	1048.6 ± 3.7	923.6 ± 5.3	0.880	978.9 ± 8.9	1002.6 ± 25.1	1.024	960.7 ± 20.1	989.7 ± 21.9	1.030
2250 ng/L	2236 ± 5.6	<LoQ	1256.8 ± 7.5	973.6 ± 13.1	0.774	1039.8 ± 13	1001 ± 15.3	0.962	1145.8 ± 16	1126.7 ± 0.23	0.983	1239.5 ± 13.8	1250.8 ± 39.8	1.009
2500 ng/L	2440 ± 5.7	<LoQ	1235.8 ± 10.1	1221.9 ± 6.9	0.988	1203.2 ± 6.7	1285.6 ± 13.9	1.068	1248.7 ± 13,1	1223.9 ± 14.9	0.980	1090.8 ± 27.7	1398.5 ± 10.6	1.282

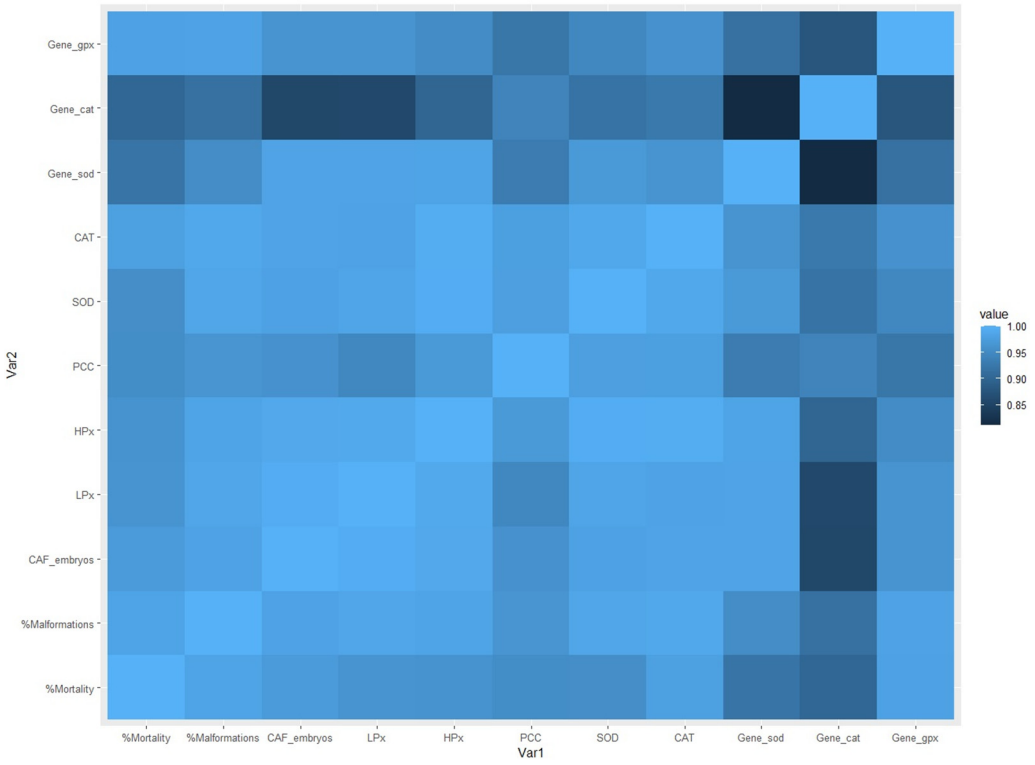


Fig. 6. Correlation between oxidative stress biomarkers, gene expression and embryolethality for CAF exposure in *C. carpio*.

formation of embryonic cardiovascular tissues, producing cardiac alterations (de Souza et al., 2016; Menezes et al., 2018).

Previous findings have identified CAF as a compound that affects cell cycle function, induces programmed cell death or apoptosis and disrupts key cell cycle regulatory proteins. Following DNA damage, the p53 protein has an influence on whether cells will live or die. Cells exposed to CAF and other chemotherapeutic agents pause their cell cycle progression to allow time for DNA repair. If DNA damage is extensive, the affected cells will undergo apoptosis (Ramkumar et al., 2001).

