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Environmentally relevant concentrations of fluconazole alter the embryonic development, oxidative status, and gene expression of *NRF1*, *NRF2*, *WNT3A*, *WNT8A*, *NRD1*, and *NRD2* of *Danio rerio* embryos

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Abstract

Up to date, there is little information published concerning fluconazole (FCZ) toxicity at environmentally relevant concentrations. Bearing in mind the above background of FCZ, we aimed to evaluate the embryotoxic effects environmentally relevant concentrations of FCZ (800-1000 ng/L) may induce in *Danio rerio*. Moreover, we also wanted to prove whether these FCZ concentrations could generate oxidative stress and alter the expression of several genes related to the antioxidant mechanisms, sterol and retinol biosynthesis, and embryogenesis. Our findings demonstrate that FCZ, at all concentrations, induced pericardial edema, yolk sac deformation, scoliosis, and tail malformation in embryos. Moreover, we also demonstrated this drug altered the redox equilibrium of fish, promoting the production of lipoperoxidation level, hydroperoxide content, and protein carbonyl content in a

concentration-dependent manner. Concerning gene expression, FCZ downregulated wingless-type MMTV integration site family member 3a (WNT3A), wingless-type MMTV integration site family member 8a (WNT8A), N-arginine dibasic convertase 1 (NRD1), and N-arginine dibasic convertase 2 (NRD2) and upregulated cytochrome P450 family 26 subfamily a member 1 (CYP26A1), cytochrome P450 family 26 subfamily a member 1B (CYP261B), nuclear respiratory factor 1 (NRF1), and nuclear respiratory factor 2 (NRF2) in *D. rerio* larvae. Collectively, our results point out that FCZ, at low concentrations, may alter the embryogenesis, oxidative status, and expression of several genes in *D. rerio* embryos via an impairment in sterol and retinol biosynthesis. Thus, our results provide some of the first evidence that FCZ, even at environmentally relevant concentrations, is harmful to aquatic species.

Keywords: Fluconazole, embryonic disruption, oxidative damage, gene alteration, zebrafish

INTRODUCTION

Fluconazole (FCZ) is a fungicide of the azole family that people use in oral and dermal medications to treat fungal infections[1]. Nonetheless, we can also find it in household products such as soap, shampoo, dermal creams, shower gels, and toothpaste[2]. In addition, FCZ is widely used as fungicide in agriculture and biocide in a variety of products[3]. FCZ enters the ecosystems from human and animal excreta after its metabolization, wastewater treatment plants, wastes coming from pharmaceutical industries, medical centers, households, and hospitals[4]. Therefore, the use of FCZ in such a broad range significantly increases its presence in the environment, especially in surface and drinking water[5]. Environmental pollution with FCZ is strictly related to its properties such as persistence in soil and water connected with the resistance of this compound towards hydrolytic, photolytic, and biological degradation[4]. Even though few authors have studied the occurrence of FCZ in the water matrix, this antifungal drug has reached concentrations up to 27,606 ng/L in wastewater, 109.6 ng/L in surface water, and 2100 ng/L in drinking water [Table 1]. Thus, it is important to keep studying the occurrence and distribution of this drug in the water matrix.

As FCZ is present in the aquatic environment and inhibits cytochrome p450 family 3 subfamily a member 4 (CYP3A4) and cytochrome p450 family 2 subfamily c member 9 (CYP2C9)[16], it may cause undesirable effects in non-target organisms. However, only three studies that we are aware of have studied the toxic effects of FCZ in aquatic species, and, in all of them, the authors have used non-environmentally relevant concentrations. Kim *et al.*[17], for instance, demonstrated that acute toxicity effects of FCZ were only observed on crustaceans (*Thamnocephalus platyurus*) and fish (*Oryzias latipes*) at concentrations over 100 mg/L. Similarly, another study found that the no observed effect concentration level and lowest observed effect concentration level required to lower the growth of green algae (*Pseudokirchneriella subcapitata*) were 15.3 and 19.6 mg/L, respectively[18].

Moreover, the authors pointed out that the growth inhibition induced in green algae was related to the capacity of FCZ to inhibit the sterol biosynthesis of algae. Thus, FCZ may also influence sterol synthesis in organisms other than bacteria. Finally, another author studied the embryotoxic effects of FCZ in rare minnow (*Gobiocypris rarus*) embryos and found this antifungal drug generated 100% death in organisms at 15 mg/L[19]. Furthermore, the authors indicated that FCZ induced body malformations, heart rate reduction, oxidative stress, and acetylcholinesterase (AChE) inhibition and alterations in the gene expression of heat shock protein 70 (hsp70), myostatin protein (mstn), metallothionein (mt), apoptosis protease-activating factor-1 (apaf1), vascular endothelial zinc finger 1 (vezf1), and cytochrome P450 family 1 subfamily A (cyp1a) from a concentration of 0.2 mg/L. In comparison to other triazole fungicides, studies have shown FCZ is more toxic[19]. However, more studies are needed to understand the risk that this drug poses to the aquatic environment.

Table 1. Occurrence of fluconazole in different water matrices

Country	Matrix	ng/L	Ref.
Brazil	Surface water	98.7	[6]
	Wastewater	63-1292	[7]
	Drinking water	35-2100	[8]
Canada	Wastewater	3-27,606	[9]
China	Surface water	109.6	[10]
South Africa	Wastewater	302.38	[11]
	Wastewater	331-9959	[12]
Spain	Wastewater	20-95	[13]
Sweden	Wastewater	< LOQ - 120	[14]
Switzerland	Wastewater	10-100	[15]

LOQ: Limit of quantification.

Therefore, we aimed to study the embryotoxic effects that FCZ may induce in the freshwater fish *D. rerio*. We exposed zebrafish embryos to nine environmentally relevant concentrations of FCZ and evaluated the mortality, malformations, and hatching rate. Moreover, we also aimed to determine whether these concentrations of FCZ may disrupt the oxidative status and the gene expression of *D. rerio* embryos.

EXPERIMENTAL

Ethical statement

All procedures performed in this study were in fulfillment of the ethical standards of The Ethics and Research Committee of the Autonomous University of the State of Mexico (approval ID: RP.UAEM.ERC.132.2020).

Reagents

We obtained FCZ (CAS number: 86386-73-4; purity > 98%) and all other compounds from Sigma-Aldrich. To produce the stock solution, we dissolved 1 g of FCZ in DMSO and completed the volume to 1 L with bi-distilled water. Moreover, to reach the desired concentrations, we performed several dilutions from the stock solution.

Zebrafish housing

For the maintenance of *D. rerio* (AB strain), we housed fish, in a ratio of 1 organism/L, in aquaria of 100 L that we provided with a UV-sterilized and charcoal-filtered water system. To ensure all aquaria fulfilled the water quality parameters throughout the experiment, we measured the levels of dissolved oxygen, nitrate, nitrite, and un-ionized ammonia in water every other day [Table 2]. Fish were fed two times a day with Spirulina flakes and supplemented once a day with *Artemia nauplii*.

