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Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of *Danio rerio*

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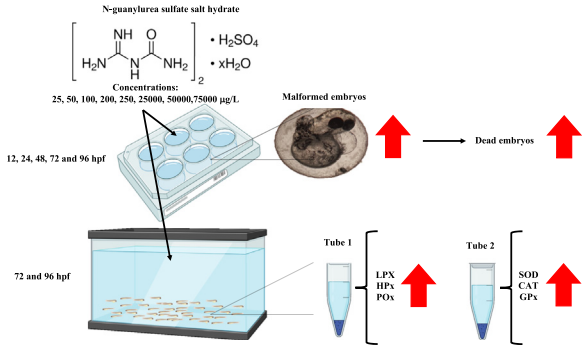
HIGHLIGHTS

- Guanylurea delayed the hatching process in all treatment groups.
- Developmental abnormalities induced by guanylurea led to the death of zebrafish embryos.
- Embryos exposed to guanylurea showed an increase in activity of antioxidant enzymes.
- Oxidative damage biomarkers levels increased as guanylurea concentration increased.
- Environmentally relevant concentrations of guanylurea pose a threat to aquatic species.

GRAPHICAL ABSTRACT



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ABSTRACT

Metformin is one the most prescribed drug to treat type 2 diabetes. In wastewater treatment plants, this drug is bacterially transformed to guanylurea, which occurs at higher concentrations in the aquatic environments than its parent compound. Since there is a huge knowledge gap about the toxicity of this metabolite on aquatic organisms, we aimed to investigate the impact of guanylurea on the embryonic development and oxidative stress biomarkers of zebrafish (*Danio rerio*). For this effect, zebrafish embryos (4 h post fertilization) were exposed to 25, 50, 100, 200, 250, 25,000, 50,000, 75,000 µg/L guanylurea until 96 h post fertilization. Guanylurea led to a significant delay in the hatching process in all exposure groups. Furthermore, this transformation product affected the embryonic development of fish, inducing severe body alterations and consequently leading to their death. The most pronounced malformations were malformation of tail, scoliosis, pericardial edema, yolk deformation and craniofacial malformation. Concerning oxidative stress response, we demonstrated that guanylurea induced the antioxidant activity of superoxide dismutase, catalase, and glutathione peroxidase in zebrafish embryos. In addition, the levels of lipid peroxidation, protein carbonyl and hydroperoxide content were also increased in the embryos exposed to this transformation product. However, the integrated biomarker response (IBR) analysis carried out in this study demonstrated that oxidative damage biomarkers got more influence over the embryos than antioxidant enzymes. Thus, we can conclude that guanylurea induces oxidative stress in zebrafish embryos, and that this transformation product impair the normal development of this freshwater organism.

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1. Introduction

Guanylurea (GUA) is the main transformation product (TP) of the antidiabetic drug metformin (MET) (Tisler and Zwiener, 2018). GUA is formed in wastewater treatment plants (WWTPs) by the aerobic degradation of its parent compound (Trautwein and Kümmerer, 2011; Scheurer et al., 2012; Tisler and Zwiener, 2019). Unfortunately, once GUA is formed, the current WWTPs technologies are not capable to completely remove it from wastewater (Scheurer et al., 2012; Briones et al., 2018), which leads to its release to recipient waters.

As a result of the biodegradation process that MET suffers in WWTPs, recent studies have found higher concentrations of GUA in effluents than its parent compound. Tisler and Zwiener (2019), for instance reported that GUA reached concentrations of up to 810 μg/L in the effluents of a WWTP, compared with 6.5 μg/L of MET. In addition, as higher concentrations of this TP are released to recipient waters compared to MET, several studies have reported higher concentrations of GUA in surface water (1–222 μg/L) in relation to its parent compound (1–33.6 μg/L) (Scheurer et al., 2012; Elliott et al., 2017; Posselt et al., 2018). Since TPs, like GUA, are concomitantly found with their parent compounds in water bodies, and as MET has been widely documented in surface waters (Blair et al., 2015; Huber et al., 2016; Kot-Wasik et al., 2016; Archer et al., 2017;Ali et al., 2017; Guzel et al., 2018; Yao et al., 2018), it is expected GUA might be as ubiquitously distributed as metformin in aquatic environments. Furthermore, as many TP remain metabolically active, GUA may be a potential threat to non-target organisms (Ussery et al., 2021).