Several studies suggest that CAF induces teratogenic effects through different mechanisms, including down-regulation of adenosine receptors (AR), inhibition of phosphodiesterases related to cellular calcium dynamics, induction of catecholamine release, and interaction with GABA receptors (Rana et al., 2010; Echeverri et al., 2010; Mustard, 2014; Cui et al., 2020; da Silva et al., 2017).

The main mechanism of action of CAF is as an adenosine receptor (AR) antagonist (Félix et al., 2021; Rana et al., 2010). When CAF reaches high plasma levels, there is a preferential blockade by A_1AR promoting an inhibition of adenylyl cyclase (AC) activity, leading to an increase in cyclic adenosine monophosphate (cAMP). The accumulation of cAMP decreases cytokine production. However, because CAF is a non-specific AR antagonist, at high concentrations, it also blocks adenosine 2 receptors (A_2AR), decreasing cAMP and increasing the transcription of proinflammatory cytokines (Rana et al., 2010).

The accumulation of cAMP is related to: 1) the inhibition of a protein kinase (ATK) involved in a wide variety of cellular processes, such as survival, growth, proliferation, migration, angiogenesis, metabolism, resistance to oxidative stress (Fisone et al., 2004; Halldner et al., 2004; Echeverri et al., 2010); and 2) activates mitogen-activated protein kinases (MAPK/ERK), which is a protein kinase capable of translocating to the nucleus and regulating transcription by modifying the activity of proteins (including transcription factors), thus modulating the expression of different genes. It is a common mechanism of transduction of extracellular information from extracellular stimuli into the intracellular space. The transduction of information leads to changes in ongoing metabolic pathways and modification of gene expression patterns. This signaling system is involved at three key times: (i) embryonic development; (ii) the early postnatal period; and (iii) adulthood (Cui et al., 2020).

Likewise, the increase of cAMP is related to the presence of intracellular Ca^{2+}. This is indispensable in embryonic cytokinesis, favors the establishment of embryonic axes, germinal layers, and provides the definition of the morphological limits of specific tissues, domains and embryonic structures. In addition, they influence other early developmental signaling pathways (Webband and Miller, 2007).

In the present study, alterations in *C. carpio* morphogenesis were observed, more frequently and more evident at higher CAF concentrations. This methylxanthine acts directly on Ca^{2+} dynamics during *C. carpio* embryogenesis. Tsuruwaka et al. (2017), report that Ca^{2+} play an important role during *D. rerio* morphogenesis in embryonic stages, in particular in the structural formation of the chorda, somitogenesis, brain division, tail elongation, muscle contraction, synaptic transmission, and cardiac nerve transmission (Webband and Miller, 2007; Tsuruwaka et al., 2017). Thus by altering Ca^{2+} dynamics, CAF may induce the alterations observed in the study in common carp.

In the present study, the biomarkers of cellular oxidation by CAF exposure during embryogenesis of *C. carpio*, was reflected with the significant increase of LPX, HPC and PCC in a concentration- and time-dependent manner ($p < 0.05$). The antioxidant response had the same effect.

Our outcomes showed that the biomarkers of cellular oxidation by CAF exposure during *C. carpio* embryogenesis reflected a significant increase of LPX, HPC and PCC in a concentration- and time-dependent manner ($p < 0.05$). The enzymatic antioxidant response had the same effect.

It is known that LPX is considered the main molecular mechanism involved in the oxidative damage of cellular structures and in the toxicity process leading to cell death. It is involved in the formation and propagation of lipid radicals, oxygen uptake, a rearrangement of double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of degradation products, including alcohols, ketones, alkanes, aldehydes and ethers (Repetto et al., 2012). These degradation products can react with proteins and nucleic acids, producing cytotoxic, genotoxic and mutagenic effects (Repetto et al., 2012). Our findings are in agreement with those obtained by Lu et al. (2008),

who mentioned that most CAF-treated cells die through apoptotic processes due to the generation of ROS. CAF triggers apoptosis in osteoblasts through activation of mitochondria-dependent cell death signaling p21-activated protein kinase 2 & c-Jun N-terminal kinase (PAK2 & JNK) and inactivation of survival signaling (ERK and Akt). ROS are an important upstream regulator of JNK and caspases activation during apoptosis.