Embryotoxicity test

The night before spawning, we located 14 adult zebrafish at a ratio of 1 female/2 male in individual reproduction chambers. Spawning and fertilization took place during sunrise, and, approximately 1 h after fertilization, we collected and washed the embryos with water and saline solution[20]. Following this, we examined the embryos under a stereoscopic microscope, as described by Kimmel *et al.*[21], and collected those at the blastula stage (2.5 hpf). Blastula stage embryos were placed in Petri-plates with ultrapure water and incubated at a temperature of 26 ± 1 °C until they reached the sphere stage (4 hpf). To assess the embryotoxic effects FCZ may induce in fish, we placed 72 embryos, at the sphere stage (4 hpf), into 24-well plates, ensuring we put 1 embryo/well. Accordingly, we used three 24-well plates for each concentration of FCZ (0, 800, 825, 850, 875, 900, 925, 950, 975, and 1000 ng/L). We chose these concentrations because all of

Table 2. Water quality parameters measured in aquaria and 24-well plates

Water parameters	Value measured
Dissolved oxygen	8.9 ± 0.4 mg/L
NO_2^-	0.023 ± 0.005 mg/L
NO_3^-	2.1 ± 0.6 mg/L
pH	7.22 ± 0.16
Un-ionized ammonia	0.009 ± 0.003 mg/L

them are environmentally relevant. Once we finished filling the plates, we incubated them at 26 ± 1 °C, ensuring all had natural light/dark periods of 12 h. To assess each endpoint, mortality, hatching rate, and malformations, we counted all dead and malformed embryos at 12, 24, 48, 72, and 96 hpf and recorded whether the embryos hatched at 72 or 96 hpf. All these endpoints were evaluated following the protocols of Kimmel *et al.*[21]. Moreover, to score the development of fish through the different times of exposure, we used the scale of Hermsen *et al.*[22]. The determination of this score consisted of performing quantitative and qualitative evaluations of each *D. rerio* embryo exposed to FCZ and was compared with the control embryo, receiving points according to its development phase with respect to time. Embryonic development was assessed considering: (1) tail development; (2) formation of somites; (3) eye development; (4) movement; (5) blood circulation; (6) heartbeat; (7) head-body pigmentation; (8) pigmentation of the tail; (9) appearance of the pectoral fin; (10) mouth protuberance; and (11) hatching. Graphs showing the major malformations induced by FCZ exposure were constructed using IBM SPSS Statistics 22 software. After 96 h of exposure, and once we finished counting the dead embryos, we performed a maximum likelihood linear regression analysis and calculated the lethal concentration 50 (LC_{50}) and the effective concentration of malformations (EC_{50}) with their 95% confidence intervals ($P < 0.05$).

Oxidative stress determination

For this experiment, we placed 10 systems in aquaria of 10 L of capacity, ensuring each system had a 1 g of embryos (approximately 1600 embryos) and following the standards of Elizalde-Velázquez *et al.*[23,24]. We exposed all systems to each of the above FCZ concentrations (Section 2.4: Embryotoxicity test) and maintained a constant temperature of 28 ± 1 °C. At 72 and 96 hpf, half of the surviving organisms were selected and homogenized in phosphate buffer (PBS pH 7.4). Next, we split the homogenate from each system into two Eppendorf tubes. Tube 1 contained 300 μL of homogenate and 300 μL of trichloroacetic acid (20%) solution and was used to evaluate the levels of protein carbonylation (POX), lipoperoxidation (LPX), and the hydroperoxide content (HPC). Tube 2 contained 700 μL of homogenate and was used to assess the antioxidant activity of the enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX). Table 3 summarizes the methods we used to measure each of the oxidative stress biomarkers.

Integrated biomarker response analysis

To calculate the *integrated biomarker response* (IBR) values of all oxidative stress, we first obtained the ratio between the biomarkers of each treatment group (Xi) and the biomarkers of the control group (Xo). Once we calculated the Xi/Xo ratio, we log-transformed their values (Yi) and then standardized them with the following formula Zi = (Yi - μ)/s, where μ and s are the mean and the standard deviation of Yi, respectively. Next, we estimated the biomarker deviation index (A) by performing a difference of Zi and Zo. Finally, we summed each absolute value of A to get IBR values. With the values of A, we depicted the integrated responses of each biomarker in a star plot.

Table 3. Methods used to assess oxidative stress biomarkers on the embryos

	Biomarker	Method used
Tube 1	LPx	[25]
	HPx	[26]
	POx	[27]
Tube 2	SOD	[28]
	GPx	[29]
	CAT	[30]

qRT-PCR

RNA was isolated from the remaining larvae of the embryotoxicity test by using the RNeasy® kit of Qiagen. After isolation, RNA concentrations were determined through the 260/280 ratio using a spectrophotometer (THERMO Scientific NanoDrop 2000/2000c). Moreover, samples purities were assessed by using agarose (1%) gel electrophoresis. We performed the reverse transcription reactions using 1.0 μg of the total RNA and the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF 205313). Reaction conditions were as follows: 42 °C for 15 min and 95 °C for 3 min. cDNA was used as a template for qRT-PCR. Genes tested [Table 4] were involved in different biological pathways connected with the toxicity of FCZ. qRT-PCR was performed using a Rotor-Gene Q (Qiagen). We performed each reaction 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. Reaction conditions were as follows: 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. B-actin was used as the housekeeping gene to normalize all the samples.

Quantification of FCZ in water

For sampling, we followed the protocols described by Elizalde-Velázquez *et al.*[23,24]. Briefly, for the embryotoxicity test, we collected 140 μL of water from each of the wells of the three plates of each concentration. Accordingly, we gathered 10 mL of water from each concentration. Concerning the oxidative stress study, we took 10 mL of water from each system. Water samples for both experiments were gathered at 0, 12, 24, 48, 72, and 96 hpf.

For analysis of water samples, we used an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS equipped with a Turbo V Ion spray source. We achieved separation by using a Gemini C18 column and a mobile phase of 70 water:30 acetonitrile (v/v). The flow rate was kept at 1 mL/min, and the injection volume was 20 μL. The calibration standards and quality control samples were prepared by spiking ultrapure water with FCZ at a concentration ranging from 0 to 1100 ng/L. The accuracy of the proposed method was confirmed by spiking ultrapure water with FCZ at three different levels: 80%, 100%, and 120%.

Statistics

We assessed our Hermsen's score, hatching rate, and oxidative stress results with a two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. For this purpose, we considered time as Factor A and concentration as Factor B and used Sigma Plot 12.3 software. In the case of healthy, dead, and malformed embryos, as well as gene expression, we performed a one-way ANOVA.

