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Antidiabetic drug metformin disrupts the embryogenesis in zebrafish through an oxidative stress mechanism

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ABSTRACT

In recent years, the consumption of metformin has increased not only due to the higher prevalence of type 2 diabetes, but also due to their usage for other indications such as cancer and polycystic ovary syndrome. Consequently, metformin is currently among the highest drug by weight released into the aquatic environments. Since the toxic effects of this drug on aquatic species has been scarcely explored, the aim of this work was to investigate the influence of metformin on the development and redox balance of zebrafish (*Danio rerio*) embryos. For this purpose, zebrafish embryos (4 hpf) were exposed to 1, 10, 20, 30, 40, 50, 75 and 100 μg/L metformin until 96 hpf. Metformin significantly accelerated the hatching process in all exposure groups. Moreover, this drug induced several morphological alterations on the embryos, affecting their integrity and consequently leading to their death. The most frequent malformations found on the embryos included malformation of tail, scoliosis, pericardial edema and yolk deformation. Regarding oxidative balance, metformin significantly induced the activity of antioxidant enzymes and the levels of oxidative damage biomarkers. However, our IBR analisis demonstrated that oxidative damage biomarkers got more influence over the embryos. Together these results demonstrated that metformin may affect the embryonic development of zebrafish and that oxidative stress may be involved in the generation of this embryotoxic process.

1. Introduction

Metformin (MET) is the most common drug prescribed to treat type 2 diabetes. Nonetheless, in recent years, numerous studies have suggested its usage for other indications such as: polycystic ovary syndrome and cancer (Zaidi et al., 2019; Bahrambeigi et al., 2020; Guan et al., 2020). In consequence, the prescriptions and consumption of this drug are increasing worldwide, leading to its presence in wastewater treatment plants (WWTPs). Once in WWTPs, MET is partially bio-transformed to guanylurea (GUA), and both compounds are released in to the aquatic environments, where they can exhibit different toxic effects in non-target organisms (Elizalde-Velázquez and Gómez-Oliván, 2020). The concentration of MET in worldwide water bodies ranges from ng/L to μg/L, with up to 33.5 μg/L detected in surface water (Elliot et al., 2017; Yao et al., 2018; Posselt et al., 2018).

Among the toxic effects that MET may induce in aquatic species are endocrine disruption and intersexuality. Niemuth et al. (2015), for instance demonstrated that 40 μg/L of this drug induced the overexpression of vitellogenin (VTG) in fathead minnow (FHM) males. Similarly, Crago et al. (2016) observed impacts on expression of VTG, estrogen receptor α (ERα) and gonadotropin releasing hormone 3 (GnRH3) in juvenile FHM at concentrations as low as 1 μg/L. In agreement with these results, Lee et al. (2019) pointed out that MET could activate AMPK by inhibiting MRC I and thereby affects the hypothalamus-pituitary-gonadal (HPG) axis, resulting in reproductive system disturbance.

Another harmful response that MET may produce in aquatic species is metabolic dysregulation. Brown trout embryos exposed to several concentrations of this drug (1 μg/L −1 000 μg/L) showed an increase in the amount of hepatic glycogen, especially in fish exposed the lowest MET concentration (Jacob et al., 2018). Analogously, early life stages of Japanese medaka exposed to a range of relevant concentrations of MET

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(1 μg/L −100 μg/L) had significantly altered metabolomes (Ussery et al., 2018). Changes in metabolite response factors and gene expression may indicate the cellular pathways affected by the presence of pollutants. In this case, MET mainly affected metabolites associated with cellular energetic and cell proliferation/growth pathways.

Regarding embryonic development toxic responses, only three studies have been carried out in fish. In the first one, environmentally relevant concentrations of MET (1 μg/L −100 μg/L) significantly reduced growth metrics and altered the expression of genes associated with cell growth (Ussery et al., 2018). According to the authors, their results may provide evidence that current environmental exposure scenarios may be sufficient to cause effects on developing fish. In disagreement with these results, Jacob et al. (2018) pointed out that mortality and development of brown trout embryos (48 dpf) were not influenced, after MET exposure (1 μg/L −1 000 μg/L). Nonetheless, they suggested that other species could react more sensitively to MET. Finally, this year, Parrot et al. (2021) investigated the chronic effects of environmentally relevant concentrations of MET (0.020 μg/L – 269 μg/L) in early life stages of fathead minnows. In their results, MET did not affect survival or growth of larval fish.

In order to fill the current knowledge gaps about the embryotoxicity effects that MET may induce in aquatic species, we carried out a study in zebrafish embryos. Our hypothesis is that environmentally relevant concentrations of MET would be capable of disrupt the embryonic development of this freshwater organism, through an oxidative stress mechanism.

2. Methods

2.1. Ethical approval

This research protocol was reviewed and approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM) to ensure that experiments are conducted in accordance with institutional standards for animal care (approval ID: RP. UAEM. ERC.132.2020). The provisions of the Mexican official standard on the breeding, care and use of laboratory animals (NOM-062-ZOO- 1999) were also taken into account.

2.2. Compounds

Metformin hydrochloride (CAS number: 1115-70-4) was purchased from Toronto Research Chemicals (Toronto, ON). Stock solutions of MET were prepared at a concentration of 1 g/L in ultrapure water.

Likewise, all other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.3. Zebrafish maintenance

Wild-type (AB strain) zebrafish embryos, were maintained at the Autonomous University of State of Mexico (Toluca, Mexico) in an open water system supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilized tap water. Feeding was performed three times a day with Spirulina flakes (Ocean Nutrition, US) supplemented with brine shrimp (*Artemia* sp. *nauplii*) to promote spawning activity. Water quality parameters are shown in Table 1. All parameters were monitored and controlled during zebrafish maintenance and breeding, as well as during subsequent experiments.

2.4. Zebrafish embryo collection

At the night before spawning, several males and females adults of zebrafish (2:1 ratio) with size 4–5 cm were chosen, and placed on individual breeding chambers. The spawning was induced by the onset of light in the morning. Embryos were collected, at 1 h post-fertilization (hpf), rinsed in embryo water and bleached according to established protocols (Westerfield, 2007; Varga, 2011). Fertilized embryos were classified under a stereoscopic microscope according to standard methods (Kimmel et al., 1995) and middle blastula stage embryos (equivalent to 2.5 hpf) were selected. After the selection, middle blastula stage embryos were kept in an incubator (27 °C± 1 °C) and left in the ultrapure water until embryos reached sphere stage (4 hpf). In Fig. 1A, we have depicted the general scheme of the procedure, and we included.

Table 1
Water quality parameters.

Parameter	Value ± SD
pH	7.35 ± 0.15
Hardness	95 ± 5 mg/L $CaCO_3$
Un-ionized ammonia	0.009 ± 0.003 mg/L
Alkalinity	95 ± 5 mg/L $CaCO_3$
Nitrate (NO^{3-})	3.1 ± 0.2 mg/L
Nitrite (NO^{2-})	0.030 ± 0.010 mg/L
Dissolved Oxygen	10.5 ± 0.5 mg/L
Conductivity	390 ± 35 μS/cm
Salinity	0.7 ± 0.2 g/L

Data represents the medium values of all replicates from all experiments ± SD.

