Science of the Total Environment 829 (2022) 154689

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Acute exposure to environmentally relevant concentrations of sucralose disrupts embryonic development and leads to an oxidative stress response in *Danio rerio*

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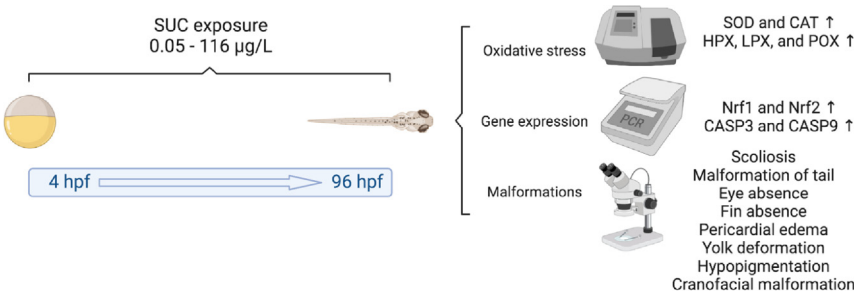
HIGHLIGHTS

- Sucralose triggered the production of several malformations in *Danio rerio* embryos.
- ROS produced by sucralose led to oxidative stress response in larvae.
- Nrf1, Nrf2, CASP3, and CASP9 were overexpressed in embryos after SUC exposure.
- Environmentally relevant concentrations of sucralose are harmful to aquatic species.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 February 2022
Received in revised form 15 March 2022
Accepted 15 March 2022
Available online 18 March 2022

Editor: Henner Hollert

Keywords:
Artificial sweetener
Zebrafish
Oxidative status
Apoptosis

ABSTRACT

Sucralose (SUC) is the most consumed artificial sweetener worldwide, not metabolized by the human body, and barely eliminated from water in wastewater treatment plants. Although different studies have reported high concentrations of this sweetener in aquatic environments, limited to no information is known about the toxic effects this drug may produce over water organisms. Moreover, most of the current studies have used non-environmentally relevant concentrations of SUC for these effects. Herein, we aimed to evaluate the harmful effects that environmentally relevant concentrations of SUC may induce in the early life stages of *Danio rerio*. According to our results, SUC altered the embryonic development of *D. rerio*, producing several malformations that led to their death. The major malformations were scoliosis, pericardial edema, yolk deformation, and tail malformation. However, embryos also got craniofacial malformations, eye absence, fin absence, dwarfism, delay of the hatching process, and hypopigmentation. SUC also generated an oxidative stress response in the embryos characterized by an increase in the levels of lipid peroxidation, hydroperoxides, and carbonyl proteins. To overcome this oxidative stress response, we observed a significant increase in the levels of antioxidant enzymes superoxide dismutase and catalase. Moreover, a significant boost in the expression of antioxidant defense-related genes, *Nuclear respiratory factor 1a (Nrf1a)* and *Nuclear respiratory factor 2a (Nrf2a)*, was also observed at all concentrations. Concerning apoptosis-related genes, we observed the expression of *Caspase 3 (CASP3)* and *Caspase 9 (CASP9)* was increased in a concentration-dependent manner. Overall, we conclude environmentally relevant concentrations of SUC are harmful to the early life stages of fish as they produce malformations, oxidative stress, and increased gene expression of apoptosis-related genes on embryos.

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http://dx.doi.org/10.1016/j.scitotenv.2022.154689

1. Introduction

Artificial sweeteners are used in food, medicines, and cosmetics to substitute sugar (Li et al., 2021). Nonetheless, they can also be used in electroplating to remove rust from metals (Van Vy et al., 2021). Such is their demand that Luo et al. (2019) estimated the global market of sweeteners would reach 1.11 trillion dollars in 2020.

Sucralose (SUC) is the fourth most consumed artificial sweetener worldwide, just after aspartame, saccharin, and acesulfame (Praveena et al., 2019). It is not readily metabolized in the human body (Xu et al., 2018), and as much as 92% of the ingested amount is excreted unchanged (Tollefsen et al., 2012). After its excretion by urine (78.3%) and feces (14.4%), SUC reaches out wastewater treatment plants (WWTPs), where conventional and advanced wastewater treatment processes have been reported to be ineffective for its elimination (Scheurer et al., 2010; Soh et al., 2011; Keen and Linden, 2013; Biel-Maeso et al., 2019; Sörengård et al., 2019). Accordingly, this artificial sweetener has been found to occur at ng/L to μg/L in aquatic environments (Table 1).

Despite the extensive usage of SUC, the safety of this sweetener has kept controversial due to its potentially harmful effects on organisms and cell cultures (Eriksson Wiklund et al., 2014; Stoddard and Huggett, 2014; Bian et al., 2017; Magnuson et al., 2017; Saucedo-Vence et al., 2017; Dhurandhar et al., 2018; Heredia-García et al., 2019; Pasqualli et al., 2020). In fish, for instance, Wiklund et al. (2012) demonstrated SUC (0–50,000 μg/L) disrupted the behavior and locomotion system in three different crustaceans (*Gobionellus oceanicus*, *Gammarus zaddachi*, *Daphnia magna*) after 14 days of exposure. Moreover, Eriksson Wiklund et al. (2014) pointed out SUC (0.0001–5 mg/L) can promote the production of reactive oxygen species (ROS) and acetylcholinesterase (AChE) in *Daphnia magna* neonates after 24 h. In accordance with both studies, Saucedo-Vence et al. (2017) reported that environmentally relevant concentrations of SUC (0.05 and 155 μg/L) induced oxidative stress in the blood, liver, brain, gill, and muscles in juvenile of *Cyprinus carpio*.

Concerning SUC toxic effects in early life stages, only three studies that we are aware of have been performed in fish. For example, Stoddard and Huggett (2014) reported the Lowest-Observed-Effect Concentration (LOEC) for SUC was >98 mg/L. Hence, they suggested that the concentrations of sucralose detected in the environment are well below those required to cause adverse effects to develop aquatic organisms. Nonetheless, in disagreement with these results, Arndorfer (2018) showed SUC (100,000 μg/L) generated scoliosis and dwarfism in *D. rerio* larvae after 96 h of exposure. Furthermore, Saputra et al. (2021) found SUC at concentrations of 100 μg/L significantly increased the heart rate frequency in *D. rerio* larvae after 12 and 72 h of exposure.

In the light of the above information and the fact that SUC toxic effects have not been evaluated in environmentally relevant concentrations, we aimed to determine whether or not SUC at concentrations previously reported in the water matrix can alter the embryonic development of *D. rerio*. Moreover, we also aimed to find out if SUC at those concentrations may disrupt redox status in the early life stages of these freshwater organisms. We hypothesize sucralose will increase the production of ROS in zebrafish embryos, triggering an oxidative stress response that will generate several malformations on them.