RESULTS AND DISCUSSION

Hermsen's score

Overall, the Hermsen's score of fish exposed to FCZ decreased in a concentration-dependent manner compared to the control group [$F(9,100) = 1241.359$; $P < 0.001$; $n = 3$]. Nonetheless, at the concentration of

Table 4. Genes used for qRT-PCR

Gene	Forward primer	Reverse primer	Ref.
Nrf1	TTT GGT TCC CGA TGA AGA CG	TGA TTA GCG TGA GAC TGA GC	[31]
Nrf2	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	[32]
CYP26A1	AGG CCA TTA TGA GGG CGT TC	AGT ACT GGC GGT GGT TTC AT	[33]
CYP26B1	GGG GCA GAG AAT GTG CGT AA	TGT TGT TCT CCT TGG CGC TT	[34]
WNT3A	TACGCCTTCTTCAAGCATCC	CTCTTTGCGCTTTTCTGTCC	[35]
WNT8A	CAAGCAAGGAAGTTGGAGATGG	CGCATTTGACTGTGCAGCAC	[36]
NRD1	CAC AAG AGC GTT CAT CAT CCT	TGG GAA ACA CCA GGA ATC AT	[37]
NRD2	GGT CGG GAG ATT CAT AGC AG	CGT CCT CGC TTG ACT TCT TT	[37]

Nrf1 and *NRf2* are genes involved in the response against free radicals; *CYP26A1* and *CYP26B1* are genes involved with retinoic acid homeostasis; and *WNT3A*, *WNT8A*, and *NRD1* are genes related to the processes of organogenesis in the early stages of development.

875 ng/L, we found an equal score to the one observed in fish exposed to 925 ng/L [Figure 1]. Moreover, we did not find significant differences between concentrations from 825 to 925 ng/L. The control group did not show any significant delay in the development of fish, reaching the highest Hermsen's score.

Dead and malformed embryos rate

Figure 2 shows the percentage of healthy, death, and malformed embryos exposed to FCZ. As with the Hermsen's score, we saw a significant decrease in the number of healthy embryos compared to the control group [healthy: $F(9,20) = 65.935$; $P < 0.001$; $n = 3$; dead: $F(9,20) = 35.988$; $P < 0.001$; $n = 3$, and malformed: $F(9,20) = 27.580$; $P < 0.001$; $n = 3$]. Thus, we found the highest percentage of healthy embryos in the control group and the lowest at the concentration of 1000 ng/L. Moreover, we also found that the percentage of dead embryos increased with the concentration. Concerning the percentage of malformed embryos, we did not see significant differences at concentrations from 825 to 900 ng/L.

Main malformations induced by FCZ

As presented in Figure 3, fish from the control group showed a minimal percentage of malformed embryos (4%), and the malformations they presented were only related to hypopigmentation. Thus, we do not consider them in this section. As shown in Figure 3, fish exposed to FCZ showed a significant increase in the prevalence of malformations compared with the control group. However, the prevalence of malformations in fish did not show a concentration-dependent trend. For instance, fish in some concentrations presented an increase in the prevalence of yolk sac deformation but in others a decreased incidence.

As shown in Figures 3 and 4, pericardial edema was the most prevalent malformation at all concentrations of FCZ, followed by yolk sac deformation. Nonetheless, in the higher concentrations of FCZ, we saw that the prevalence of scoliosis and tail deformation increased compared to the control group.

Hatching rate

Besides the malformations above, fish exposed to FCZ showed a significant delay in the hatching process at 72 and 96 hpf compared to the control group [$F(9,40) = 56.988$; $P < 0.001$; $n = 3$]. The delay in the hatching process was in a concentration-dependent manner for both time points. However, this alteration was even more noticeable at 72 hpf compared to 96 hpf [Figure 5]. Between treatment groups, it is noteworthy to say we did not find significant differences among concentrations from 800 to 875 ng/L. At a concentration of 1000 ng/L, less than 25% of fish hatched at 72 hpf, while, at 96 hpf, only 76% of fish hatched at the same concentration.

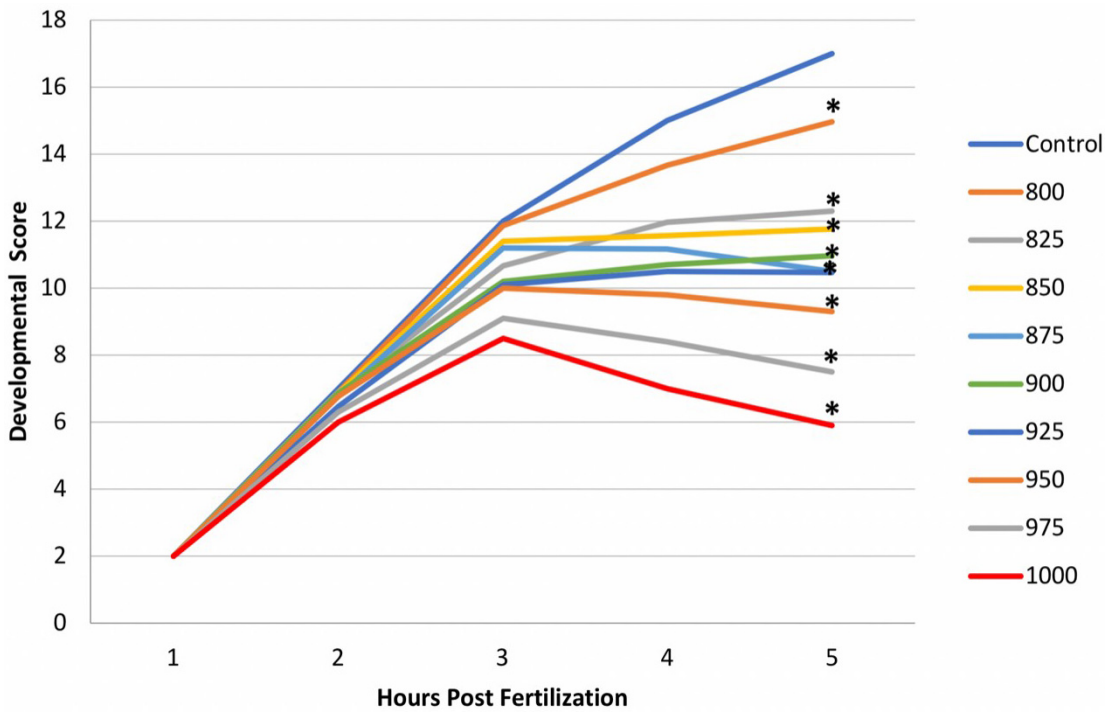

Figure 1. Hermsen's score of embryos exposed to FCZ. Data represent mean ± standard deviation. *All FCZ concentrations were significantly different from the control group ($P < 0.05$). FCZ: Fluconazole.