2.5. Zebrafish embryo toxicity test

Seventy two embryos at the sphere stage (4 hpf) were selected and randomly distributed into 24-well plates (1 embryo per well). Each plate contained either a control solution (ultrapure water) or a test solution of MET (1, 10, 20, 30, 40, 50, 75, 100 μg/L). Three 24-well plates were used per test solution of MET as is shown in Fig. 1B. Furthermore, the exposure of embryos to each test solution of MET was carried out per triplicate, in three independent experiments to guarantee the reproducibility of results. The concentrations used on this experiments were based on pre-experiments (Elizalde-Velázquez and Gómez-Oliván, 2020). Sample size was calculated according to the OECD 236 Guidelines, which indicate to perform the test in 24-well plates. Furthermore, in this study, we opted to use three 24-well plates per treatment to guarantee statistical significance. In order to minimize the biases in this research, we considered the following points: 1) the main researcher of this research would know the treatments at which embryos were exposed; 2) one lab technician would be in charge of preparing the test solutions and the maintenance of the systems; and 3) another lab technician would be in charge of the randomization process. This way, lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Randomization was carried out as follows. Total of embryos were divided in 9 groups (72 embryos per group) that represented the control solution and each of the test solutions of MET. Next, all groups were assigned with a number from 1 to 9 using the standard = INT (RAND ()) function in Microsoft Excel. This way, numbers represented the treatment at which embryos were exposed. Plates were maintained at 27 °C± 1 °C, and under same light/dark periods (14:10). Lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Embryo mortality, hatching rate and malformation rate were assessed at different times (12, 24, 48, 72 and 96 hpf) during the course of MET exposure. Malformation rate was given as the percentage of embryos with at least one malformation with regard to the control. A graphic with the principal body alterations induced by MET exposure on embryos of *Danio rerio* was created using IBM SPSS Statistics 22 software. Mortality of embryos was also evaluated using a stereoscopic microscope, and dead embryos were removed daily. At the end of exposure time (96 hpf), live dead and malformed embryos were counted and a maximum likelihood linear regression analysis was done to determine LC_{50} and EC_{50m} with their 95% confidence intervals ($p < 0.05$). Spearman-Karber method trimmed was used (US-EPA software ver 1.5). In order to depict the cumulative

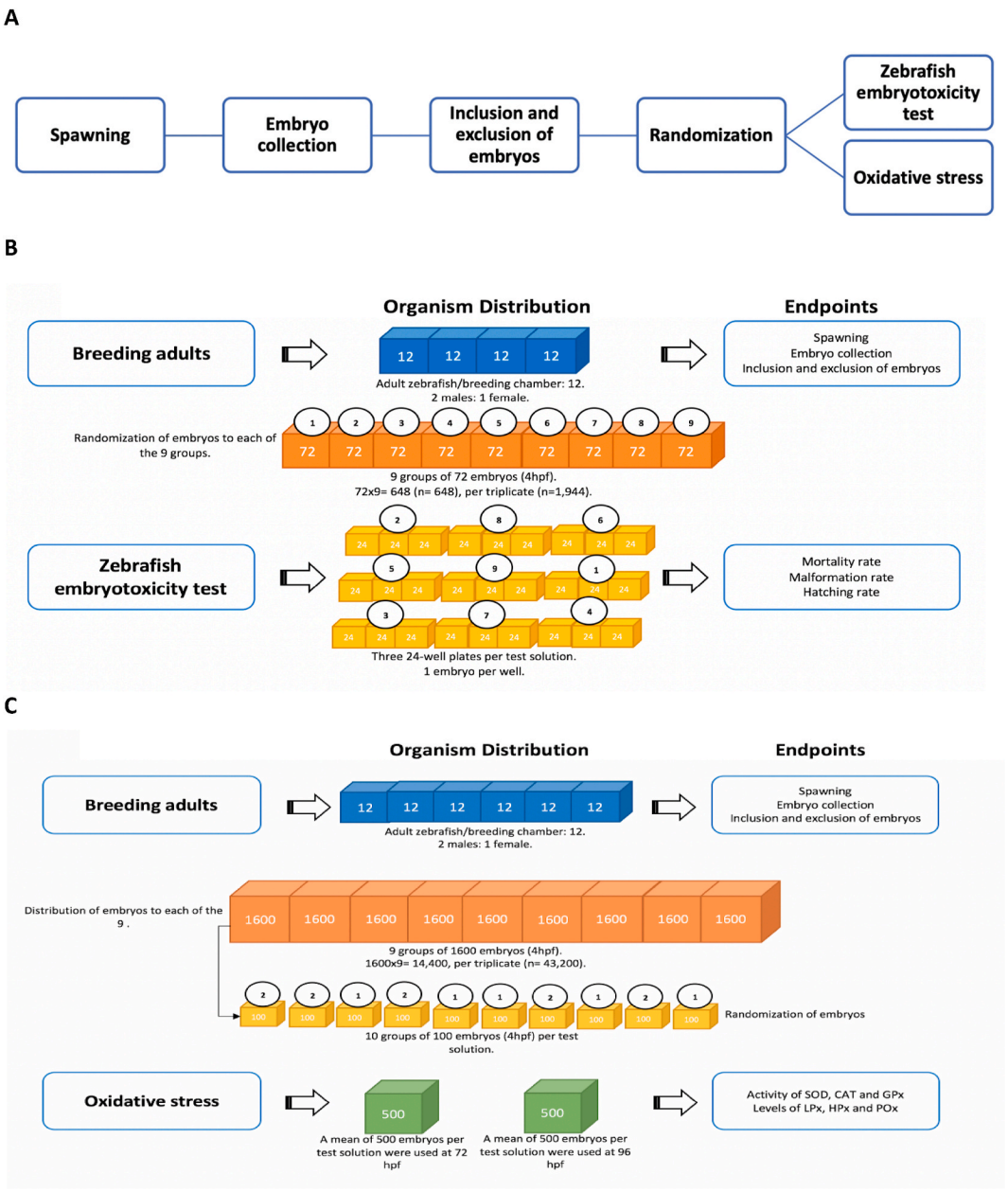

Fig. 1. Experimental design. A general scheme of the procedure B zebrafish embryo toxicity test procedure C oxidative stress procedure.

morality rate of the embryos at 12, 24, 48, 72 and 96 hpf, we performed a Kaplan-Meier analysis, using the SigmaPlot 12.3 software.