The protective barriers due to CAF exposure are exceeded by the presence of ROS, with the increase of the biomarkers LPX, HPC and PCC. ROS production is associated with the occurrence of apoptosis and plays an important role in altering embryonic development, as our results have shown.

During particular periods of fish development, the embryo is more susceptible to oxidative stress and teratogens, which can modify the redox state and thus alter development (Dennery, 2007). Depending on certain periods in the development of the fish the embryo may be more or less susceptible to oxidative stress. For example in early development the embryo depends on the Krebs cycle as a metabolic source, in the blastocytic stage on glycolysis and ananerobic pathways. Once the circulatory system is developed there is a greater dependence on oxidative and aerobic metabolism, more ROS are formed that can generate important alterations in the development of the embryo (Hansen, 2006). In development, the delicate balance between oxidants and antioxidants can be altered by exogenous agents such as CAF, which induce the production of ROS and cause an imbalance, as demonstrated in the present study, with increased LPX, HPC, PCC, SOD & CAT activity.

Antioxidant enzymes during embryogenesis in fish are important for understanding mechanisms against ROS and changes associated with environmental stress, such as temperature, oxygen, and pollution (Faggio et al., 2014; Burgos-Aceves et al., 2018; Blahova et al., 2021; Mukherjee et al., 2022). Animals have evolved mechanisms that utilize the toxic properties of ROS to resist pathogens. In addition, a wide range of antioxidant mechanisms are present in fish, the main antioxidant enzymes being superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione transferase (GT). These provide the first defense; a second line of defense is provided by antioxidants provided by nutritional supplements (Repetto et al., 2012).

The outcomes obtained in the present study showed that, although the activity of both antioxidant enzymes increased significantly with increasing CAF concentration, this defense strategy was not able to effectively eliminate excess ROS to prevent *C. carpio* embryos from cellular oxidation. Defense mechanisms can be activated when cell damage is present, and not only depend on the species, but also on the xenobiotic and concentration (Cruz et al., 2016). This may explain why, in the present study, at the lowest CAF concentrations, there were no significant differences with respect to the control group, i.e. the defense system was able to eliminate reactive species, but as the CAF concentration increased, ROS were overcome by antioxidant mechanism.

Under stressful conditions, such as the presence of pollutants and seasonal variations, organisms can increase their metabolic activity and decrease their energy reserves (glycogen content) to activate antioxidant and detoxifying defense mechanisms (Almeida et al., 2015; Dickinson et al., 2012; Smolders et al., 2004). The present study demonstrated that *C. carpio* embryos exposed to CAF increased their antioxidant enzyme activity (SOD & CAT), in an attempt to fight oxidative stress with the expenditure of their energy reserves.

ROS not only have a direct effect on cells but also act as second messengers, regulating transcription factors that alter gene expression in embryos. Among the many transcription factors that are sensitive to the redox process: hypoxia-inducing factor (HIF-1); nuclear factor κB (NF-kB); activating protein factor 1 (AP-1); redox effector factor (Ref-1); nuclear factor (NF)-E2-related factor 1 (Nrf-1), and the integration site for mouse mammary tumor virus (Wnt), are vital in cell signaling pathways that dictate proliferation, differentiation, and apoptosis and play a significant role in embryonic development (Dennery, 2007).

In this study, up-regulation of *sod*, *cat* and *gpx* gene expression associated with altered oxidative status in the embryo was observed. These results are consistent with those found by Wiegand et al. (2000) in *D. rerio* and Zengin et al. (2015) in *O. mykiss*. They refer that, since ontogenesis, embryos possess detoxification enzymes such as glutathione S-transferase (GST) and GPX. These enzymes appear to be constitutive in embryos, even with activity from fertilized and unfertilized oocytes, showing the need to cope with oxidative stress.

The results of altered gene expression and oxidative stress due to exposure to CAF observed in *C. carpio* embryos are directly related to the embryological alterations presented in common carp and put the life of this species at risk.