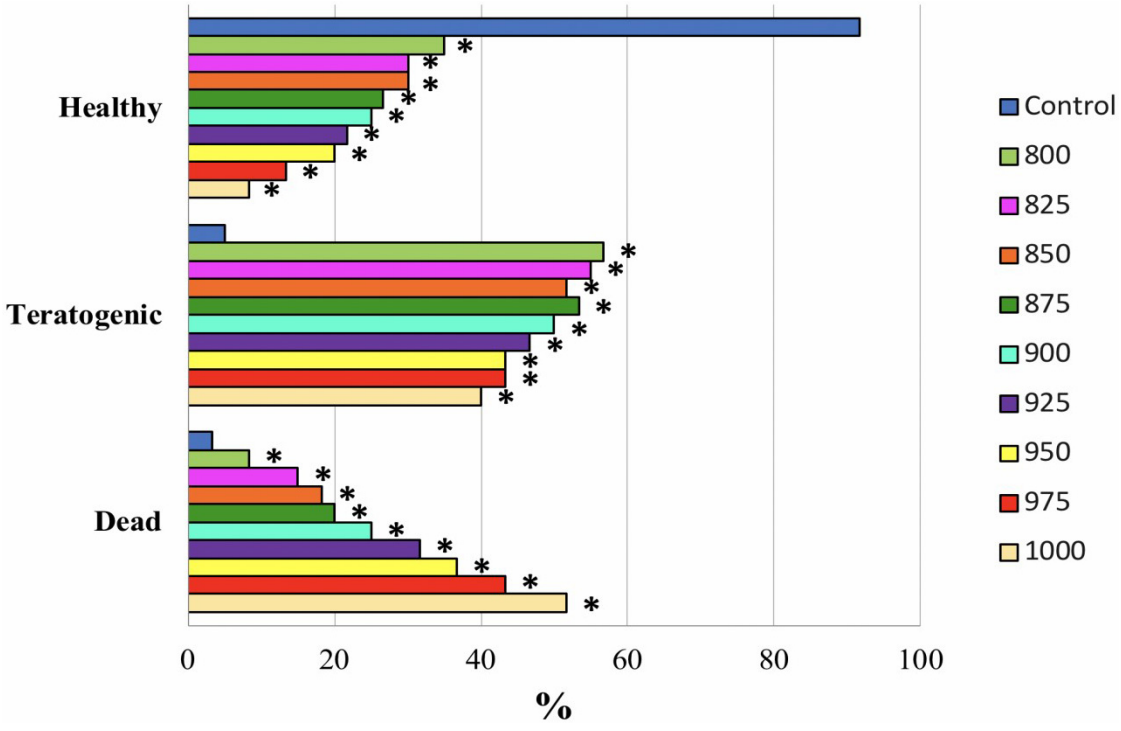

Figure 2. Percentage of dead, teratogenic, and healthy embryos exposed to FCZ. Data represent mean ± standard deviation. *All FCZ concentrations were significantly different from the control group ($P < 0.05$). FCZ: Fluconazole.

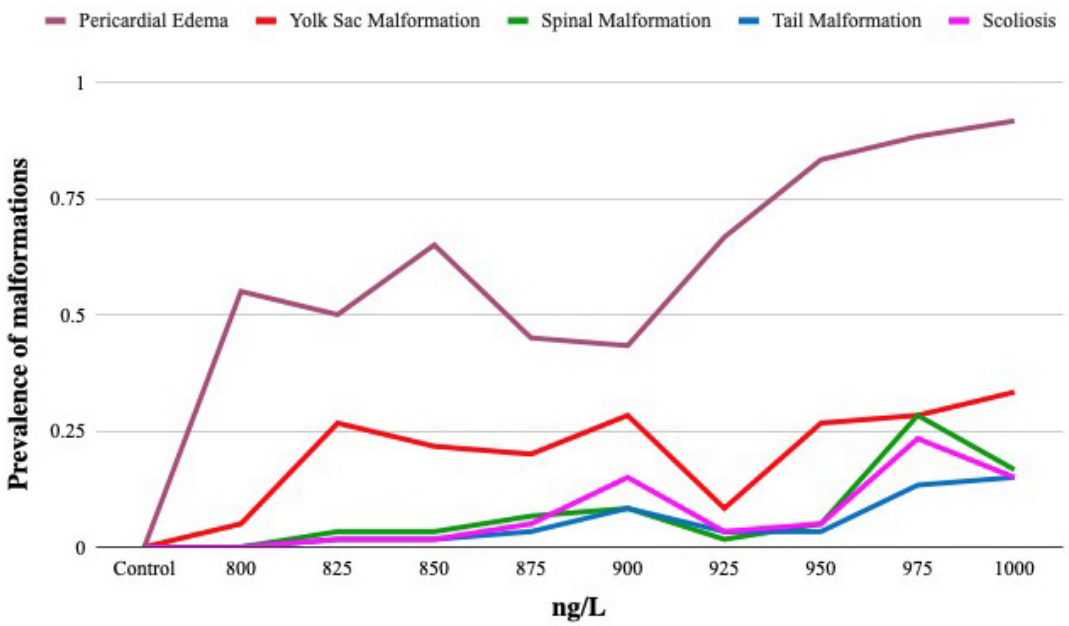

Figure 3. Prevalence of malformations on embryos exposed to FCZ. Data represent mean ± standard deviation. FCZ: Fluconazole.

Oxidative stress

According to our results, the enzymatic activity of SOD and CAT in fish exposed to FCZ increased in a concentration-dependent manner compared to the control group [SOD: F (9,40) = 54.821; $P < 0.001$; $n = 3$ and CAT: F (9,40) = 50.763; $P < 0.001$; $n = 3$]. Thus, we found significant differences between all the treatment groups and the control group in SOD and CAT, except for the lowest concentration [Figure 6]. Concerning oxidative damage biomarkers, we found that the levels of LPX, HPX, and POX increased with the concentration. Moreover, as with the antioxidant enzymes, we also found significant differences between all the treatment groups and the control group in LPX, HPX, and POX, excluding the lowest concentration [LPX: F (9,40) = 57.560; $P < 0.001$; $n = 3$, HPX: F (9,40) = 42.927; $P < 0.001$; $n = 3$; POX: F (9,40) = 44.709; $P < 0.001$; $n = 3$]. For all oxidative stress biomarkers, we found significant differences between 72 and 96 hpf at all concentrations of FCZ.