2.6. Oxidative stress in zebrafish embryos

Nine systems, each with 1600 *Danio rerio* at the sphere stage, were formed in aquariums of 4 L capacity. From these, eight were exposed to every MET concentrations and the last one was exposed to the control solution as is shown in Fig. 1C. Sample size was established based on pre-experiments (Nogueira et al., 2019). Briefly, for enzymatic determinations, they used a total of 700 unhatched zebrafish embryos at the beginning of each exposure. Here, we opted to use 800 embryos considering the high rates of mortality reached by MET during the fish embryotoxicity test. Furthermore, in this study we duplicate the number of embryos due to samples were taken up at 72 hpf and 96 hpf. These endpoint times were selected because at that time the zebrafish embryos had already hatched and their enzymatic system was already working. All along the exposure period, temperature (27 °C± 1 °C) and light/dark cycles (14:10) were kept constant in all the systems. For sampling, a mean of 500 embryos were randomly selected at 72 hpf and 96 hpf, and homogenized in 1 mL of phosphate buffer solution (pH 7.4). In order to minimize the biases in this research, we considered the following points: 1) the main researcher of this research would know the treatments at which embryos were exposed; 2) one lab technician would be in charge of preparing the test solutions and the maintenance of the systems; and 3) another lab technician would be in charge of the randomization process. This way, lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Randomization was performed as follows. Embryos from each system were divided into 10 groups (a mean of 100 embryos per group), and groups were assigned with a number between 1 and 2 using the standard = INT (RAND () function in Microsoft Excel. This way, 1 represents the groups that were used at 72 hpf, while 2 symbolizes the groups used at 96 hpf. Lab technicians were blinded to treatment during systems maintenance, while researchers were blinded to treatment during randomization. Samples were separated in two

Eppendorf tubes. On one hand, tube 1 contained 300 μL from the homogenate and 300 μL of a solution of trichloroacetic acid (TCA, 20%). On the other hand, tube 2 contained 700 μL from the homogenate. All tubes were maintained at −20 °C until they were use. Tube 1 was centrifugated at 11 495 rpm for 15 min at 4 °C and the precipitate was used to assess the protein carbonyl content (POx), while the supernatant was used to measure the levels of lipid peroxidation (LPX) and the hydroperoxide content (HPx). Tube 2 was centrifugated at 12 500 rpm for 15 min at 4 °C and the supernatant was used to determine the activity of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx).

Oxidative damage biomarkers and antioxidant enzymes activity were determined by different methods. The thiobarbituric acid assay for instance, was used to determine the LPX levels on fish (Buege and Aust, 1978). Analogously, the determination of POx was carried out by the method of Levine et al. (1994), which is centered on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine. In addition, for the quantification of HPx, we used the ferrous oxidation-xylenol assay described by Jiang et al. (1992).

Regarding antioxidant enzymes, CAT was evaluated by the method of Radi et al. (1991) based on the consumption of exogenous H_20_2. For SOD determination, we used the enzymatic assay of Misra and Fridovich (1972) that measure the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2. Finally, the method of Gunzler and Flohe-Clairborne (1985) was used to assess the GPx activity, based on the net reduction of glutathione *S*-transferase in 1 min at 37^{O}C and pH 7.

Results from all biomarkers were normalized against total proteins, which were analyzed with the method of Bradford (1976). Furthermore, the experiments were replicated three times.

2.7. Metformin determination

For the zebrafish embryo toxicity test, water samples from all of the seventy two wells for each treatment group were collected and stored at −20 °C until their quantification. Similarly, for the oxidative stress test, water samples from each of the nine systems were collected and stored at −20 °C until their quantification. Water samples for both studies were taken up at 0 time, as well as at 12, 24, 48 and 96 hpf. MET determination in water samples was perfomed on an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS equipped with a Turbo V Ion spray source. Positive ESI parameters were optimized to the following conditions: nebulizer gas 310 kPa; turbo gas 448 kPa; ion spay voltage 4.5 kV; collision gas: medium; curtain gas 172 kPa; and a ion source temperature 550 °C. >98% nitrogen was used as deolvation nebulizer and collision gas. Instrument control, data acquisition and data processing were performed with Analyst 1.6 software. Separation was performed using a Xbridge Phenyl column (150 mm × 2.1 mm, particle size 3.5 μm). The mobile phase consisted of a mixture of water with 5 mM ammonium formate as eluent A and MeOH 100% with 5 mM ammonium formate as eluent B. The flow rate was of 100 μL/min and the injection volume was of 50 μL.

2.8. Integrated biomarker response index (IBR)

Oxidative stress results were applied into the "Integrated Biomarker Response (IBR) Index" described by Sanchez et al. (2013). This tool can be used to integrate multi-biomarker responses (Sanchez et al., 2013). For the IBR calculation, all biomarkers from each treatment group (Xi) were compared to biomarkers of the control group (Xo). The ratio between Xi and Xo was log transformed (Yi) to reduce variance. Next, Yi values were standardized applying the following formula Zi=(Yi-μ)/s and using the mean (μ) and standard deviation (s) of Yi. Thereafter, the biomarker deviation index (A) were calculated thought the difference between Zi and Z0. Finally, A values were pictured in a star plot that represents the integrate biomarker responses. Furthermore, the absolute value of A from each biomarker was summed to get the IBR values.

2.9. Statistical analysis

Once all endpoints were evaluated, results were analyzed by a researcher who did not know the treatment at which embryos were exposed. Results, from all three replicates of each experiment were pooled and evaluated as follow: Oxidative stress biomarkers data was examined using a two-way analysis of variance (ANOVA), considering time as factor A and concentration as factor B. Additionally, variations between the means were examined with the Student-Newman-Keuls method, using SigmPlot 12.3 software. All oxidative stress biomarkers passed the normality test, excluding LPX. Thus, we opted to use a dot plot to show the distribution of data of this biomarker. This graphic was done in StatGraphic Centurion 16.02.04 and an ANOVA non-parametric was also carried out. Please head over supplementary material to see this graphic (Fig. 1S). Embryotoxic and teratogenic effects data were evaluated by Fisher's exact test. Significance was accepted when $p < 0.05$, using SigmPlot 12.3 software. Statistical analysis was blinded to researchers.

3. Results

3.1. Mortality test

The cumulative mortality rate of zebrafish embryos exposed to each MET concentration is showed in Fig. 2A. When compared with the control group, MET significantly increased the mortality rate in zebrafish embryos in a time and concentration dependent manner, reaching the highest number of dead embryos at the concentration of 75 μg/L. Although, for the concentration of 100 μg/L, the mortality rate was significantly higher than the controls, this was much lower than in the rest of the concentrations. As same as in mortality rate, MET considerably increased the malformation rate, reaching the maximum peak at the concentration of 75 μg/L (Fig. 2B). After this concentration, MET showed an important decrease in the number of malformed embryos. Taking into account this data, LC_{50} and EC_{50m} were calculated, getting a value of 3.25 μg/L and 0.37 μg/L, respectively. Furthermore, the teratogenic index of MET in *Danio rerio* got a value of 8.8. According to the criteria of Weigt et al. (2011), MET should be classified as teratogenic.