2. Method

2.1. Compounds

Sucralose (>98%; CAS number: 56038-13-2) and all further reagents were acquired from Sigma-Aldrich (St. Louis, MO).

2.2. Danio rerio upkeep

A hundred five-month-old *D. rerio* adults (AB strain) were kept in two aquaria of 50 L provided with charcoal-filtered and UV-sterilized tap water. One of the aquaria housed the females and the other one the males (1 fish: 1 L ratio). Feeding was performed three times a day with Spirulina flakes (Ocean Nutrition, US). Moreover, every other day we gave female fish brine shrimp (*Artemia* sp. *nauplii*) to promote spawning activity. Water quality parameters were controlled and monitored during fish maintenance: alkalinity (94 ± 3 mg/L $CaCO_3$), conductivity (385 ± 31 μS/cm), dissolved oxygen (10.1 ± 0.3 mg/L), nitrate (2.9 ± 0.3 mg/L), nitrite (0.028 ± 0.009 mg/L) and pH (7.29 ± 0.11).

2.3. Danio rerio spawning and embryo collection

On the late-night before spawning, twenty-four *D. rerio* adults were placed on eight individual breeding chambers. Each chamber was assigned with two female fish and a male. The onset of light on next morning promoted the spawn and fertilization of embryos, which were collected and rinsed according to protocols of Westerfield (2007) and Varga (2011). Next, following Kimmel et al. (1995) method, fertilized embryos were classified using a stereomicroscope (Zeiss Stemi 305). Only middle blastula stage embryos (2.5 hpf) were selected and incubated at 27 ± 1 °C until they reached the sphere stage (4 hpf).

Table 1
Worldwide occurrence of sucralose in aquatic environments.

	Country	Aquatic matrix	Concentration (μg/L)	References
Asia	China	Ground water	0.0129	Gan et al., 2013a
		Tap water	0.113–0.171	Gan et al., 2013a
		Surface water	0.287–0.311	Gan et al., 2013b
		Surface water	0.05	Sang et al., 2014
		Surface water	0.89–20.6	Fu et al., 2020
	Singapore	Ground water	<0.03	Tran et al., 2013
		Surface water	>0.05	Tran et al., 2013
Europe	Finland	Surface water	<0.2–1	Perkola and Sainio, 2014
	Spain	Waste water	0.5	Ordóñez et al., 2012
		Surface water	25	Ordóñez et al., 2012
		Waste water	0.05–155	Arbeláez et al., 2015
		Surface water	3.57	Arbeláez et al., 2015
	Sweden	Surface water	0.004	Gan et al., 2013a
	Switzerland	Surface water	<0.01–0.949	Berset and Ochsenbein, 2012
North America	EE. UU.	Waste water	27	Oppenheimer et al., 2011
		Surface water	0.12–10	Oppenheimer et al., 2011
		Waste water	77	Cantwell et al., 2019
		Surface water	0.018–3.18	Cantwell et al., 2019
		Waste water	0.9176–2.0312	Yang et al., 2021
		Surface water	2.07	Yang et al., 2021
		Drinking water	0.2881–0.5053	Yang et al., 2021

2.4. Embryotoxicity test

Seventy-two sphere stage (4 hpf) embryos per environmentally relevant concentration of SUC (0, 0.05, 19.3, 48.4, 77.5, 96.97, 116.25 μg/L) were selected and allocated into 24-well plates. Each well contained one embryo; thus, we used three 24-well plates per SUC concentration. All plates were incubated at 27 ± 1 °C and maintained under the same light/dark periods (14 h:10 h ratio). Malformation and mortality rates were estimated and assessed at different times (12, 24, 48, 72, and 96 hpf) during the course of SUC exposure. Malformation rate was given as the % of embryos with at least one body deformation compared to the control group. The experiments were replicated three times in independent experiments. Considering mortality data, we performed a maximum likelihood linear regression analysis to determine lethal concentration 50 (LC_{50}) and effective concentration 50 of malformations (EC_{50m}) with their 95% confidence intervals ($p < 0.05$). Moreover, by using SigmaPlot 12.3 software, a Kaplan-Meier analysis was performed. Finally, using IBM SPSS Statistics 22 software, we performed a chart with principal alterations induced by SUC on the embryos.

2.5. Oxidative stress

Seven systems, each with 1600 sphere stage (4 hpf) embryos, were allocated in aquaria of 5 L of capacity. Each system was assigned with one of the seven concentrations of SUC (0, 0.05, 19.3, 48.4, 77.5, 96.97, 116.25 μg/L). Temperature (27 ± 1 °C) and light/dark cycles (14 h:10 h ratio) were kept constant in all systems during the exposure period. At 72 hpf and 96 hpf, respectively, a mean of 500 larvae (SD: ±25 larvae) was selected and homogenized in 1 mL of phosphate buffer solution (pH 7.4). Those endpoints were selected because, at that time, embryos had hatched and their antioxidant enzymes were functioning (Elizalde-Velázquez et al., 2021a, 2021b). Homogenate was split into two Eppendorf tubes. Tube one contained 300 μL of trichloroacetic acid (20%) and 300 μL of homogenate, and tube two only enclosed 700 μL of the latter. For quantification of lipid peroxidation (LPX) by Buege and Aust (1978) method, protein carbonyl content (POX) by Levine et al. (1994), and hydroperoxide content (HPX) by Jiang et al. (1992). Meanwhile, to assess antioxidant enzymes activity of superoxide dismutase (SOD) by Misra and Fridovich (1972) method and catalase (CAT) by Radi et al. (1991) we used tube two. Results from all biomarkers were normalized against total proteins by the method of Bradford (1976). Oxidative stress experiment was performed three times, and samples from each experiment were analyzed per triplicate. Accordingly, we got nine results for each biomarker, which we then used to perform statistical analyses and charts (n = 9).

2.6. Gene expression

For ribonucleic acid (RNA) isolation, we used a mean of 100 larvae (SD: ±25 larvae) per concentration of SUC and the RNeasy® kit off Qiagen. Once isolated, RNA concentration and purity were determined through the 260/280 ratio and gel electrophoresis techniques, respectively. 1 μg of the total RNA and the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF 205313) were used to perform the reverse transcription under the following conditions: 42 °C for 15 min and 95 °C for 3 min. qRT-PCR was performed using 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 30s. Each reaction was carried out in a 50 μL solution containing 0.3 μmol primers, 25 μL 2× SYBER Green QuantiTec® (QIAGEN, Hilden, Germany), and 500 ng of cDNA template. B-actin was used as housekeeping gene to normalize all the samples. The housekeeping gene was evaluated for each organism in both control and exposed organisms. No change in expression was observed in those organisms that received treatment. Since for gene expression experiment, we used the remaining larvae from the oxidative stress experiment, and such experiment was performed three times, we also analyzed three different samples per SUC concentration for this experiment. Moreover, each sample was analyzed per triplicate. So, we got nine results per gene, which we then used to perform statistical analysis and charts (n = 9). Genes used for qRT-PCR are related to oxidative stress response and cell apoptosis (Table 2).