IBR

Since the levels of oxidative damage biomarkers and antioxidant enzymes in fish exposed to FCZ increased, we performed an IBR analysis to determine their tendency. At all concentrations of FCZ, the star plots showed a tendency towards oxidative damage biomarkers [Figure 7]. In addition, even though the IBR values increased from 2.82 to 8.77, we did not observe any changes in the IBR values among the middle concentrations (825-925 ng/L). Moreover, we also observed a significant decrease in the IBR value of the concentration of 1000 ng/L compared to the 975 ng/L. Intriguingly, we did not observe significant differences between the IBR values and star plots of 72 and 96 hpf.

qRT-PCR

The gene expression of *NRF1* and *NRF2* increased in a concentration-dependent manner compared to the control group [F (9,20) = 320.471; $P < 0.001$; $n = 3$]. Moreover, we also observed that the expression of *CYP26A1* and *CYP26B1* increased with the concentration in comparison with the control group [F (9,20) = 291.731; $P < 0.001$; $n = 3$] [Figure 8] Thus, we found significant differences among the treatments as well as between treatments and the control groups for all these genes. Concerning genes *WNT3A*, *WNT8A*, *NRD1*, and *NRD2*, their expression decreased in a concentration-dependent manner compared to the control

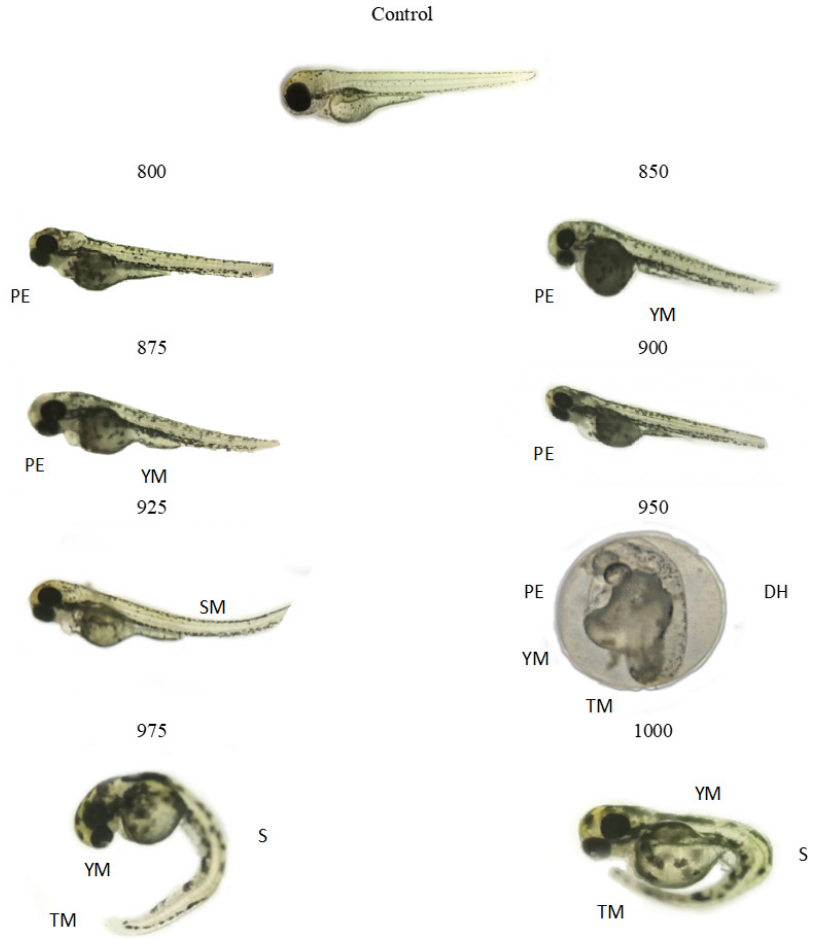

Figure 4. Main malformations found in embryos exposed to FCZ. DH: Delayed hatching; PE: pericardial edema; S: scoliosis; TM: tail malformation; YM: yolk sac malformation; FCZ: fluconazole.

group, with significant differences among all treatment groups [F (9,20) = 345.195; $P < 0.001$; $n = 3$; F (9,20) = 301.963; $P < 0.001$; $n = 3$] [Figure 8].

FCZ determination in water samples

For both experiments, the concentration of FCZ decreased after 96 h of exposure compared to the nominal concentration. Nonetheless, between the two experiments, the concentrations of FCZ in the oxidative stress experiment decreased more than in the embryotoxicity test [Table 5]. In the oxidative stress experiment and the embryotoxicity test, the concentration of FCZ in the control group was below the limit of quantification. Since the measured concentrations of FCZ did not decrease more than 20% compared to the nominal concentration, we analyzed all results based on the latter.

Herein, we aimed to determine whether FCZ at environmentally relevant concentrations may alter the development, oxidative status, and the gene expression of *NRF1*, *NRF2*, *WNT3A*, *WNT8A*, *NRD1*, and *NRD2* of *D. rerio* embryos. Overall, all biomarkers showed either a significant increase or decrease compared to the control group. Below, we discuss each of the results found in this study.

Table 5. Measured concentrations of FCZ in the water of the embryotoxicity test and the oxidative stress experiment

Nominal Concentration	Measured concentration Embryotoxicity test	Measured concentration Oxidative stress experiment
Control	< LOQ	< LOQ
800 ng/L	718.2 ng/L	703.2 ng/L
825 ng/L	734.5 ng/L	719.1 ng/L
850 ng/L	750.6 ng/L	732.4 ng/L
875 ng/L	773.1 ng/L	754.5 ng/L
900 ng/L	801.2 ng/L	781.3 ng/L
925 ng/L	818.9 ng/L	801.7 ng/L
950 ng/L	849.5 ng/L	820.6 ng/L
975 ng/L	863.7 ng/L	854.3 ng/L
1000 ng/L	884.4 ng/L	870.6 ng/L

Data represent mean ± standard deviation. LOQ: Limit of quantification (10 ng/L); LOD: limit of detection (5 ng/L).

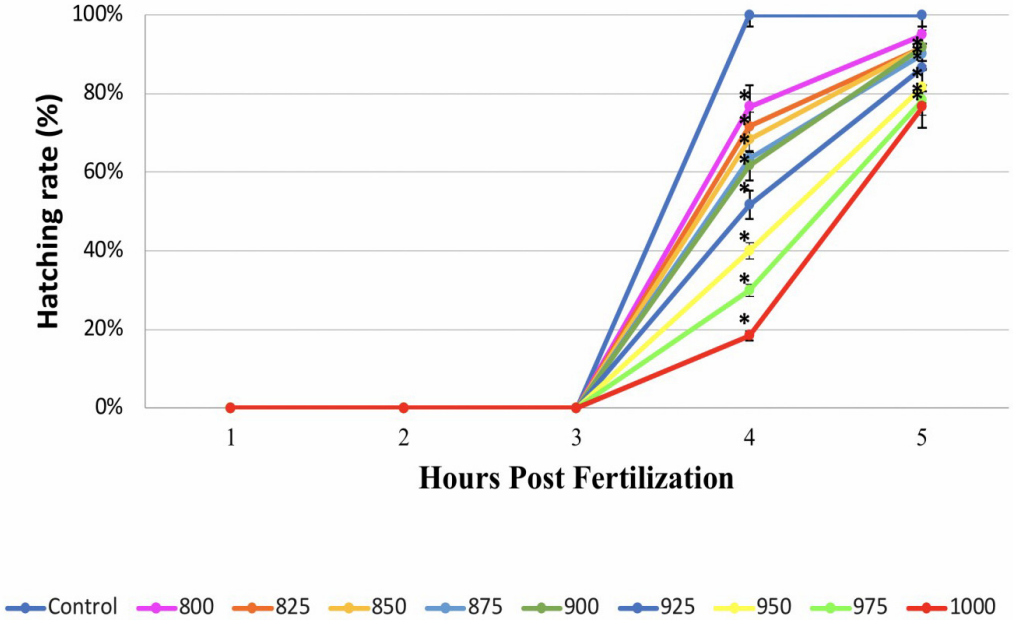

Figure 5. Hatching rate of embryos exposed to FCZ. Data represent mean ± standard deviation. *Denotes significant difference compared to control group. FCZ: Fluconazole.