3.2. Hatching rate

Hatching rates of zebrafish embryos exposed to MET and ultrapure water are depicted in Fig. 2C. As can be seen from this figure, at 24 hpf, embryos exposed to 10 μg/L, 20 μg/L and 30 μg/L of MET started to emerge from chorion. However, this process was only found to be statistically significant for 10 μg/L and 20 μg/L dose of MET. At 48 hpf, the hatching rate for all exposure groups was significantly increased compared with the control group. The highest number of embryos dechorionated was obtained for the concentration of 75 μg/L of MET. Furthermore, at the concentration of 100 μg/L, the hatching rate notably decreased compared with the rest of the treatment groups. 24 h later, most of the embryos of the control group and the MET treated group had hatched. At this moment, none of the treatment groups showed a significant difference compared with the control group. Finally, at 96 hpf, all of the embryos from the control and treatment groups had hatched.

3.3. Teratogenic effects induced by MET

During all the exposure period, development of the embryos in the control group was normal. Unlike control group, MET caused body malformations in all treatment groups and exposure time (Fig. 3A). The observed malformations included malformation of tail, scoliosis, pericardial edema, yolk deformation, hypopigmentation, delay of the hatching process, fin absent, eye absent and craniofacial malformation. Among these, the most pronounced malformations were malformation of tail and scoliosis (Fig. 3B). As can be seen in Fig. 3, the sternness of

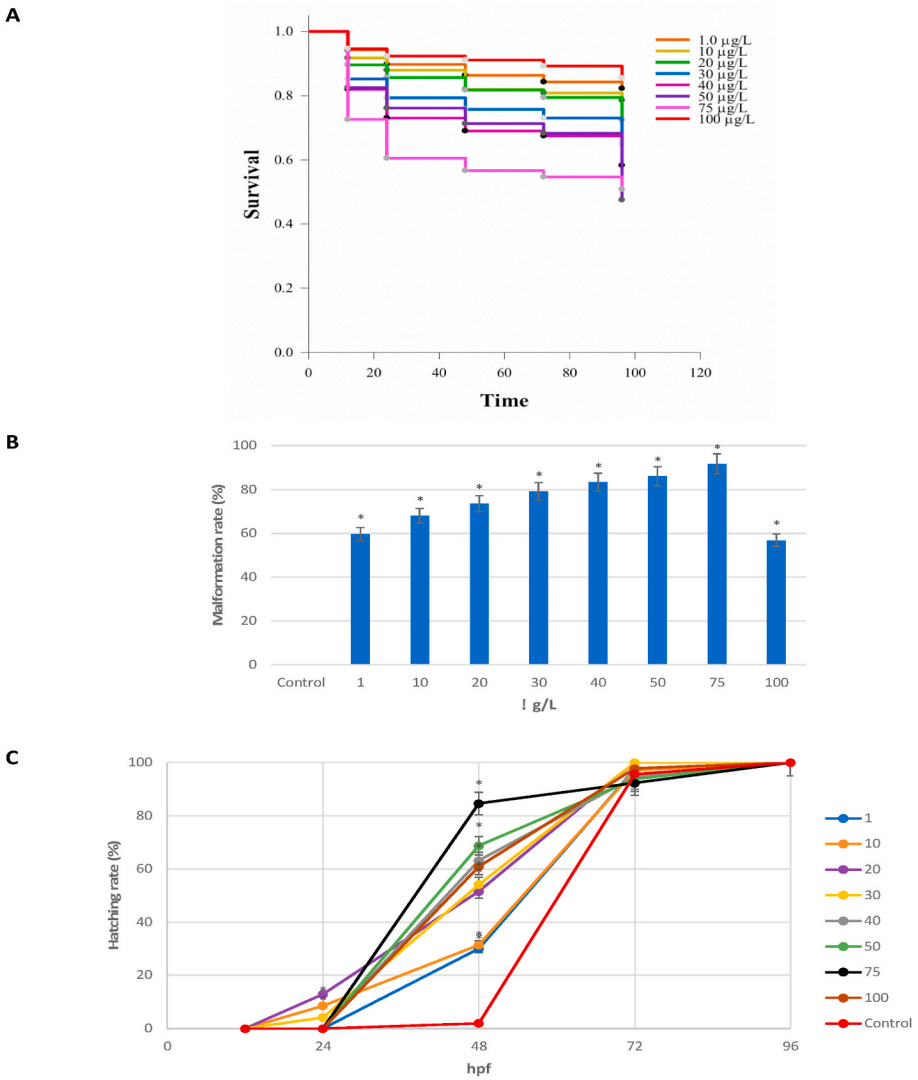

Fig. 2. Mortality, malformations and hatching rate in *Danio rerio* embryos exposed to MET (µg/L). **A** represents the cumulative mortality rate of zebrafish embryos exposed to each MET concentration at 12,24,48,72 and 96 hpf. **B** represents the cumulative malformations rate of zebrafish embryos exposed to each MET concentration at 96 hpf. **C** represents the cumulative hatching rate of zebrafish embryos exposed to each MET concentration at 12, 24, 48, 72 and 96 hpf. Data are expressed as means ± standard error (SEM) from three independent experiments. * denote a significant change with $P < 0.05$.

malformations was more evident as concentration increase, showing a higher incidence of embryos with pericardial edema, yolk deformation, eye absent and craniofacial malformation. The most severe malformations were observed for the embryos exposed to 75 µg/L. After this concentration, MET showed a significant decrease in the severity of malformations.

3.4. Antioxidant activity induced by MET

The antioxidant activity of SOD, CAT and GPx, in *Danio rerio* embryos exposed to MET is depicted in Fig. 4. As can be seen in this figure, the activity of all enzymes, in all exposure groups, showed a significant increase compared with control group. Furthermore, as the concentration increased, the activity of the enzymes also increased, reaching the maximum peak at the concentration of 75 µg/L. After this concentration, the activity of all enzymes significantly declined compared with rest of the treatment groups. Regarding exposure time, no significant differences were found in the activity of SOD. Nonetheless, at the concentration of 50 µg/L, the enzymatic activity of CAT showed a significant increase at 96 hpf compared to 72 hpf. Moreover, the enzymatic activity of GPx was significantly increased at the concentrations of 40 µg/L, 50 µg/L and 75 µg/L in a time dependent manner.

3.5. Oxidative damage induced by MET

The levels of LPX, HPx and POx in *Danio rerio* embryos exposed to MET are shown in Fig. 5. As same as in the antioxidant enzymes, the levels of all oxidative damage biomarkers, in all exposure groups, exhibited a significant increase compared with the control group. Additionally, the levels of all oxidative damage biomarkers increased as the concentration did, reaching the highest peak at a dose of 75 µg/L of MET. On the other hand, at a concentration of 100 µg/L, the levels of LPX, HPx and POx considerably decreased compared with the rest of the treatment groups. Regarding exposure time, the levels of LPX and HPx significantly increased at the concentrations of 50 µg/L and 75 µg/L. However, no differences were found in the levels of POx at 96 hpf compared to 72 hpf.