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 Polymerase Chain Reaction (PCR) reaction was repeated three times to minimize experimental error.

2.7. Sucralose quantification

Stock solution of SUC standard was prepared by dissolution of pure compound in methanol and ultrapure water (50%/50%) at a concentration of 1000 μg/L and then stored at −20 °C in amber glass bottles. Sampling was performed as Elizalde-Velázquez et al. (2021a, 2021b) described before. Briefly, in the case of the embryotoxicity test, 140 μL of water was collected and pooled from each of the 72 wells. Thus, we got water samples of 10 mL for each of the SUC concentrations. In the oxidative stress experiment case, on the other hand, we just collected 10 mL of water from each of the systems of SUC. Extraction of SUC from water samples was performed following the method established by Saucedo-Vence et al. (2017). Briefly, 10 mL of water samples were acidified with HCl (1 M). Next, SUC was extracted from 1 mL of the acidified water with 5 mL of methanol: water (1:1 ratio). The mixture was then centrifugated at 1800 ×*g* for 10 min, and the organic layer obtained was used for SUC quantification. Quantification of SUC was performed in an Agilent 1290 Infinity HPLC unit coupled to an Agilent 6430 Triple Quadruple MS equipped with electrospray. Separation was achieved using a RRHD Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm). The mobile phase consisted of a mixture of water with formic acid (pH 2.5) and acetonitrile. The flow rate was 0.4 mL/min, and the injection volume was 50 μL. Analyses were performed in negative mode employing Multiple Reaction monitoring (MRM). Negative ESI was optimized to the following conditions: nebulizer pressure 45 psi, drying gas (N_2) flow of 11 L/min, drying gas temperature of 350 °C, and capillary voltage 4000 V. The accuracy of the proposed method was confirmed by the control spiking method, which was carried out by spiking an ultrapure water-methanol solution (50:50 ratio) with SUC at three different levels 80%, 100%, and 120%. Samples of both experiments were evaluated per triplicate. To evaluate the usefulness for quantitative analyses of SUC in water, the analytical performance of the proposed method was studied and validated in terms of recovery, linearity, limits of detection, limits of quantitation and inter-day precision of the technique. Values for the coefficient of determination R^2 was >0.99. Recoveries in % (RSD) was 83, LOD: limit of detection (15 ng/L). LOQ: limit of quantification (10 ng/L).

Table 2
Genes used for qRT-PCR.

Gene	Forward primer	Reverse primer	Reference
Nrf1a	TTT GGT TCC CGA TGA AGA CG	TGA TTA GCG TGA GAC TGA GC	Sant et al., 2017
Nrf2a	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	Sant et al., 2017
CASP3	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT	Félix et al., 2018
CASP 9	CGG AGG TGA GAA GGA TAT	TCC AGC ACA CGA TCA AGA TT	Jiang et al., 2014

Nuclear respiratory factor 1a = Nrf1a; *Nuclear respiratory factor a2 = Nrf2a*; *Caspase 3 = CASP3*; *Caspase 9 = CASP9*. Primer efficiency was evaluated by Primer-Blast NCBI (Ye et al., 2012).

The inter-day precision and repeatability of the method were also evaluated on water samples at different concentration levels. The RSD (n = 5) values for intra-day analyses were in the range 1–3% and the RSD for inter-day (n = 5) values were between 3 and 8% showing good reproducibility of the methodology.

2.8. Statistical analysis

Data from all experiments were expressed as the mean ± standard deviation (SD). Significant differences between means were determined through a Student Newman Keuls test. Furthermore, a one-way ANOVA test with 95% confidence intervals was carried out to assess the significance of differences between concentrations (SigmaPlot 12.3). Normality was evaluated with the Shapiro-Wilk test. A principal component analysis to correlate all variables was done using R software and considering $p < 0.05$.

3. Results

3.1. Embryotoxicity test

After 96 h of exposure, mortality ($F(6,14) = 322.784$, $p < 0.001$) and malformation rates ($F(6,14) = 217.806$, $p < 0.001$) showed a statistically significant concentration-dependent increase (Fig. 1A–B). Thus, the highest number of dead and malformed embryos was observed at the highest concentration of SUC (116.5 μg/L). From Fig. 1A, it can also be seen that as time elapsed, the number of dead embryos also increased. However, it is noteworthy to say more deaths occurred in the last 24 h of exposure. Significant differences were observed between treatment groups, including the control group. LC_{50} and EC_{50} of malformations were calculated, and their values were 123.155 μg/L and 4.88 μg/L, respectively.

3.2. Teratogenic effects

At 96 hpf, we observe three embryos with hypopigmentation in the control group. However, unlike the control group, SUC-exposed fish showed a higher volume of malformations, and all of them were much more severe. Among the malformations induced by SUC on embryos, we found malformation of the tail, scoliosis, pericardial edema, yolk deformation, craniofacial malformations, eye absence, fin absence, dwarfism, delay of the hatching process, and hypopigmentation (Fig. 2A and B). Additionally, it is noteworthy to say that as the concentration of SUC started to increase, the severity of malformations also increased, and the proportion of malformations per embryo (Fig. 2B). Thus, at the concentration of 116.5 μg/L of SUC, we observed embryos presented a higher incidence of scoliosis, pericardial edema, yolk deformation, and craniofacial malformation compared to other SUC concentrations (Fig. 2C). Fig. 2D shows the concentration-response curves for morphological scoring performed from all concentrations of SUC. For SUC, there was a clear shift between the concentration-response curves, suggesting a progressive effect on the development of alterations at all concentrations tested.

3.3. Oxidative stress

As same as the mortality and malformation rate, we observed antioxidant enzymes and oxidative damage biomarkers increased in a concentration-dependent manner (Fig. 3A–E). However, it is noteworthy that the activity of SOD (Fig. 3A1 and A2) ($F(6,56) = 890.416$, $p < 0.001$) and CAT (Fig. 3B1 and B2) ($F(6,56) = 1009.612$, $p < 0.001$) was not increased as much as the levels of HPX (Fig. 3E1 and E2) ($F(6,56) = 1979.350$, $p < 0.001$), POX (Fig. 3D1 and D2) ($F(6,56) = 366.539$, $p < 0.001$), and LPX (Fig. 3C1 and 3C2) ($F(6,56) = 1538.233$, $p < 0.001$). For example, in the case of SOD and CAT, it is observed that values from SUC concentrations