Previously, only one study that we are aware of assessed the embryotoxic effects of FCZ in fish. In this study, the authors pointed out malformations in *Gobiocypris rarus* embryos become apparent at 1 mg/L of FCZ; however, they did not mention the type of malformations embryos presented[22]. Unlike this study, we demonstrated embryos showed the incidence of pericardial edema, yolk sac deformation, scoliosis, and tail deformation after their exposure to low concentrations of FCZ (800-1000 ng/L). At the concentrations of 825, 875, and 925 ng/L, we found similar prevalences of hypopigmentation and developmental delay. Furthermore, we found no significant differences between the concentrations of 825 and 925 ng/L because the malformations and developmental delay of the fish were similar. Moreover, we also demonstrated that FCZ significantly delayed the hatching process of *D. rerio* embryos. Alterations in the hatching process are vital to embryos because an anticipated hatch may make organisms more vulnerable to environmental hazards, such as mechanical and osmotic stress and other toxic pollutants present in water[23]. Meanwhile, a

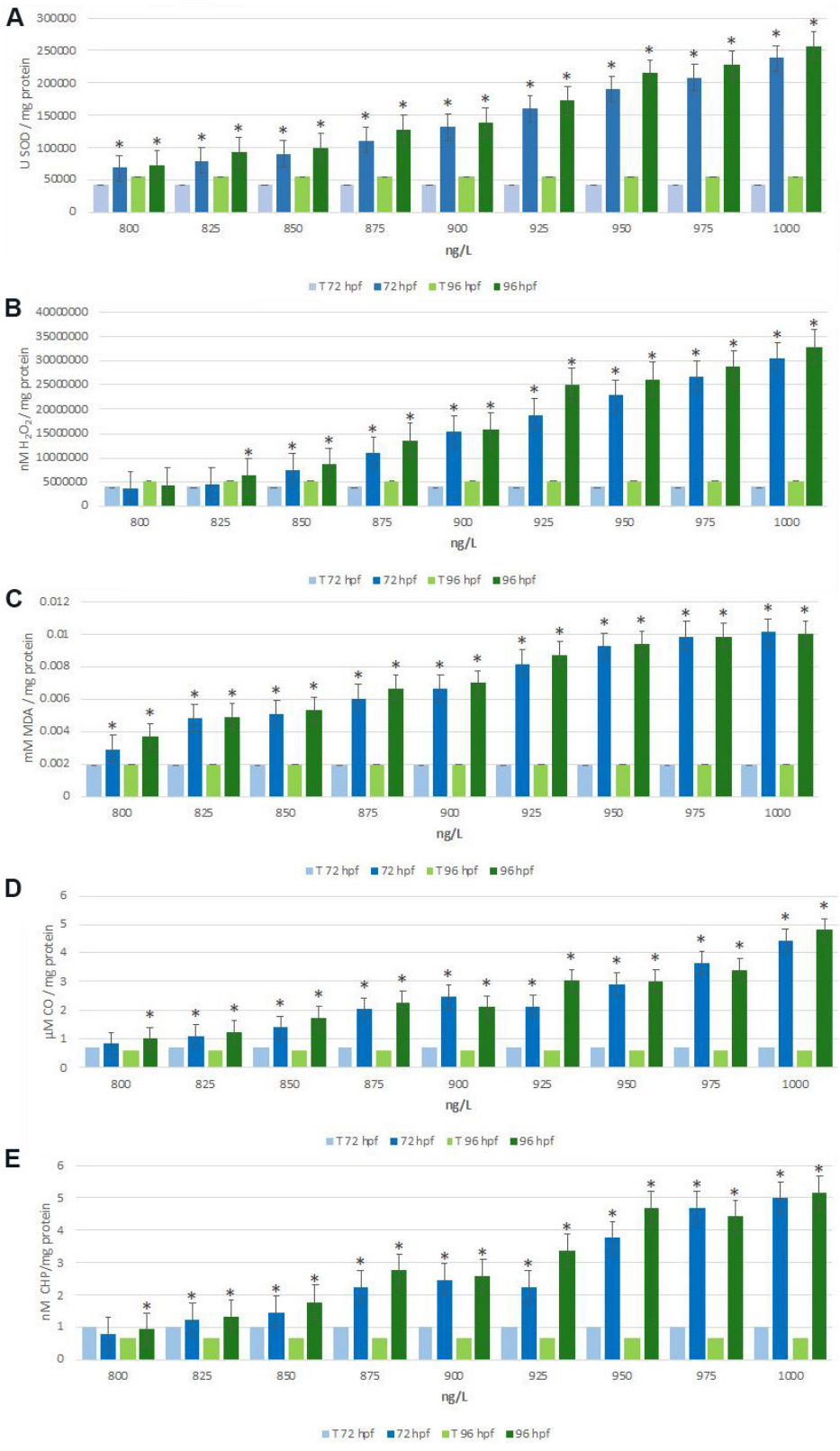

Figure 6. Oxidative stress biomarkers evaluated in fish exposed to FCZ: (A) SOD; (B) CAT; (C) LPX; (D) POX; and (E) HPX. Data represent mean ± standard deviation. *Denotes significant difference compared to control group. FCZ: Fluconazole; SOD: superoxide dismutase; CAT: catalase; LPX: lipoperoxidation; POX: protein carbonylation.