3.6. IBR

IBR values increased as MET concentrations increased, indicating that high concentrations of this drug induced more pronounced effects in the embryos, with the exception of the 100 µg/L concentration (Fig. 6). These effects were mainly focused on oxidative damage as the star plot was mainly deviated to these biomarkers. At the lowest concentration, for instance the oxidative damage biomarker that more impacted the embryos was POx. Nonetheless, as the concentrations

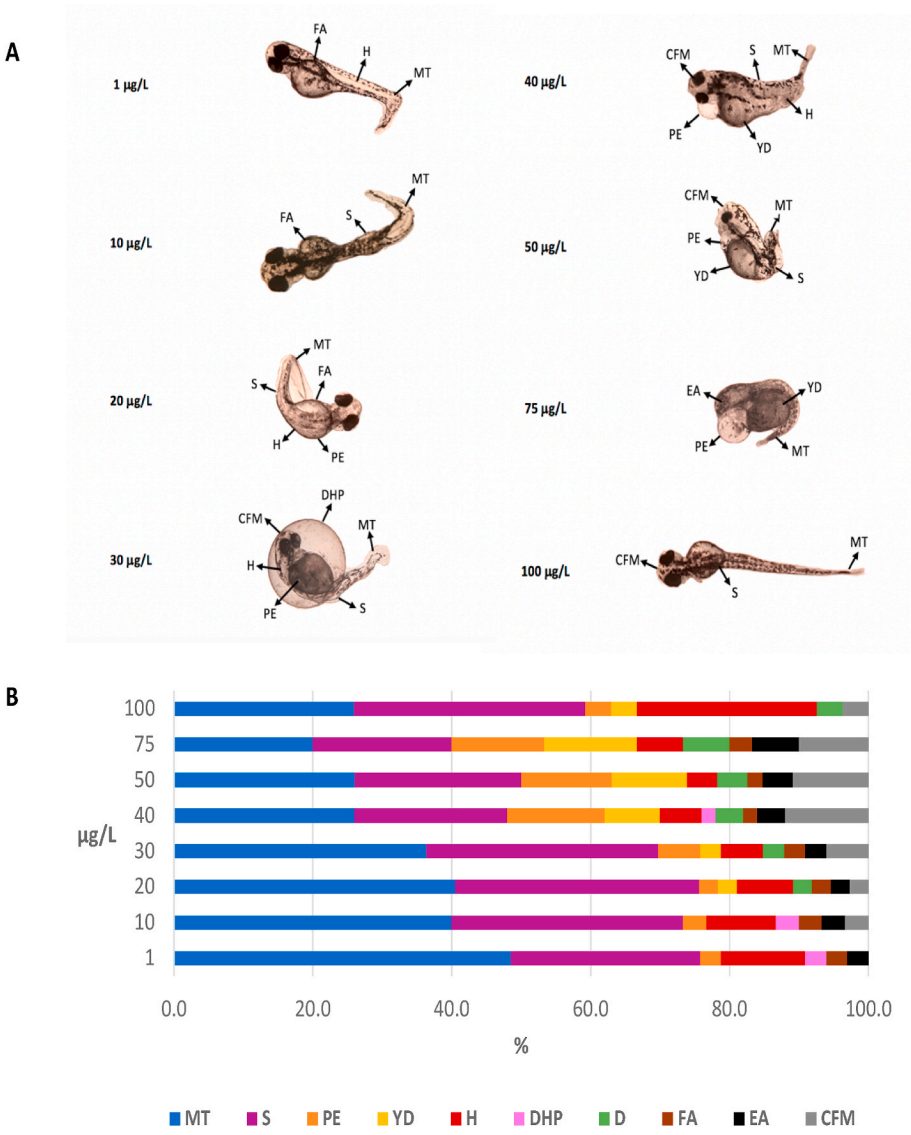

Fig. 3. Main malformations induced by each concentration of MET in *Danio rerio* embryos. **A** Representative photos of morphological abnormalities in zebrafish embryos exposed to environmentally relevant concentrations of MET at 96 hpf. Arrows indicate all malformations found on each embryo. **B** Cumulative incidence of each malformation found in zebrafish embryos exposed to MET, expressed in percentage. Data are expressed as means ± standard error (SEM) from three independent experiments. MT: malformation of tail; S: scoliosis; PE: pericardial edema; YD: yolk deformation; H: hypopigmentation; DHP: delay of the hatching process; FA: fin absent; EA: eye absent; CFM: craniofacial malformation.

increased, the HPx and LPx biomarkers got more influence over the embryo. Regarding exposure time, the concentration of 50 µg/L and 75 µg/L showed a significant increase in the mean IBR values.

3.7. Metfromin determination

For both test, MET concentrations in water samples was in accordance with the nominal MET concentrations (Table 2 and Table 3). Since the concentration of MET was mantained above 80% for all samples, analyses of the results were based on nominal values.

4. Discussion

In the present study, the harmful effects of the antidiabetic drug MET on the embryonic development of *Danio rerio* were investigated. Our results demonstrated that environmentally relevant concentrations of MET may increase the mortality and malformation rate in zebrafish embryos. Nonetheless, these results are not in agreement with those found by Jacob et al. (2018), who pointed out that MET concentrations from 1 µg/L to1000 µg/L did not affect the mortality rate of brown trout embryos. Differences between studies may be explained due to the dissimilar incubation temperatures. Brown trout embryos, for instance were incubated at 7 °C and 11 °C, while zebrafish embryos were kept in climate chambers at 27 °C. This is noteworthy, as Jacob et al. (2018) found higher concentrations of MET in brown trout embryos exposed to 11 °C compared to the lower temperature. Thus, it is suggested that temperature may enhance the uptake of this drug. Another factor that may influence the results of both studies are the differences in the stages of embryonic development. For example, in our study, zebrafish embryos exposed to MET were at the sphere stage (4 hpf), while brown trout embryos exposed to this drug were in the eyed stage (48 dpf). It is well known that as time passes the structure of chorion change and the toughness of the embryo membrane begins to raise, leading to a low permeability. Ussery et al. (2018), for instance demonstrated that Japanese medaka embryos exposed to 10 µg/L of ^{14}C-MET prior to hardening (<6 hpf) had significantly more ^{14}C-MET relative to embryos exposed to this compound post-chorion hardening (24 hpf). Since the embryonic development of Japanese medaka and zebrafish are comparable (Furutani-Seikia and Wittbrod, 2004), we suggest MET had a similar uptake behavior in zebrafish. Therefore, MET could be easily absorbed by zebrafish at sphere stage, but no in brown trout in eyed stage, leading to a different toxic response, with the latter being more severe in zebrafish.