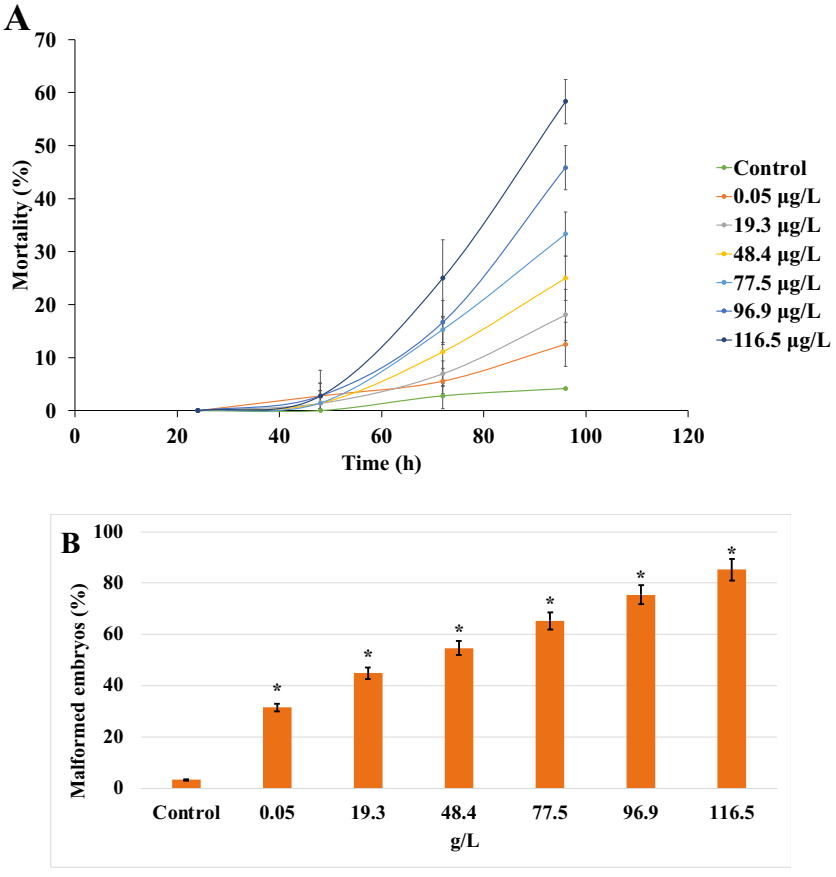
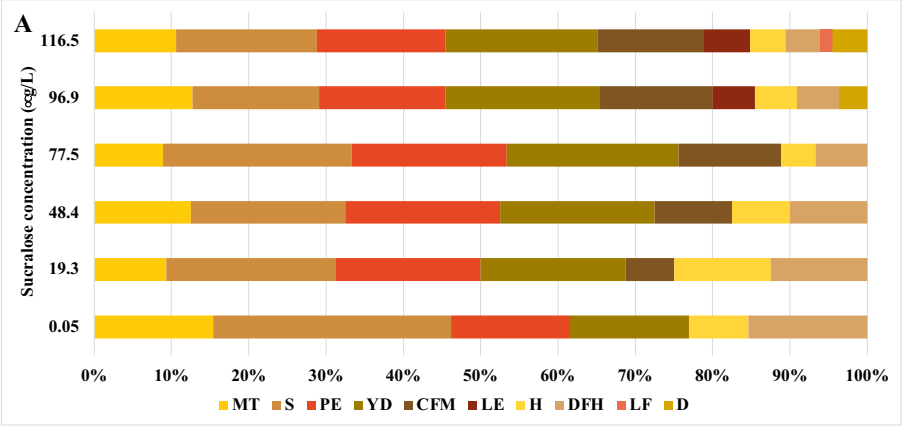


Fig. 1. Mortality (A) and malformation (B) rates of *D. rerio* larvae exposed to SUC. Data represents mean ± standard deviation (SD) (n = 3). * indicates significant difference compared to control group.

barely overpass the values from the control group. Meanwhile, in the case of LPX, POX, and HPX, levels from SUC exposed fish were up to four times higher than the levels of the control group. Thus, we believe SOD and CAT were not able to overcome the oxidative damage produced by SUC. Significant differences between treatment groups, including the control group, were found for all biomarkers. Concerning times of exposure, we saw

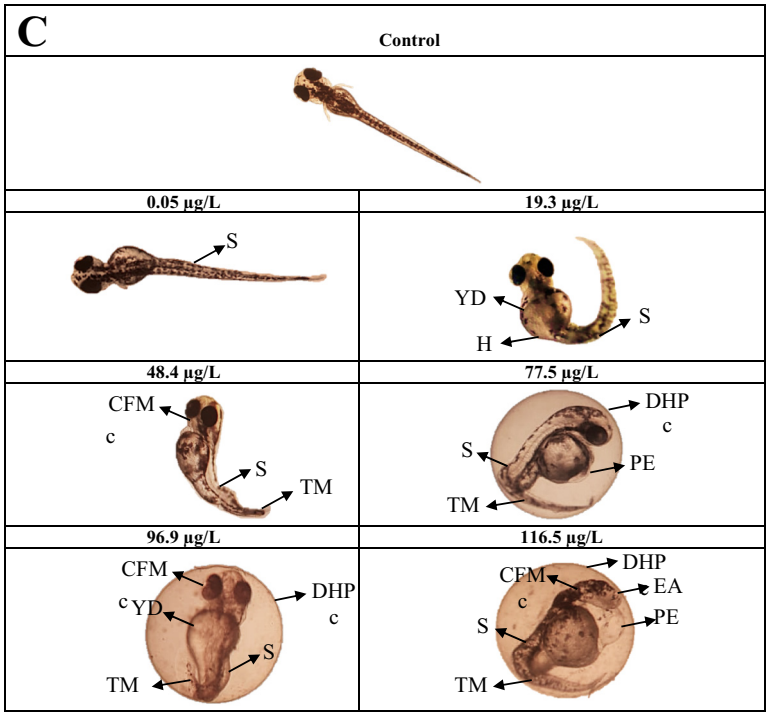
A



B

Sucralose concentration (µg/L)	Incidence of malformations										
	MT	S	PE	YD	CFM	LE	H	DFH	LF	D	Total
Control	0/72	1/72	0/72	0/72	0/72	0/72	3/72	0/72	0/72	0/72	4/72
0.05	4/72	7/72	4/72	4/72	0/72	0/72	2/72	4/72	0/72	0/72	26/72
19.3	3/72	8/72	6/72	6/72	2/72	0/72	4/72	4/72	0/72	0/72	32/72
48.4	5/72	8/72	8/72	8/72	4/72	0/72	3/72	4/72	0/72	0/72	40/72
77.5	4/72	11/72	9/72	10/72	6/72	0/72	2/72	3/72	0/72	0/72	45/72
96.9	7/72	9/72	9/72	11/72	8/72	3/72	3/72	3/72	0/72	2/72	55/72
116.5	7/72	12/72	11/72	13/72	9/72	4/72	3/72	3/72	1/72	3/72	66/72