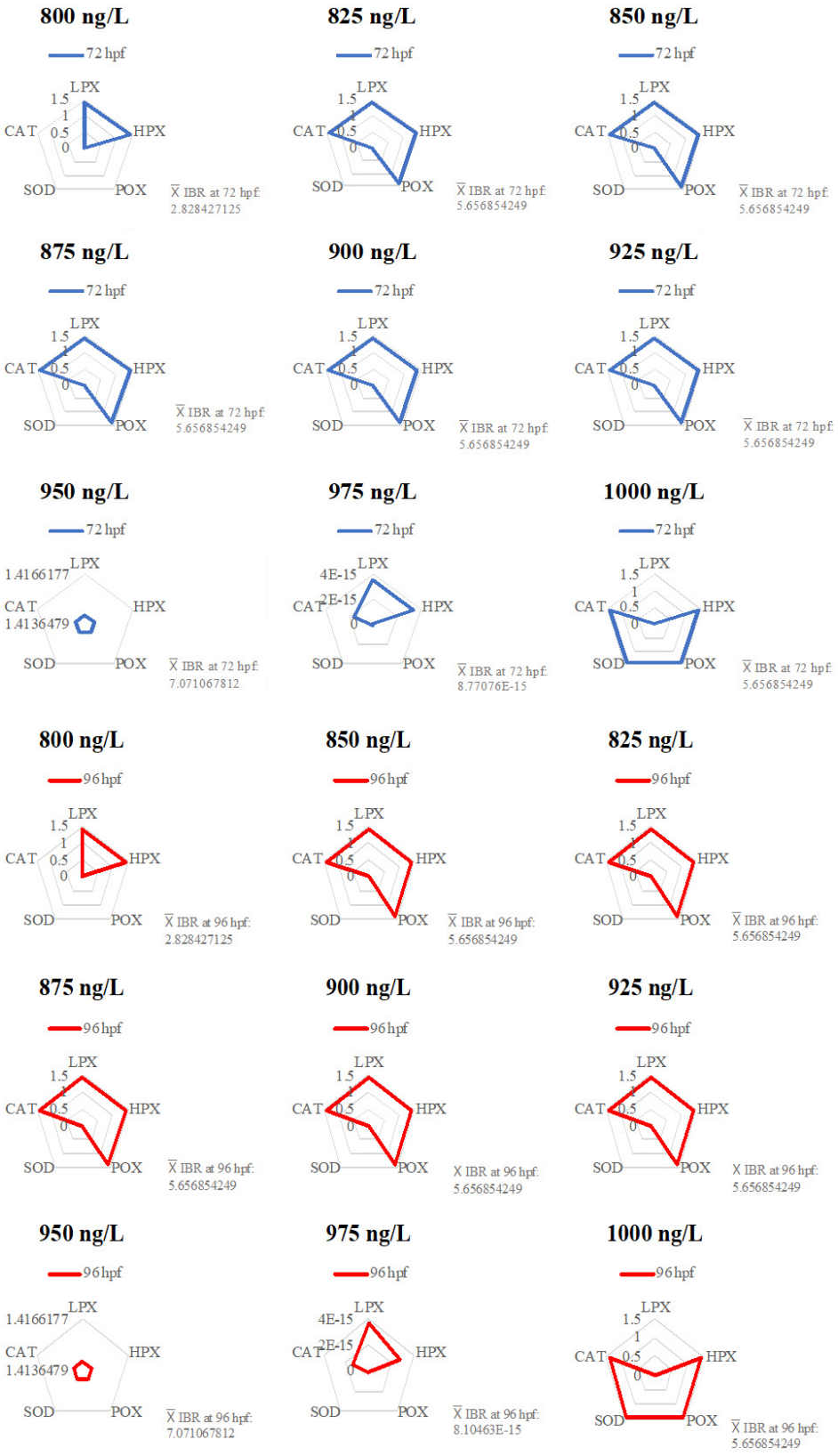

Figure 7. IBR and star charts of oxidative stress biomarkers. IBR: Integrated biomarker response.

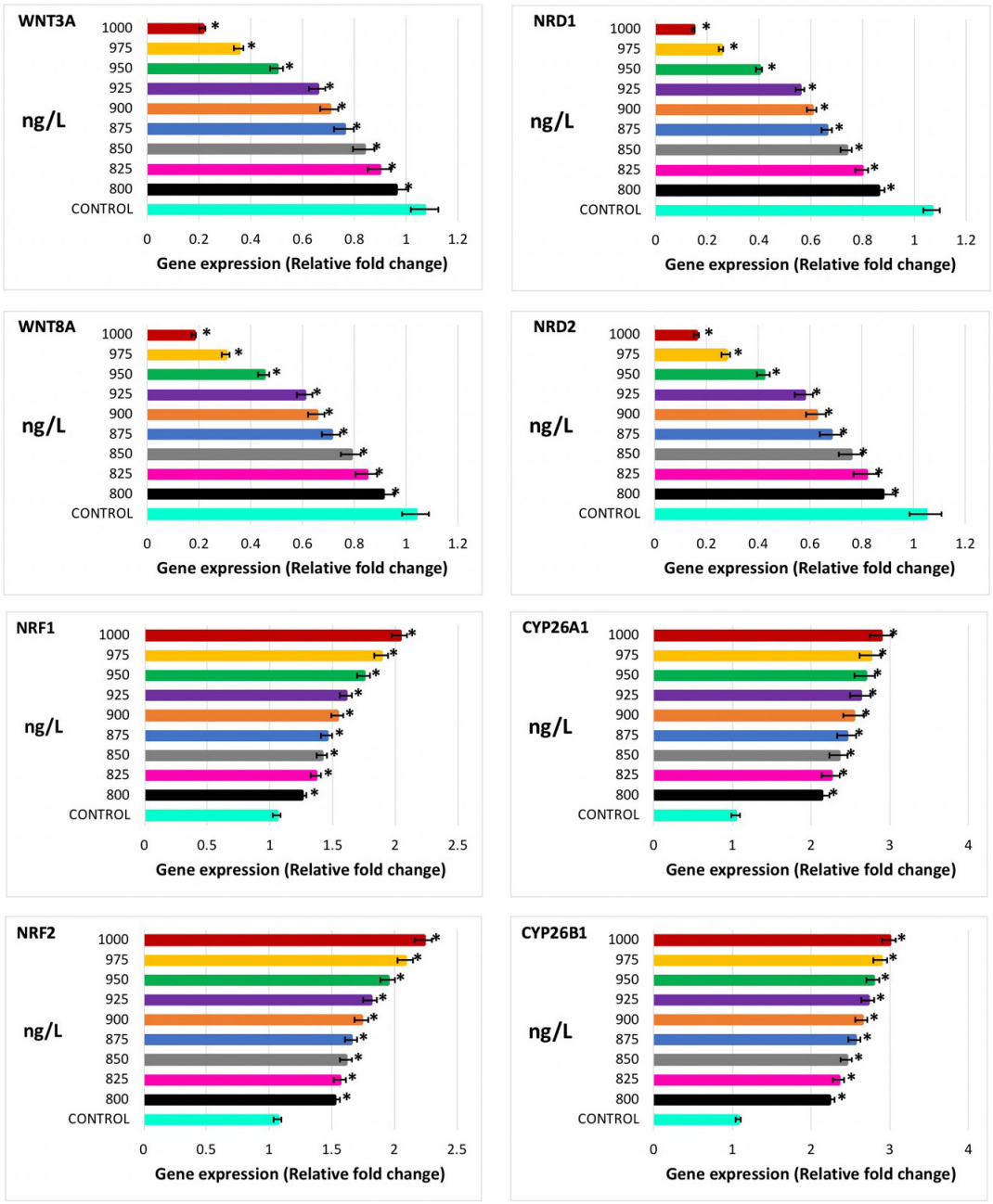

Figure 8. Gene expression of *NRF1*, *NRF2*, *CYP26A1*, *CYP26B1*, *WNT3A*, *WNT8A*, *NRD1*, and *NRD2* in larvae of *D. rerio* exposed of FCZ. *Denotes significant difference compared to control group.

delay in the hatching process could make embryos more susceptible to other predators. Up to date, authors have indicated that hatching disruptions in fish may be the result of different exogenous and endogenous factors such as oxygen availability, chemical modulators of CNS, hormonal levels, the release of proteolytic enzymes, and toxic agents[35]. As the FCZ mechanism of action is related to the inhibition of sterol biosynthesis and sterols are needed for the production of hormones[1,38], we believe FCZ-induced hormonal disruptions are likely to be the mechanism by which this drug delays the hatching process of fish. Nonetheless, future studies are needed to elucidate the mechanism by which FCZ inhibits the hatching process in fish. In agreement with the above-described results, Delattin *et al.*[39] found that triazoles

triadimefon, triadimenol, and free triazole (1,2,4-T) inhibit zebrafish hatching by blocking the secretory function of hatching gland cells. Nonetheless, they found this process was rescued by co-incubation with a dopamine D2 receptor antagonist. Thus, this may be another pathway by which FCZ alters the hatching process of fish.