In Fig. 7, we refer to our proposed mechanism of action through which CAF exerts its embryological effect on *C. carpio*. CAF acts as an antagonist of adenosine receptors, promoting the inhibition of AC activity. Likewise, CAF causes the inhibition of phosphodiesterase's, which will trigger an accumulation of cAMP. Also, CAF influences intracellular Ca^{2+} homeostasis in two

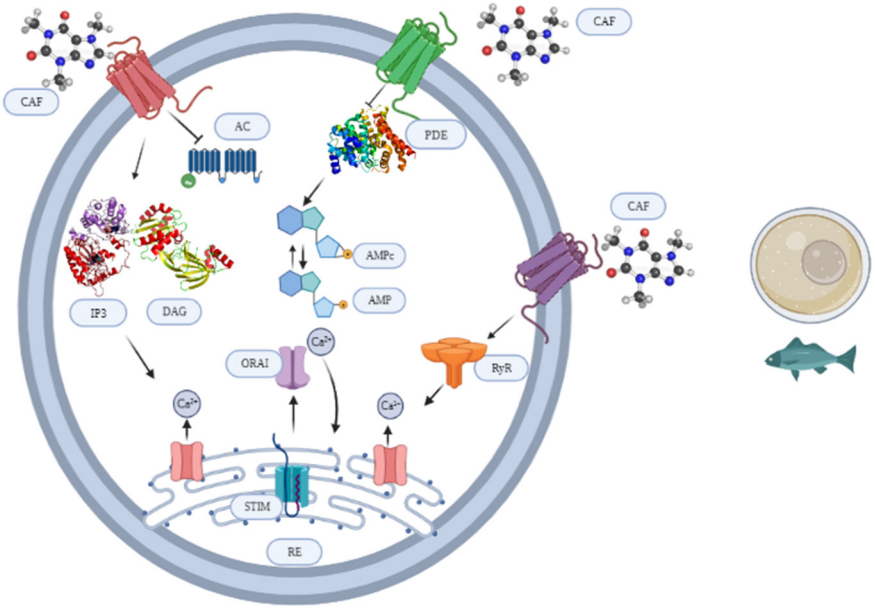


Fig. 7. Proposed mechanism through which CAF exerts embryological effects. CAF: caffeine; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; IP3: inositol 1,4,5-trisphosphate; DAG: diacylglycerol; PDE: phosphodiesterases; RyR: ryanodine receptor; ER: endoplasmic reticulum.

different ways: one is by inhibiting the activation of IP3 receptors, through which it suppresses the release of Ca^{2+} from intracellular stores. However, CAF can increase Ca^{2+} levels, by activation of (RyR), which causes an increase in Ca^{2+} release from the endoplasmic reticulum. Depletion of ER Ca^{2+} stores, activates the stromal interaction molecule (STIM) of the censor protein to open ORAI Ca^{2+} channels. The mechanism of action of CAF is involved in different cellular processes, signaling pathways and transcription factors leading to modification of patterns in gene expression.

5. Conclusions

The results obtained clearly demonstrated that CAF induced embryological alterations in *C. carpio*. The main alterations identified by CAF exposure were cardiac malformations, somite alterations, pericardial edema and chorda malformations. CAF, even at environmentally relevant concentrations, had the ability to alter cellular oxidation biomarkers such as LPX, HPC and PCC, as well as a significant increase in antioxidant enzymes such as SOD & CAT. An upward expression of genes related to antioxidant status *sod*, *cat* and *gpx* was observed in *C. carpio* embryos. CAF is a potentially hazardous compound to aquatic organisms, including *C. carpio*.

Further investigation is warranted to explore the effects of CAF during early and adult stages, as the complete understanding of its mechanism of action, including signaling pathways, molecular targets, cellular factors, and genes involved, remains incomplete.

CRediT authorship contribution statement

Idalia Casas-Hinojosa, Veronica Margarita Gutierrez-Noya & Karina Elisa Rosales-Pérez performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Idalia Casas-Hinojosa were involved in the conception.

Leobardo Manuel Gómez-Oliván, Idalia Casas-Hinojosa and Sandra García-Medina, were involved in the design and interpretation of the data and the writing of the manuscript with input from José Manuel Orozco-Hernández, Gustavo Axel Elizalde-Velázquez, Marcela Galar-Martínez, Octavio Dublán-García, María Dolores Hernández-Navarro, & Hariz Islas-Flores.

Data availability

Data will be made available on request.

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.

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