C



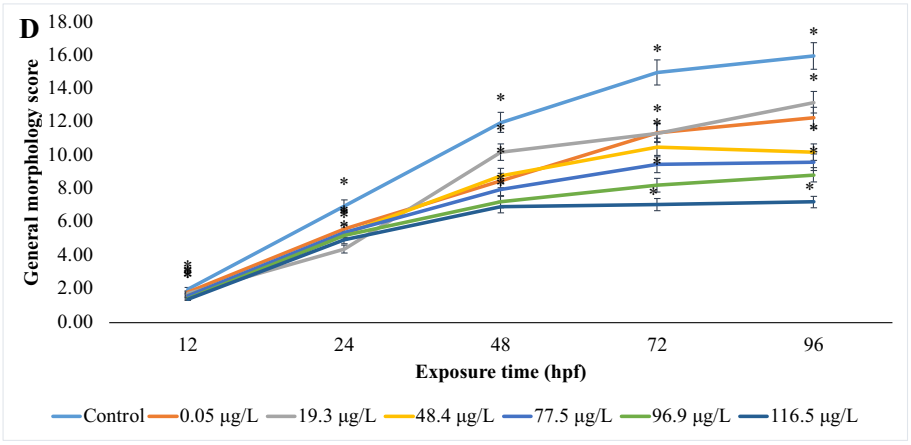


Fig. 2. Main malformations (A), incidence of malformations (B), examples of the main embryonic alterations (C) and concentration-response curves (D) induced by SUC on *Danio rerio* embryos. Data represents mean ± standard deviation (SD) (n = 3). MT: malformation of the tail. S: scoliosis. PE: pericardial edema. YD: yolk deformation. CFM: craniofacial malformation. EA: eye absence. H: hypopigmentation. DHP: delay of the hatching process. FA: fin absence. D: dwarfism.

those values of all biomarkers at 96 hpf increased compared to those observed at 72 hpf. Thus, we observed significant differences between times of exposure at all biomarkers and concentrations, except for LPX and POX at 96.9 and 116.5 µg/L concentrations.

3.4. Gene expression

Antioxidant defense-related genes, *Nrf1a* and *Nrf2a*, increased in a concentration-dependent manner. Nonetheless, the increase in the *Nrf2a* ($F(6,56) = 791.134$, $p < 0.001$) expression was higher compared to the *Nrf1a* ($F(6,56) = 895.419$, $p < 0.001$). Concerning apoptosis-related genes, *CASP3* and *CASP9*, we also observed a significant increase in their expression compared to the control group. From both genes, *CASP9* ($F(6,56) = 951.932$, $p < 0.001$) increased much more its gene expression than *CASP3* ($F(6,56) = 1013.212$, $p < 0.001$). Significant differences between treatment groups, including the control group, were found for all genes (Fig. 4).

3.5. SUC quantification

Measured SUC concentrations in both experiments, embryotoxicity and oxidative stress tests, significantly decreased compared to the nominal concentrations (Table 3). Moreover, concentrations of SUC in the control group remained below the limit of quantification (<LOQ). It is noteworthy to say that none of the measured concentrations of SUC decreased beyond 80% of nominal concentration. Thus, our results were based on nominal values of this sweetener.

3.6. Principal component analysis

As can be seen from Fig. 5, the rate of malformed embryos is positively correlated to the antioxidant defense- (*Nrf1a* and *Nrf2a*) and apoptosis-related genes (*CASP3* and *CASP9*). Moreover, SOD and all oxidative damage biomarkers (HPX, LPX, and POX) are closely related to the rate of death. Thus, all variables are positively correlated with each other.

4. Discussion

SUC is one of the most consumed sweeteners around the world, the human body cannot metabolize it, so it reaches WWTPs unaltered. Once in WWTPs, several authors have reported that neither conventional nor advanced treatments can remove SUC from water, leading to its spill out into the aquatic environment. Nowadays, this sweetener has been reported in effluents and surface waters in concentrations up to 155 µg/L and 25 µg/L, respectively (Ordóñez et al., 2012; Arbeláez et al., 2015). However, limited information is known about the harmful effects this drug may induce in aquatic species, especially at environmentally relevant concentrations. Herein, we aimed to provide new insights into the toxic effects this sweetener may induce in the early life stages of fish. Likewise, to evaluate the expression of important genes in embryonic developmental alterations. Firstly, *Nrf1a* & *Nrf2a* which are genes involved in the response against free radicals and also *CASP3* and *9* genes involved in apoptosis mechanisms. According to our results, SUC altered the normal development of *D. rerio* embryos, leading to cerebral malformations on them. Such malformations included malformation of the tail, scoliosis, dwarfism, pericardial edema, yolk deformation, eye absence, fin absence, and on. Malformations found in this study are related to those reported in previous studies. For example, Arndorfer (2018) indicated SUC generated scoliosis and dwarfism in embryos of *D. rerio*. Nonetheless, the concentrations used by the author were not environmentally relevant (100,000 µg/L) and badly overpassed the ones used here (0.05–116.25 µg/L). Moreover, unlike the results reported by Saputra et al. (2021), who indicated SUC (100 µg/L) did not induce cardiac deformations such as edema, we observed pericardial edema was one of the most common malformations observed in the embryos. These differences may be explained by the time of exposure. Herein, for instance, we exposed fish to SUC from the sphere stage (4 hpf), where embryos are more susceptible to pollutants because they do not have an antioxidant defense mechanism against oxidative stress, and their organs are under development (Kimmel et al., 1995; Dennery, 2007). Meanwhile, they exposed *D. rerio* larvae from the pec-fin stage (60 hpf), at which their organs and circulatory system are fully established, and antioxidant enzymes are available to counteract and detoxify ROS (Hansen, 2006).