Concerning its toxic effects, only four studies that we aware of have evaluated the effects of GUA on aquatic organisms. Markiewicz et al. (2017) for instance, exposed *Daphnia magna* neonates to several concentrations of this TP (5–150 mg/L) for 48 h. At the end of exposure, the number of immobilized or dead organisms was checked. According to their results GUA reached an immobilization EC_{50} of 40 mg/L. Furthermore, as the EC_{50} of immobilization of MET was reported to be 64 mg/L, they pointed out that both, parent compound (MET) and its metabolite (GUA) have comparable toxicities. Similarly, Ussery et al. (2019) exposed embryos of Japanese medaka (*Oryzias latipes*) to five concentrations (1, 3.2, 10, 32 and 100 ng/L) of GUA. After 28 days of exposure, a significant decrease in mean body weight and mean fish length were detected in all GUA exposed fish. Moreover, as they previously described the effects of MET on the growth of Japanese medaka (Ussery et al., 2018), they concluded that GUA induces similar growth effects to those of Japanese medaka exposed to its parent compound. Nonetheless, these effects occurred at three orders of magnitude lower that the effects concentrations for MET. This is noteworthy, as several studies have demonstrated that environmentally relevant concentrations of MET may induce endocrine disruption, intersexuality, oxidative stress and embryotoxicity on different aquatic species (Niemuth et al., 2015; Crago et al., 2016; Lee et al., 2019; Ussery et al., 2018).

In disagreement with the aforementioned studies, Jacob et al. (2019) exposed eggs of brown trout (*Salmo trutta f. fario*) (46 dpf) to four concentrations of GUA (0, 10, 100 and 1000 μg/L) for 91 d. The parameters they evaluated were mortality, length, weight, tissue integrity (liver and kidney), levels of lipid peroxides and behavioral endpoints. However, they did not find significant changes in any of these parameters for all the tested concentrations. Nonetheless, in a most recent study, Ussery et al. (2021) investigated the potential mechanisms by which 1 ng/L of GUA may affect the development of early life stages (ELS) of Japanese medaka. After 28 days of exposure, they found GUA altered many important pathways involved in the general health of fish. Among the pathways affected by GUA, it is included nervous system function and development, cellular metabolism, cellular communication and structure, and detoxification of reactive oxygen species (ROS).

ROS can act as second messengers to regulate signal transduction pathways that control different signaling cascades involved in function, proliferation, differentiation and apoptosis of cells (Pašková et al., 2011). However, when the balance between ROS and antioxidant defense systems is disturbed by the action exogenous agents (Nita and Grzybowski, 2016), oxidative damage to cellular macromolecules may occur, leading to cell death (Rai et al., 2015; Kreuz and Fischle, 2016). Even though apoptosis play an important role in development of embryos, cell death can severely impact formation a growth of cells during the embryonic development by actively proliferating cells or by expanding the area of cells already programmed for apoptosis (Hansen, 2006; Hansen and Harris, 2013).

Taken together this data, and the fact that there is a huge knowledge gap about the toxic effects that GUA may induce in aquatic species, we carried out a study in ELS of zebrafish (*Danio rerio*). Our hypothesis is that GUA 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

N-Guanylurea sulfate salt hydrate (CAS number: 207300-86-5) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of GUA were prepared at a concentration of 10 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 adults, were maintained at the Autonomous University of State of Mexico (Toluca, Mexico) in an open water system (5 zebrafish per liter of water), supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilized tap water (27 °C ± 1 °C, 14 h light:10 h dark). 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. Feeding was performed three times a day with Spirulina flakes (Ocean Nutrition, US) supplemented with brine shrimp (*Artemia* sp. *nauplii*) to promote spawning activity.

2.4. Zebrafish embryo collection

At the night before spawning, twelve males and females adults of zebrafish (2:1 ratio) with size 4–5 cm were chosen, and placed on individual breeding chambers. Spawning was stimulated by the onset of light in the morning. Oocytes were collected, at 1 hour post-fertilization (hpf), rinsed with ultrapure water and bleached according to the methods of Westerfield (2007) and Varga (2011). Fertilized oocytes were classified under a stereoscopic microscope (Zeiss Stemi 305 at 10× of magnification) according to the protocol of Kimmel et al. (1995) and middle blastula stage oocytes (equivalent to 2.5 hpf) were selected. After the selection, middle blastula stage oocytes were kept in an incubator (27 °C ± 1 °C) and left in the ultrapure water until embryos reached sphere stage (4 hpf).