Interestingly, MET not only increased the mortality and malformations rate of zebrafish, but also accelerate the hatching process in fish. These findings are consistent with those reported by Flores et al. (2020),

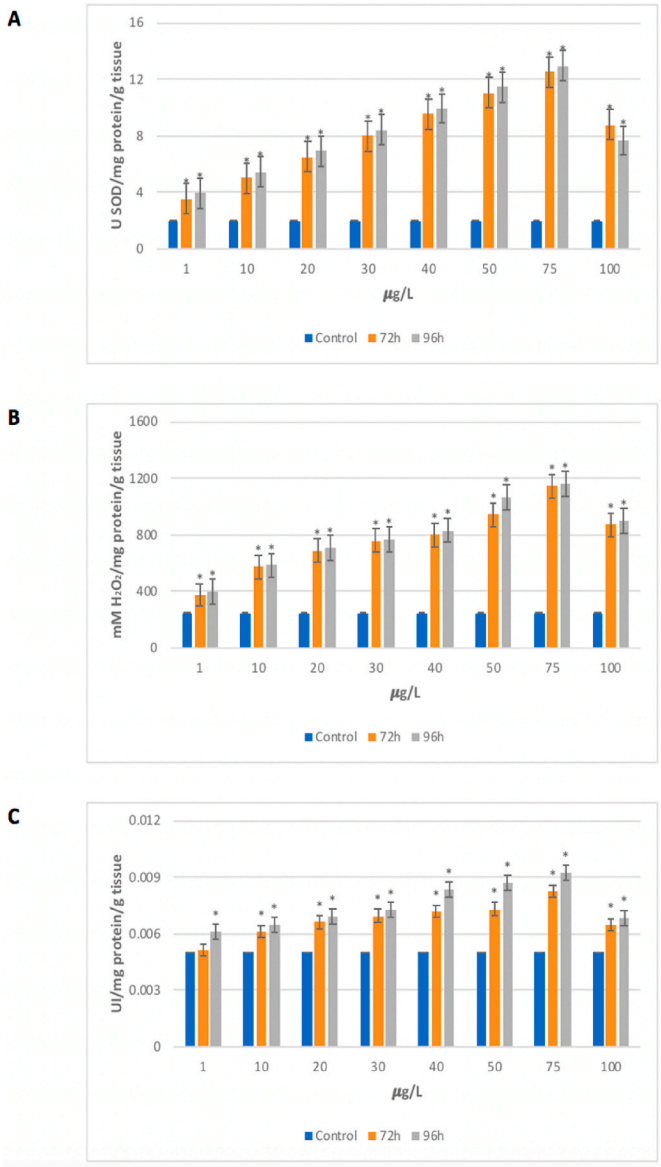

Fig. 4. Activity of antioxidant enzymes, SOD ($F(8,144) = 6797.101$; $n = 9$; $p < 0.001$) **A**, CAT ($F(8,144) = 630.279$; $n = 9$; $p < 0.001$) **B** and GPx ($F(8,144) = 56.674$; $n = 9$; $p < 0.001$) **C**, in *Danio rerio* embryos exposed to MET at 72 hpf and 96 hpf. Data are expressed as the mean of three replicates ± standard error (SEM). * indicate a significant change with $P < 0.05$.

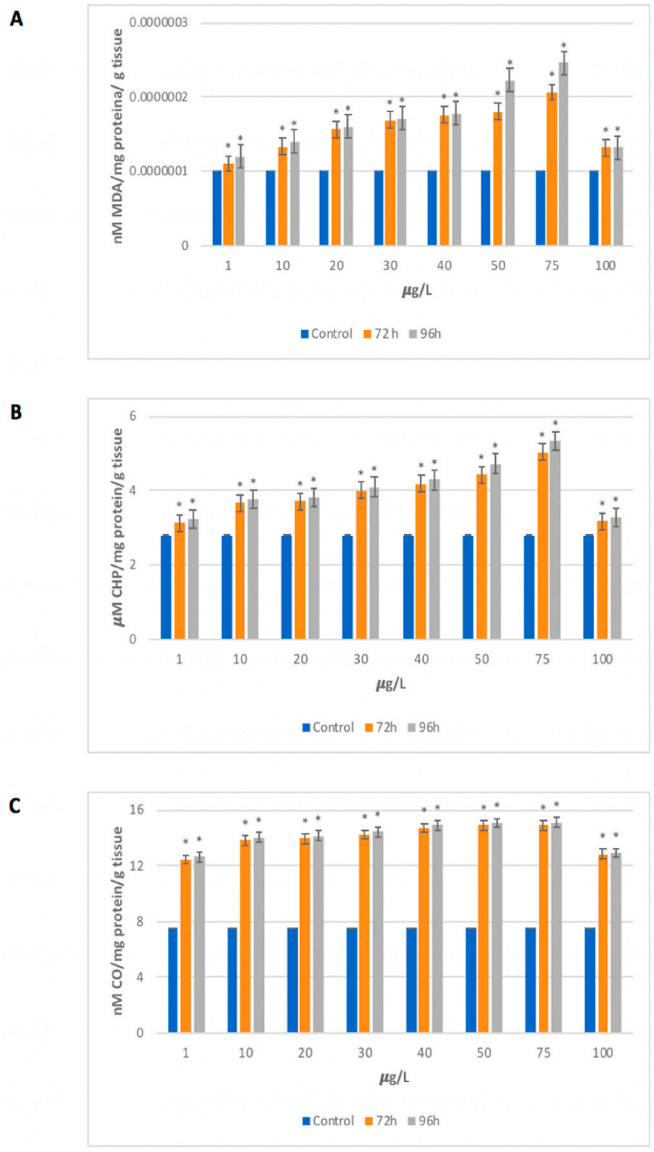

Fig. 5. Levels of LPX ($F(8,144) = 467.969$; $n = 9$; $p < 0.001$) **A**, HPx ($F(8,144) = 243.986$; $n = 9$; $p < 0.001$) **B** and POx ($F(8,144) = 92.710$; $n = 9$; $p < 0.001$) **C**, in *Danio rerio* embryos exposed to MET at 72 hpf and 96 hpf. Data are expressed as the mean of three replicates ± standard error (SEM). * indicate a significant change with $P < 0.05$.

who demonstrated that MET induced an anticipated hatching (48 hpf) in zebrafish embryos exposed to several concentrations (10 µg/L–2000000 µg/L) of this drug. Analogously, in a randomized comparative study, Rowan et al. (2008) exhibited the rates of spontaneous and iatrogenic preterm births were higher in women treated with metformin than those with insulin. Anticipated hatching in fish is important as hatched fish are more vulnerable to predators, mechanical and osmotic stress, and toxic agents present in the water bodies, leading to more severe alterations in its development. Hatching alterations in fish can be induced by several endogenous and exogenous factors, including toxic agents, oxygen availability, central nervous system chemical modulators, release of proteolytic enzymes and hormonal levels (De la Paz et al., 2017). Nonetheless the mechanisms and pathways involved has not been completely elucidated. Since previous studies have demonstrated that MET may be an endocrine disruptor (Niemuth and Kapler, 2015; Lee, 2017; Monshi, 2017; Niemuth et al., 2018; Lee et al., 2019), it could be suggested that the hormonal disturbances on fish might be involved in the accelerate hatching process. Nevertheless, future studies should elucidate the mechanism by which this drug may alter the hatching process of fish.