Organogenesis is the period at which embryos are more susceptible to ROS (Al-Gubory et al., 2010). ROS play a key role in cell development as they act as primary and secondary messengers that regulate different cell signaling pathways related to proliferation, differentiation, and apoptosis (Elizalde-Velázquez et al., 2021a). Nevertheless, over the course of embryonic development, the frail stability between antioxidants and oxidants can be disrupted by non-endogenous agents that boost the production of ROS and generate oxidative stress. Herein, we demonstrated that SUC at environmentally relevant concentrations (0.05–116.25 µg/L) induced oxidative stress on *D. rerio* embryos. In accordance with our results, Saucedo-Vence et al. (2017) showed that 0.05 and 155 µg/L of SUC generated oxidative stress in the blood, gills, liver, brain, and muscles of *Cyprinus carpio*. Similarly, Eriksson Wiklund et al. (2014) stated that SUC induced lipid peroxidation (LPX) in 3 day-olds *Daphnia magna* after 24 h of exposure. Although the mechanism by which SUC produces oxidative stress is still being studied, authors have suggested the most likely via is through energy disruption this drug production in cells (Schiffman and Rother, 2013;

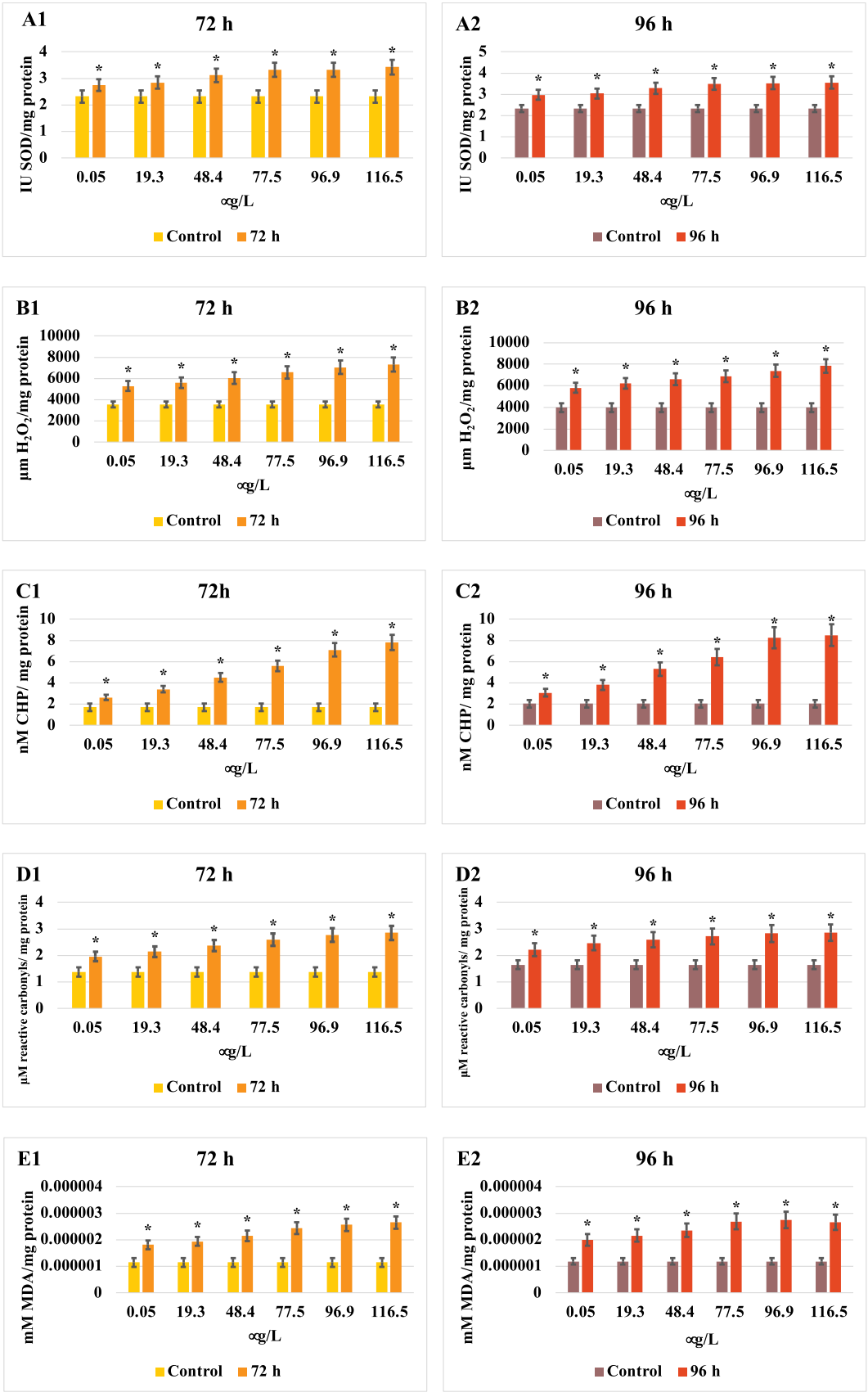


Fig. 3. Oxidative stress (A1: SOD 72 h; A2: SOD 96 h; B1: CAT 72 h; B2: CAT 96 h; C1: HPX 72 h; C2: HPX 96 h; D1: POX 72 h; D2: POX 96 h; E1: LPX 72 h and E2: LPX 96 h) response produced by SUC on *D. rerio* embryos. Data represents mean ± standard deviation (SD) (n = 9). * indicates significant difference compared to control group.