Table 1
Water quality parameters.

Parameter	Value ± SD
pH	7.30 ± 0.13
Hardness	91 ± 3 mg/L $CaCO_3$
Un-ionized ammonia	0.008 ± 0.004 mg/L
Alkalinity	91 ± 3 mg/L $CaCO_3$
Nitrate (NO^{3-})	2.9 ± 0.3 mg/L
Nitrite (NO^{2-})	0.030 ± 0.009 mg/L
Dissolved oxygen	10.1 ± 0.5 mg/L
Conductivity	370 ± 30 μS/cm
Salinity	0.8 ± 0.2 g/L

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 GUA (25, 50, 100, 200, 250, 25,000, 50,000, 75,000 μg/L). Three 24-well plates were used per test solution of GUA. Furthermore, experiment was carried out per triplicate, in three independent experiments, to assess the reproducibility of results. Reproducibility of results was assessed calculating the precision of them. Precision was calculated using the following formula: C.V. $= \left(\frac{\sigma}{\overline{X}}\right) \times 100\%$. The concentrations used on this experiment span concentrations that have been measured in the environment (0.004 μg/L–222 μg/L) (Elizalde-Velázquez and Gómez-Oliván, 2020) and extend to higher concentrations that have been found to induce toxic effects similar to other studies (5 mg/L–150 mg/L) (Markiewicz et al., 2017). Plates were maintained at 27 °C ± 1 °C, and under same light/dark periods (14 h:10 h). Solutions were not renewed throughout the exposure. Embryo mortality, hatching rate and malformations rate were assessed using a stereoscopic microscope (Zeiss Stemi 305, US) at different moments (12, 24, 48, 72 and 96 hpf) during the course of GUA exposure. Malformation rate was expressed as the percentage of embryos with at least one malformation compared to the control. A graphic with the principal body malformations produced by GUA on embryos of zebrafish was constructed using IBM SPSS Statistics 22 software. 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_{50} of malformations with their 95% confidence intervals ($p < 0.05$). The teratogenic index (TI) of GUA was calculated through the quotient between LC_{50} and EC_{50} of malformations. If the value of TI is >1, substance should be considered teratogenic, but if this is <1, substance should be categorized as embryo-lethal (Weigt et al., 2011).

2.6. Oxidative stress in zebrafish embryos

Nine lots, each with approximately 1800 zebrafish at the sphere stage (4 hpf), were distributed to aquariums of 4 L capacity. Each lot was assigned one of the eight GUA treatment concentrations with one lot assigned as the control treatment. All along the exposure period, temperature (27 °C ± 1 °C) and light/dark cycles (14 h:10 h) were kept constant in all lots. At each endpoint time (72 hpf and 96 hpf), a mean of 800 embryos (SD: ±50 embryos) were randomly selected and homogenized in 1 mL of phosphate buffer solution (PBS, pH 7.4) using a rotor-stator homogenizer (IKA T 10 basic ULTRA-TURRAX, US). These endpoint times were selected because at that time the zebrafish embryo had hatched and their enzymatic system was functioning. Randomization was performed as follows. Embryos from each lot were divided into 10 groups (a mean of 100 embryos per group, SD ± 10 embryos), 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. Samples were separated in two Eppendorf tubes. Tube 1 contained 300 μL from the homogenate and 300 μL of a solution of trichloroacetic acid (TCA, 20%). Tube 2 contained 700 μL from the homogenate. All tubes were maintained at −20 °C until they were use. Tube 1 was centrifugated at 11495 rpm for 15 min at 4 °C (Centurion Scientific K241R, UK) and the precipitate was used to ascertain the protein carbonyl content (POx), while the supernatant was used to determine the degree of lipid peroxidation (LPX) and the hydroperoxide content (HPx). Tube 2 was centrifugated at 12500 rpm for 15 min at 4 °C (Centurion Scientific K241R, UK) and the supernatant was used to determine the activity of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx).