Besides the alterations to embryonic development, FCZ also impaired the redox status of embryos by increasing the levels of SOD, CAT, LPX, POX, and HPX. In agreement with our results, Zhu *et al.*[19] demonstrated that 0.2 mg/L of FCZ significantly increased the enzymatic levels of SOD and glutathione s-transferase in *Gobiocypris rarus* embryos. Moreover, several other studies have indicated that FCZ induced ROS on different fungi[40-43]. Although the mechanism by which FCZ induces oxidative stress in organisms is not fully understood, Uthman *et al.*[41] suggested this process is related to the capacity of FCZ to inhibit the expression of genes encoding metallothioneins (MTs). MTs may control the ROS production via copper binding capacity and by donating electrons[42]. However, Peng *et al.*[40] indicated that FCZ did not affect the gene expression of MT and MT2 in *Cryptococcus neoformans*. Unlike the above mechanism, we believe FCZ-induced oxidative stress in fish is likely to be due to its inhibiting sterol biosynthesis capacity. Herein, we demonstrated FCZ upregulated the expression of cytochrome P450 family 26 subfamily A member 1 (CYP26A1) and cytochrome P450 family 26 subfamily B member 1 (CYP26B1) in embryos, which might be a response of fish against FCZ sterol biosynthesis inhibition[44]. Sterols play a vital role in stabilizing the plasma membrane; however, previous findings demonstrate this is also vital for mitochondrial function and stress tolerance. Thus, inhibition of sterol may impair the function of mitochondria causing ATP depletion and reactive oxygen species (ROS) production. In addition to the downregulation of MT and MT2, we also demonstrated that FCZ upregulated the expression of NRF1 and NRF2, which may be activated by the increased production of ROS. Gureev *et al.*[45], for instance, pointed out ROS, particularly H_2O_2, are strong Nrf2 activators. The ability to mount an efficient response against the continuous threat posed by exogenous oxidants such as FCZ is essential for cellular homeostasis and survival. Oxidative stress activates transcription of a variety of antioxidant genes through a cis-acting sequence known as antioxidant response element (ARE). Members of the Cap-N-Collar family of transcription factors that bind ARE, including Nrf1 and Nrf2, have been identified. Nrf1 and Nrf2 are expressed in a wide range of tissues and cell types, and both bind ARE as heterodimers with small-Maf proteins. Both Nrf1 and Nrf2 are responsible for regulating the expression of many antioxidant genes including peroxiredoxin-1 (Prx-1), thioredoxin-1 (Txn-1), GCLC (glutamate cysteine ligase catalytic subunit - an enzyme responsible for catalyzing the formation of glutathione), glutathione peroxidase (GPX-1), drug metabolizing enzymes (cytochrome P-450s), and several ATP binding cassette (ABC) transporters that are responsible for drug efflux. All of these genes are essential to the maintenance of oxidative homeostasis[46-48].

Once FCZ enters the cell, it inhibits CYP26, promoting the production of retinoic acid (RA), which in excess induces the upregulation of CYP26A1 and CYP26B1 to ensure RA homeostasis[49]. RA is essential in embryonic development, as organisms need it for the development of the spinal cord, somites, and eye[50,51]. Nonetheless, when RA exceeds the normal levels, it may cause many of the same embryonic developmental defects seen with vitamin A deficiency[52]. RA does not function alone in controlling the development of embryos; it interacts with other signaling pathways such as WNT, nodal growth differentiation factor (NODAL), fibroblast growth factor, sonic hedgehog, and bone morphogenetic protein. Our results demonstrate that FCZ downregulated, in a concentration-dependent manner, the gene expression of *WNT3A*, *WNT8A*, *NRD1*, and *NRD2* in embryos. WNT genes are related to the processes of organogenesis in the early stages of development[53]. Meanwhile, NODAL-related genes (NRD) genes encode a secreted ligand of the transforming growth factor-beta (TGF-β) superfamily of proteins that regulates early

embryonic development[54]. This teratogenic pathway is in line with prior findings that demonstrate RA reduced the gene expression of TGF-β in rodent embryos[55]. Moreover, El Zein *et al.*[55] pointed out that dysregulation of retinoic acid receptor alpha leads to an impairment of non-canonical WNT signaling, promoting abnormal cell proliferation in mice. Collectively, we believe FCZ-induced embryotoxicity, oxidative stress, and impaired gene expression in fish are the result of the alterations to sterol biosynthesis and RA signaling pathway. Nonetheless, future studies are needed to better understand the mechanism by which FCZ increases the production of ROS and disrupts embryonic development in zebrafish, as well as how these findings affect the fitness and health of fish at environmentally relevant concentrations.

CONCLUSIONS

FCZ is a triazole fungicide used in multiple medications and personal care products, which, due to its consumption, enters into the aquatic environment. However, the scientific community has barely studied the harmful effects this drug may pose to aquatic species. In this study, we demonstrated that environmentally relevant concentrations of FCZ altered the embryonic development of fish, producing several malformations in them. Moreover, we also demonstrated acute exposure to FCZ impaired the oxidative status of fish, promoting the production of LPX, POX, and HPX in the embryos. Since sterol is implicated in the normal function of mitochondria, we believe FCZ-induced oxidative stress may be the result of altered sterol biosynthesis; however, future studies are needed to elucidate whether this is the mechanism by which FCZ affects fish redox balance. Our results also indicate FCZ altered the expression of WNT, NRD, and CYP26 in the early life stages of *D. rerio*. Downregulation of these genes could be related to increased production of retinoic acid, as it is implicated in different signaling pathways related to embryogenesis. Increased retinoic acid production is likely to be the mechanism by which FCZ altered the gene expression of the above genes and induced malformations in embryos.

DECLARATIONS

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Authors' contributions

Performed all the exposure experiments: Escobar-Huerfano F, Elizalde-Velázquez GA
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Involved in the design and interpretation of the data: Gómez-Oliván LM, Escobar-Huerfano F, Elizalde-Velázquez GA, Orozco-Hernández JM, Rosales-Pérez KE
The writing of the manuscript with input: Islas-Flores H, Hernández-Navarro MD

Availability of data and materials

The data will be available in the uaemex repository.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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.132.2020).

Consent for publication

Not applicable.

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