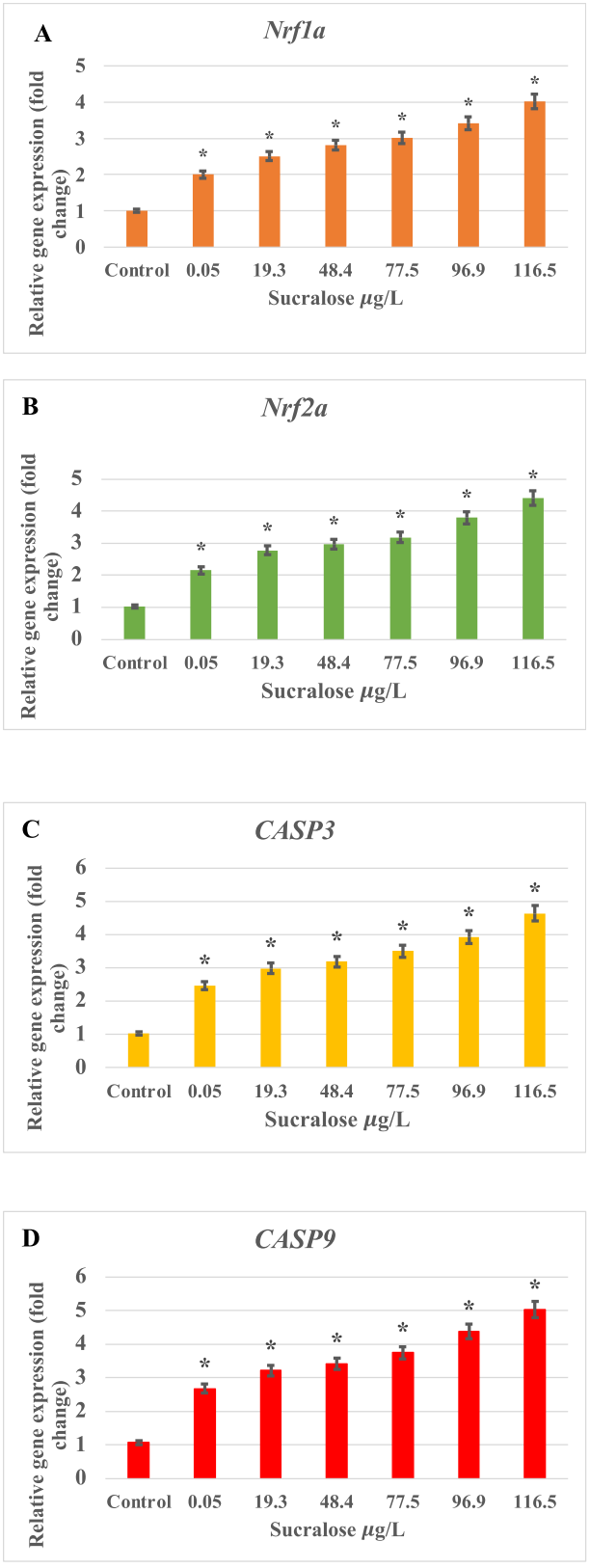


Fig. 4. Gene expression of A) *Nrf1a*, B) *Nrf2a*, C) *CASP3*, and D) *CASP9* in *D. rerio* embryos (early larval period exposed to SUC). Data represents mean ± standard deviation (SD) (n = 9). * indicates significant difference compared to control group.

Saucedo-Vence et al., 2017). However, further studies are needed to comprehend the mechanism by which this sweetener generates this harmful response in aquatic species.

Table 3
SUC concentrations in the systems.

	Nominal concentration (μg/L)	Measured concentration (μg/L)
Embryotoxicity test	Control	<LOQ
	0.05	0.044 ± 0.02
	19.3	16.98 ± 0.54
	48.4	40.05 ± 0.73
	77.5	63.53 ± 1.01
	96.9	80.42 ± 1.26
	116.5	101.35 ± 2.05
Oxidative stress test	Control	<LOQ
	0.05	0.042 ± 0.03
	19.3	15.98 ± 0.76
	48.4	38.99 ± 0.81
	77.5	62.09 ± 0.90
	96.9	78.24 ± 1.11
	116.5	99.67 ± 1.93

LOD: limit of detection (15 ng/L). LOQ: limit of quantification (10 ng/L). Data represents the mean values of all replicates ± SD.

In addition to the increased production of SOD and CAT observed in larvae exposed to SUC, we also demonstrated this sweetener promoted the gene expression of *Nrf1a* and *Nrf2a*. Both genes are members of the nuclear factor erythroid 2 *(Nfe2)* family. *Nrf1a* upregulates the antioxidant response by increasing glutathione biosynthesis (Sant et al., 2017). Although *Nrf1a* and *Nrf2a* might be activated via the activated protein kinase (AMPK) pathway, there is controversy about whether or not SUC can activate AMPK. For example, Wang et al. (2016) showed SUC stimulates the hypothalamic response to fasting through the activation of AMPK. However, Bórquez et al. (2021) stand out that they did not observe changes in AMPK activation after cell line Caco-2 was exposed to SUC. Thus, we believe oxidative stress played a huge role in the increased expression of *Nrf1a* and *Nrf2a* genes. Gureev et al. (2019), for instance, pointed out ROS, particularly H_2O_2, are strong *Nrf2a* activators. Moreover, several authors have indicated that oxidative stress triggers a response that activates the *Nrf1* and *Nrf2* pathways in different organs, cells, and organisms (Sajadimajd and Khazaei, 2018; Haque et al., 2020; Bardallo et al., 2021; Sekine and Motohashi, 2021; Elizalde-Velázquez et al., 2022). Oxidative stress may also be implicated in the activation of *CASP3* and *CASP9*. Our findings, for instance, demonstrated SUC at environmentally relevant concentrations increased the gene expression of *CASP3* and *CASP9* in *D. rerio* larvae. Similarly, Heredia-García et al. (2019) demonstrated that SUC (0.05 and 155 μg/L) promote the enzymatic activity of *CASP3* in white blood cells of *Cyprinus carpio*. *CASP9* is an initial caspase in the mitochondrial apoptotic cascade that, under oxidative cell, conditions go cleavage *CASP3* and several other caspases (Zuo et al., 2009). *CASP3* is an apoptosis executor enzyme that, thus, coordinates the destruction of cellular structures (Heredia-García et al., 2019). Both, our results and those reported by Heredia-García et al. (2019), point out *CASP3* is activated through the intrinsic pathway after SUC exposure. Nonetheless, future studies should elucidate whether or not SUC may also use the intrinsic pathway to induce apoptosis and asses other transcription factors such as tumor suppressor protein (*p53*) and c-Jun N-terminal kinases (*JNK*) to determine their role in this toxic response.

In summary with the previous findings and those identified in this study, we propose a mechanism through which SUC induces alterations to embryonic development and oxidative stress in zebrafish. SUC penetrates the embryo membrane: 1) First, by *CYP3A* action it is biotransformed to oxidized metabolites, in this process ROS such as H_2O_2 and superoxide anion radical are generated; 2) Last, SUC can generate energetic disruption by activating the polyol pathway and decreasing NADPH and glutathione equivalents stimulating ROS formation. ROS are able to generate an increase of antioxidant enzymes (SOD and CAT) and cellular oxidation biomarkers (HPX, POX and LPX) inducing oxidative stress. This may stimulate an up-regulation of *CASP3* and CASP9 and lead to apoptosis. In addition, ROS are able to up-regulate *Nrf1a* and *Nrf2a* stimulating the antioxidant response in the embryo (Fig. 6).