For the quantification of all oxidative stress biomarkers different enzymatic and colorimetric methods were used. The thiobarbituric acid assay for instance, was used to determine the extent of LPX reaction (Buege and Aust, 1978). Briefly, malondialdehyde, formed from the breakdown of polyunsaturated fatty acids during LPX, reacts with thiobarbituric acid to give a red species absorbing at 535 nm. Analogously, the determination of POx was carried out by the method of Levine et al. (1994), which is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydarzine to form a 2,4-dinitrophenylhydrazone. The hydrazone formed was determined spectrophotometrically by its absorbance at 370 nm. In addition, for the quantification of HPx, we used the ferrous oxidation-xylenol assay described by Jiang et al. (1992). This method is based on the oxidation of Fe^{2+} to Fe^{3+}, which in the presence of xylenol orange forms a complex which can be measured at 560 nm.

Concerning antioxidant enzymes, the method of Radi et al. (1991) was performed to evaluate the activity of CAT. This method is based on the formation and detoxification of H_2O_2 by tissue, and the reaction can be measured at 240 nm. 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. The rate of autoxidation of epinephrine and the sensitivity of this autoxidation to inhibition by superoxide dismutase were measured at 480 nm. Finally, GPX was ascertained using the procedure described by Gunzler and Flohe-Clairborne (1985). Briefly, this method evaluate the net reduction of glutathione S-transferase in 1 min at 37 °C and pH 7, and the reaction can be measured at 340 nm.

Oxidative stress results were normalized against total proteins, which were measured with the method of Bradford (1976). All samples were evaluated per triplicate. Furthermore, experiment was carried out three times, in three independent experiments, to assess the reproducibility of results. Reproducibility of results was assessed calculating the precision of them. Precision was calculated using the following formula: C.V. $= \left(\frac{s}{\mu}\right) \times 100\%$.

2.7. Guanylurea determination

For zebrafish embryo toxicity test, 140 μL of water from each of the 72 wells were collected and pooled, for each treatment group. Accordingly, water samples of 10.8 mL were obtained for each test solution of GUA. For oxidative stress experiment, 10 mL of water from each of the nine lots were collected. Once collected, water samples were stored at −20 °C, until their quantification. Water samples, for both experiments, were taken up at 0, 12, 24, 48, 72 and 96 hpf. Thus, 6 water samples (0, 12, 24, 48, 72 and 96 hpf) for each treatment group were quantified, per experiment. In order to confirm exposure, water samples from each of the 3 experiments were measured ($N = 18$). GUA determination in water samples was performed 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 an ion source temperature 550 °C. Analytical grade nitrogen was used as desolvation nebulizer and collision gas. Instrument control, data acquisition and

data processing were performed with Analyst 1.6 software. Separation was performed using an 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. A stock solution containing GUA at a concentration of 10 g/L in ultrapure water was prepared. GUA was quantified using normal standard quantitation as no internal standard was available. A five point calibration curve was established by spiking ultrapure water with GUA at a concentration ranging from 0 μg/L–80,000 μg/L. The accuracy and precision of analytical method were <15% of the nominal concentration. Accuracy of the proposed method was confirmed by control spiking method, which was carried out by spiking ultrapure water with GUA at three different levels 80%, 100% and 120%. Triplicate determination of these 3 levels have been recorded to obtain the %RSD.

2.8. Integrated biomarker response (IBR) index

Our results from oxidative stress biomarkers were applied into the IBR index, previously reported by Sanchez et al. (2013). IBR index is used to identify impacts of environmental stress on organism, and provide an integrated approach for assessing the health status of organisms (Sanchez et al., 2013). In this study, we used this tool for quantitative monitoring of the GUA-induced stress levels of fish. For IBR determination, we obtained the ratio between all biomarker from each treatment group (Xi) and the biomarkers of the control group (Xo). This ratio (Xi/Xo) was then log transformed (Yi) to reduce variance. In order to standardize Yi values, the following formula $Zi = (Yi - \mu) / s$ was used, with the mean (μ) and standard deviation (s) of Yi. Next, the biomarker deviation index (A) was calculated via the difference between Zi and Z0. 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

Oxidative stress biomarkers data was evaluated with a two-way analysis of variance (ANOVA), considering time as factor A and concentration as factor B. Variations between the means were assessed with the Student-Newman-Keuls method. Embryotoxic and teratogenic effects data was evaluated using the Fisher's exact test. Significance was accepted when $p < 0.05$, using IBM SPSS Statistics 22 software. LC_{50} and EC_{50} of malformations were calculated, using the trimmed Spearman-Karber method (US-EPA software ver 1.5).