During all the exposure period, MET induced several malformations on zebrafish embryos. Among these, it is included malformation of tail, scoliosis, pericardial edema, yolk deformation, hypopigmentation, fin absent, eye absent and craniofacial malformation. In agreement with these results, a study carried out in chick embryo showed that multiple concentrations (0.1 mg/ml - 5 mg/ml) of MET displayed a delay in closure of anterior and posterior neuropores, leading to brain abnormalities, absence of limb buds, as well as a delay in the eye, otocyst, heart, branchial and somites formation (Siripattanaphol et al., 2020). Furthermore, Flores et al. (2020) pointed out that MET may cause microcephaly and decreased tail length in zebrafish embryos exposed to multiple concentrations (10 µg/L - 2000000 µg/L) of MET. One possible mechanism through which MET may induce these malformations on fish may be oxidative stress. As it is known, reactive oxygen species (ROS)

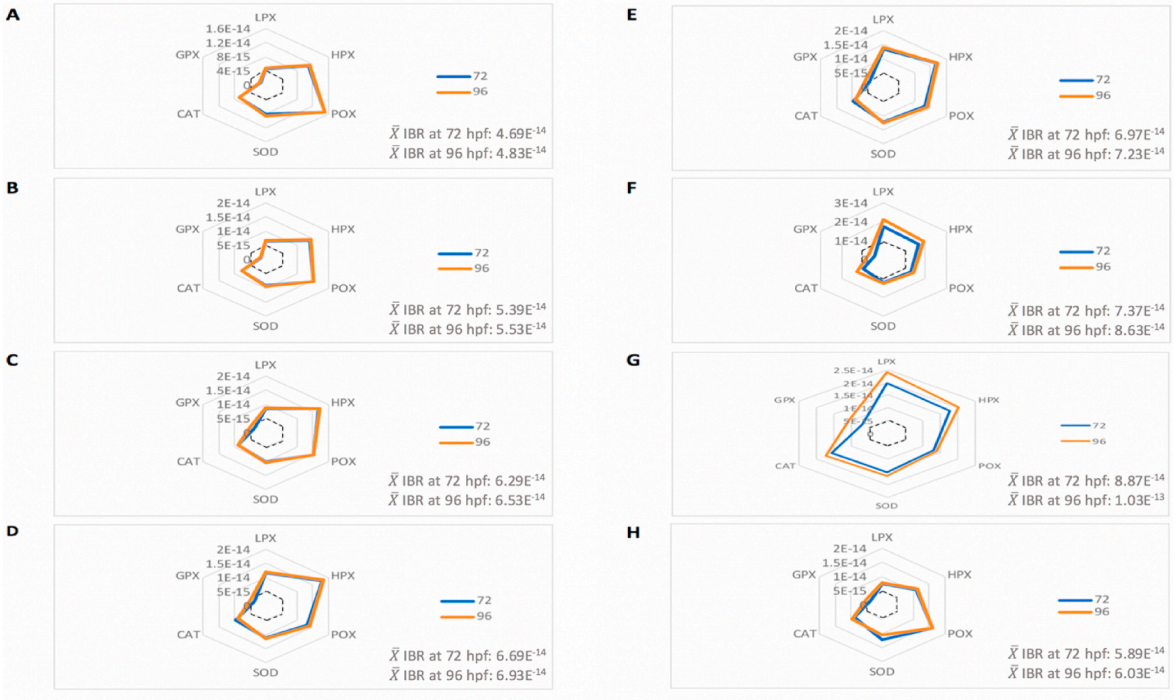

Fig. 6. IBR values and star plots of oxidative stress response of *Danio rerio* embryos exposed to MET. Biomarkers from each treatment groups (colors lines) were compared to those of the control group (dashed line). The area above zero indicate the production of the biomarker and under zero reflects the reduction of the biomarker. Letters (A–H) represent each of the MET concentrations tested in this study, arranged from the lowest to the highest. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Measured concentrations of MET in the zebrafish embryo toxicity test.

Nominal concentrations of MET	Measured MET concentrations at diferent exposure times (μg/L)					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
1 μg/L	0.98 ± 0.03	0.89 ± 0.02	0.89 ± 0.02	0.88 ± 0.02	0.88 ± 0.01	0.87 ± 0.01
10 μg/L	9.97 ± 0.06	9.05 ± 0.05	9.01 ± 0.04	8.99 ± 0.04	8.92 ± 0.05	8.85 ± 0.04
20 μg/L	19.93 ± 0.12	18.21 ± 0.09	18.07 ± 0.10	17.92 ± 0.10	17.77 ± 0.10	17.61 ± 0.09
30 μg/L	30.11 ± 0.21	28.54 ± 0.19	28.10 ± 0.17	27.63 ± 0.15	27.35 ± 0.15	27.02 ± 0.16
40 μg/L	40.02 ± 0.50	37.33 ± 0.43	37.01 ± 0.40	36.95 ± 0.42	36.81 ± 0.40	36.77 ± 0.42
50 μg/L	50.05 ± 0.64	48.20 ± 0.58	47.34 ± 0.60	47.01 ± 0.61	46.73 ± 0.59	46.48 ± 0.62
75 μg/L	75.01 ± 0.73	72.20 ± 0.70	71.83 ± 0.69	71.04 ± 0.70	70.42 ± 0.69	69.90 ± 0.71
100 μg/L	100.45 ± 0.91	96.02 ± 0.85	95.18 ± 0.83	94.31 ± 0.84	92.83 ± 0.80	91.60 ± 0.82

Values reprenset mean ± standard deviation of each concentration. LoQ: limit of quantification (500 ng/L).

Table 3
Measured concentrations of MET in the oxidative stress test.

Nominal concentrations of MET	Measured MET concentrations at diferent exposure times (μg/L)					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
1 μg/L	1.01 ± 0.01	0.91 ± 0.01	0.90 ± 0.01	0.90 ± 0.01	0.88 ± 0.02	0.86 ± 0.01
10 μg/L	10.03 ± 0.02	9.30 ± 0.02	9.29 ± 0.01	9.28 ± 0.01	9.26 ± 0.02	9.21 ± 0.02
20 μg/L	20.04 ± 0.05	19.01 ± 0.04	18.98 ± 0.03	18.85 ± 0.03	18.80 ± 0.04	18.73 ± 0.03
30 μg/L	30.03 ± 0.05	28.53 ± 0.04	28.30 ± 0.03	28.19 ± 0.03	27.96 ± 0.04	27.94 ± 0.03
40 μg/L	40.05 ± 0.05	38.25 ± 0.05	38.04 ± 0.04	37.93 ± 0.04	37.40 ± 0.03	37.08 ± 0.03
50 μg/L	50.01 ± 0.06	49.51 ± 0.04	49.04 ± 0.04	48.78 ± 0.03	48.46 ± 0.03	48.02 ± 0.05
75 μg/L	75.02 ± 0.03	74.18 ± 0.05	73.75 ± 0.03	73.44 ± 0.03	72.84 ± 0.03	72.39 ± 0.04
100 μg/L	100.04 ± 0.09	98.73 ± 0.10	98.20 ± 0.10	98.03 ± 0.08	97.93 ± 0.08	97.90 ± 0.09