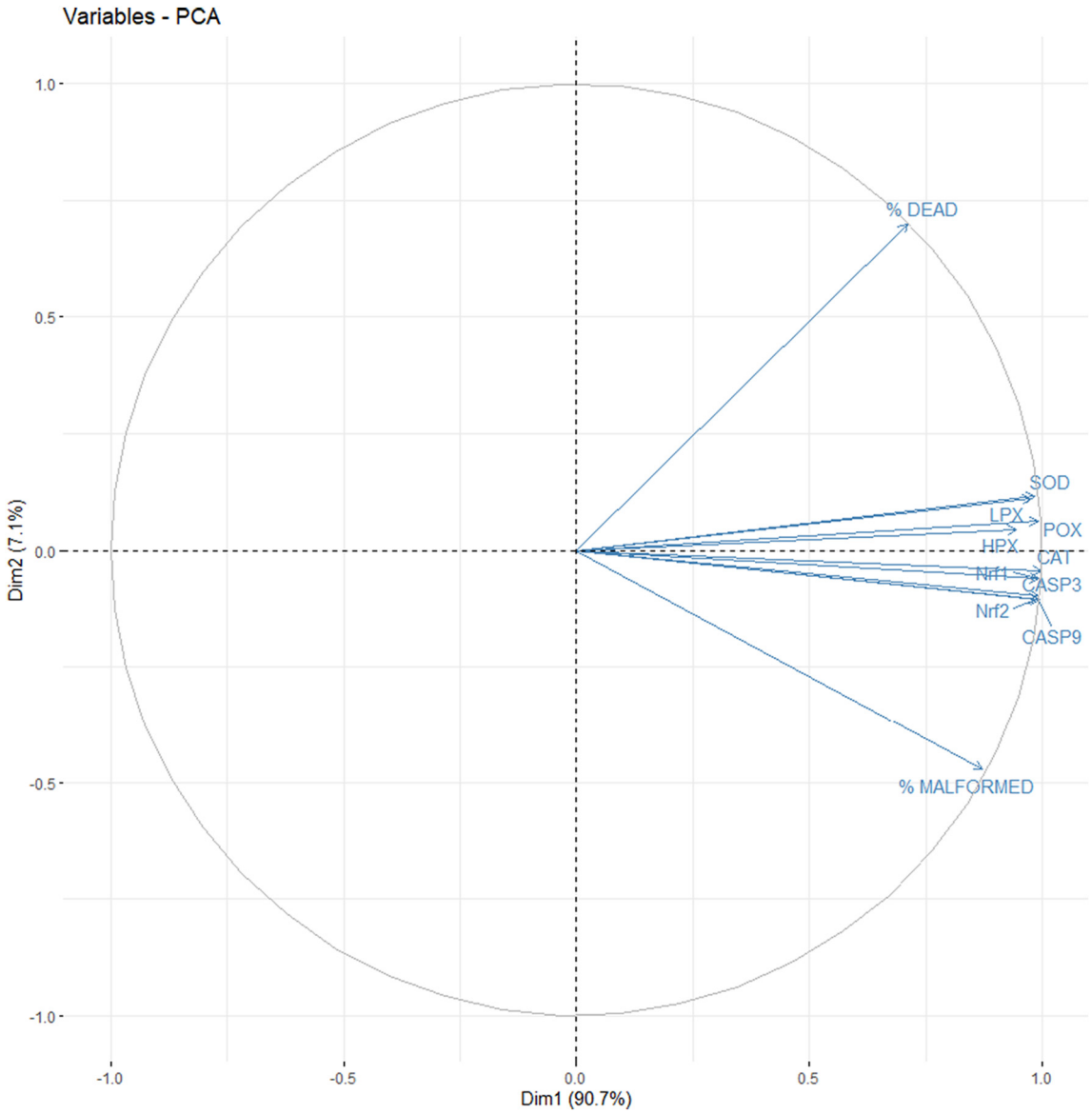


Fig. 5. Principal Component Analysis of biomarkers evaluated in *D. rerio* embryos.

5. Conclusions

Environmentally relevant concentrations of SUC induced the generation of different body malformations in *D. rerio* embryos. These malformations include scoliosis, pericardial edema, yolk deformation, and tail malformation. As malformations of the embryos were harmful to organisms, we also observed a significant increase in the mortality rate, which reached a maximum of up to 56% deaths at the highest concentration. SUC also led to an oxidative stress response, which in turn triggered a significant increase in the expression of antioxidant defense (*Nrf1a* and *Nrf2a*)- and apoptosis (*CASP3* and *CASP9*)-related genes. Since oxidative stress is responsible for several toxic effects in cells, we believe it generated all malformations observed in the embryos. Nonetheless, more studies are needed to elucidate the whole mechanism of toxicity of this sweetener. Overall, our findings support that SUC at environmentally relevant concentrations is harmful to aquatic species, and it can induce embryotoxicity and oxidative stress in the early life stages of fish.

Financial support and sponsorship

This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología México (CONACyT, Project 300727).

Ethical approval and consent to participate

Study protocols and design were approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (approval ID: RP.UAEM.ERC.145.2021).

CRediT authorship contribution statement

Karla Colín-García performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Gustavo Elizalde-Velázquez were involved in the conception.

Leobardo Manuel Gómez-Oliván, Gustavo Elizalde-Velázquez and Karla Colín-García were involved in the design and interpretation of the data and the writing of the manuscript with input from Hariz Islas-Flores, Sandra García-Medina and Marcela Galar-Martínez.

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.

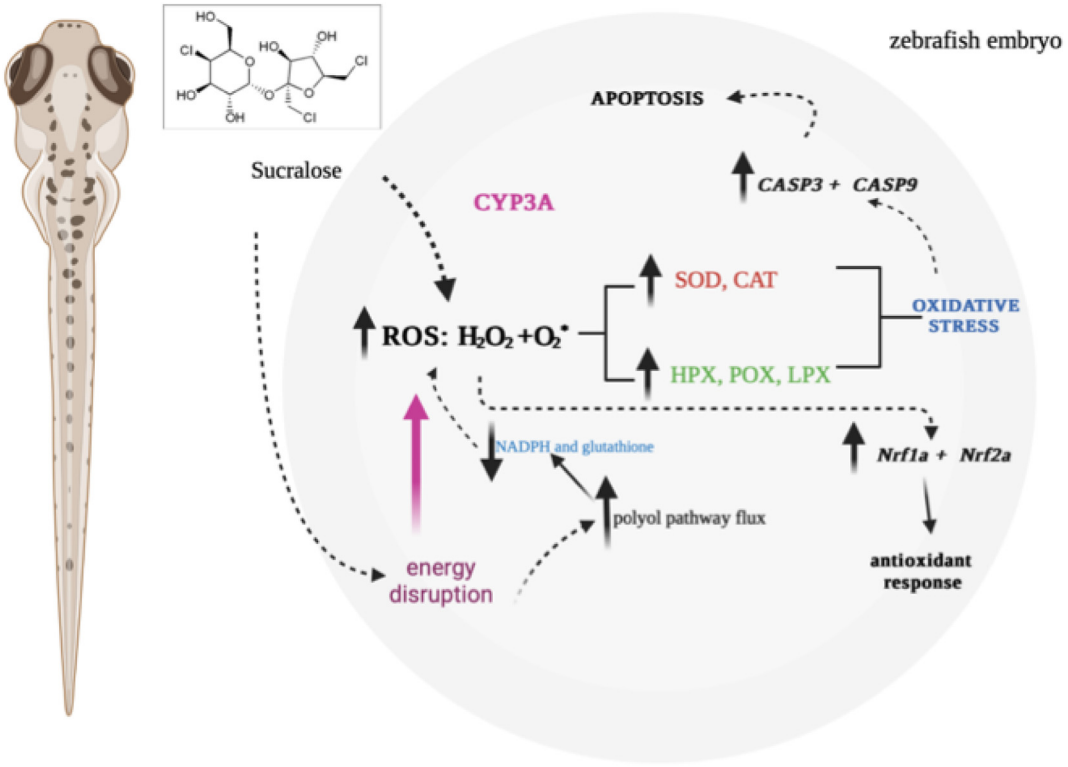


Fig. 6. Proposed mechanism by which SUC induces embryonic development and oxidative stress in *D. rerio*.

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