Values reprenset mean ± standard deviation of each concentration. LoQ: limit of quantification (500 ng/L).

act as a primary and secondary messengers to induce the cell growth or death. Furthermore, redox system control different transcriptions factors that regulate cell signaling pathways involved in proliferation, differentiation and apoptosis (Pašková et al., 2011). Therefore, oxidative stress may disrupt several important reactions that affect the development of the embryos. Here, we demonstrated that after an acute exposure to MET on early life stages of zebrafish, this drug may induce an oxidative stress response on the embryos. Furthermore, our IBR analyses indicated that as MET concentrations increased, mean IBR values also increased. Therefore, oxidative stress responses in the organisms were more pronounced as the concentration increased. In agreement with these findings, Anedda et al. (2008) indicated that MET increased the levels of ROS and lowers the aconitase activity in 3T3-L1 cells. Similarly, Queiroz et al. (2014) demonstrated that MET induced cell cycle arrest and increased cell apoptosis in MCF-7 cells treated with 10 mM of this drug. Moreover, they explained these responses were mediated by oxidative stress, and a treatment with SOD and CAT improved cell viability. Finally, in a more recent study, Lee et al. (2019) evaluated the MET aquatic toxicity under a multi-generational exposure regimen, and found that this drug increased the ROS content and decreased the GSH activity in F0 fish. Furthermore, CAT activity was also significantly increased under MET treatment, suggesting that

environmentally relevant concentrations of this pollutant may cause oxidative stress in *Oryzias latipes*.

Mitochondria are an important source of ROS and as we aforementioned, these are involved in several cell signaling pathways. Nonetheless, ROS may also produce to oxidative damage in the proteins, membrane and DNA of mitochondria, affecting the mitochondrial capacity to synthesize ATP and to carry out their metabolic functions (Adam-Vizi and Chinopoulos, 2006). This is noteworthy, as mitochondrial DNA (mDNA) alterations may disturb respiratory chain elements or ribosomal and transfer RNAs, causing several diseases (Tuppen et al., 2010). Additionally, the accumulation of dysfunctional mitochondria may increase the undesirable effects of electron-transport chain (ETC) complex I inhibitors on cell survival (Espada et al., 2020).

One of the metformin's primary functions is the inhibition of complex I of the mitochondrial ETC, leading to different changes in the mitochondrial membrane and ATP production (Andrzejewski et al., 2014; Cameron et al., 2018). These changes may result in an increased production of ROS, affecting the mitochondrial integrity, and triggering the activation of the cell's apoptotic machinery. Therefore, the body abnormalities and the accelerate hatching process found in this study may be a consequence of the inhibition of complex I of the mitochondrial ETC. In agreement with our results, a recent study investigated the toxic effects that mitochondrial inhibitors drugs induced in zebrafish embryos. In their results pointed out that different concentrations (0.3 nM–10 mM) of complex I and II inhibitors induced several malformations in embryos of this fish. These malformations included edema, eye, head and skeletal defects, hypopigmentation, gastrula arrest, yolk sac necrosis and bleeding (Pinho et al., 2013).

There are two mechanisms by which mitochondria increase the production of superoxide. In the first one, the NADH pool is reduced, for example by alterations in the respiratory chain, low ATP demand and loss of cytochrome *c* trough the apoptosis. This leads to the formation of superoxide, at the flavin mononucleotide (FMN), in a rate that is established by the amount of FMN reduced (Kushnareva et al., 2002; Liu et al., 2002; Kussmaul and Hirst, 2006). In the second one, there is no ATP production and there is a high protonmotive force and a reduced coenzyme Q (CoQ) which leads to a reverse electron transport through complex I, producing large amount of superoxide (St-Pierre et al., 2002; Liu et al., 2002). Until now, it is believed that the inhibition of mitochondrial complex I by MET may disrupt the electron flow, and cause the superoxide generation by an FMN reduction (Lee et al., 2019). Nonetheless, more studies are needed to better understand the mechanism by which this drug increases the production of superoxide in the mitochondria.

At low concentrations (1 μg/L–75 μg/L), MET increased the mortality, malformations severity, hatching rate, and oxidative stress biomarkers in a concentration dependent manner. Nonetheless, at a concentration of 100 μg/L, all the endpoints evaluated in this work were significantly declined with regard the other treatment groups. Interestingly, this is not the first study that found these variations at higher concentrations of this drug. Jacob et al. (2018) for instance, demonstrated that the quantity of hepatic glycogen was increased in brown trout embryos exposed to MET. Nonetheless, this increase was specifically higher in fish exposed to the lowest MET dose. Meanwhile, at higher doses, the glycogen content in the liver showed a high inconsistency. Similarly, Flores et al. (2020) pointed out that at the highest concentration of MET, 100% of the embryos showed an anticipated hatching. However, at the same concentration, MET conceived a 0% of teratogenicity. Therefore, we suggest MET could experience a biphasic dose-response. Hormesis is a dose-response phenomenon, characterized by a low dose response that is opposite in effect to that seen at high doses (Ray et al., 2014). Furthermore, this is highly generalizable, being independent of biological model, endpoint measured, chemical class, and interindividual variability (Calabrese and Mattson, 2017).

5. Conclusion

Embryos exposed to environmentally relevant concentrations of MET showed an anticipated hatching process, making them more vulnerable to other stressors present in the environment. MET also induced several morphological alterations on the embryos, affecting their integrity and consequently leading to their death. Among the main malformations induced by MET, it is included malformation of tail, scoliosis, pericardial edema, craniofacial malformation and yolk deformation. Since oxidative damage biomarkers (LPX, HPx, POx) increased in the embryos exposed to MET, we suggest that the embryotoxic effects of this drug may be induce through an oxidative stress mechanism. To the date is believed that this oxidative stress response is closely related to the metformin capacity of inhibit the mitochondrial complex I. Nonetheless, more studies are needed to fully comprehend the mechanism by which MET may induce oxidative stress on fish. A significant decline in the mortality, malformations severity, hatching rate, and oxidative damage was found at the highest concentration used in this study. Variability in the concentration-effect relation in MET is also indicated in other studies, and this might be awarded to the hormesis phenomena that are common in environmental toxicology. However, mechanistic explanations for this phenomenon are still lacking, and need to be further analyzed to better understand the environmental impact of this drug.

Author statement

Gustavo Axel Elizalde-Velázquez performed all the exposure experiments, Leobardo Manuel Gómez-Oliván was involved in the conception, Sandra García-Medina conducted statistical analyses, Leobardo Manuel Gómez-Oliván, Gustavo Axel Elizalde-Velázquez, Sandra García-Medina and Hariz Islas Flores were involved in the interpretation of the data and the writing of the manuscript with input from María Dolores Hernández-Navarro 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.

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Appendix A. Supplementary data

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