3. Results

3.1. Mortality test

Comparing with the control group, GUA increased the mortality and malformations rate in a concentration-dependent manner (Fig. 1A–B). The highest mortality and malformations rate were obtained at the concentration of 75,000 μg/L of GUA. Although, some of the concentrations used in this study are not environmentally relevant (25,000–75,000 μg/L), it is noteworthy to say that at environmentally relevant concentrations (25–250 μg/L) the mortality rate got a minimum value of 27.7%. Moreover, this value increased as the concentration did, reaching a maximum value of 73.6%. Concerning malformations rate, at environmentally relevant concentrations (25–250 μg/L), this reached a minimum and a maximum value of 36.1% and 91.2%, respectively.

LC_{50} and EC_{50} of malformations were calculated, getting a value of 38.5 μg/L (1.25 μg/L, 197.23 μg/L) and 18.5 μg/L (0.408 μg/L, 56.247 μg/L), respectively. Furthermore, the teratogenic index of GUA in zebrafish got a value of 2.1. Taking into account the criteria of Weigt et al. (2011), GUA should be categorized as teratogenic.

3.2. Hatching rate

Differences in the hatching rates between the zebrafish embryos exposed to GUA and the control group are shown in Fig. 1C. When compared with the control group, a significant decline in the hatching rate was observed in all exposure groups, at 72 and 96 hpf ($p < 0.001$). Furthermore, this decline in the hatching rate showed to be in a concentration dependent manner, reaching the lowest number of dechorionated embryos at the concentration of 75,000 μg/L. Regarding environmentally relevant concentrations (25–250 μg/L), GUA also showed a significant decline in the ratio of hatched embryos ($p < 0.001$). At 72 hpf for instance, these range of concentrations delayed the hatching process in the embryos between a 43.1%–56.7% compared with the control group. Meanwhile at 96 hpf, a percentage between 5.7%–16% of the embryos had not hatched in relation to the control group.

3.3. Teratogenic effects induced by GUA

Throughout the exposure, development of the embryos in the control group was normal. Contrarily, GUA caused several body malformations in all treatment groups and exposure time (Fig. 2A). 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. 2B). Furthermore, it is noteworthy that the sternness of malformations was more evident as concentration increase. For example, as the concentration increased the incidence of embryos with pericardial edema, yolk deformation, fin absent, eye absent and craniofacial malformation also increased. The most severe malformations were observed for the embryos exposed to 75,000 μg/L. Nonetheless, at environmentally relevant concentrations (25–250 μg/L), GUA also induced malformations that may affect the integrity of the fish and may lead to their death. These malformations included malformation of tail, scoliosis, pericardial edema and yolk deformation.

3.4. Antioxidant activity induced by GUA

The antioxidant activity of SOD, CAT and GPx in zebrafish embryos exposed to GUA is depicted in Fig. 3. From this picture, it can be seen that the antioxidant activity of all enzymes, in all exposure groups, showed a significant increase compared with control group. Furthermore, our statistical analysis indicated that the antioxidant activity of SOD ($F(8,144) = 636.926$; $n = 9$; $p < 0.001$), CAT ($F(8,144) = 2.138$; $n = 9$; $p = 0.036$) and GPX ($F(8,144) = 87.838$; $n = 9$; $p < 0.001$) was significantly different between the exposure groups. Regarding exposure time, no significant differences were found in the activity of CAT between the 72 hpf and 96 hpf. Nonetheless, compared to 72 hpf, the enzymatic activity of SOD and GPX showed an important increase at 96 hpf, in all the treatment groups. The antioxidant activity of the enzymes increased in a concentration dependent manner, reaching the maximum antioxidant activity at the concentration of 75,000 μg/L.

3.5. Oxidative damage induced by GUA

The levels of LPX, HPx and POx in zebrafish embryos exposed to GUA are shown in Fig. 4. 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. Moreover, according to our statistical analysis, the levels of LPX ($F(8,144) = 130.361$; $n = 9$; $p < 0.001$), HPx ($F(8,144) = 69.817$; $n = 9$; $p < 0.001$) and POx ($F(8,144) = 19.297$; $n = 9$; $p < 0.001$) were significantly different between the concentrations tested. Concerning differences between times of exposure, levels of LPX increased in time dependent manner, showing a significant increase between 72 and 96 hpf. However, compared to 72 hpf, no significant differences were found in the levels of

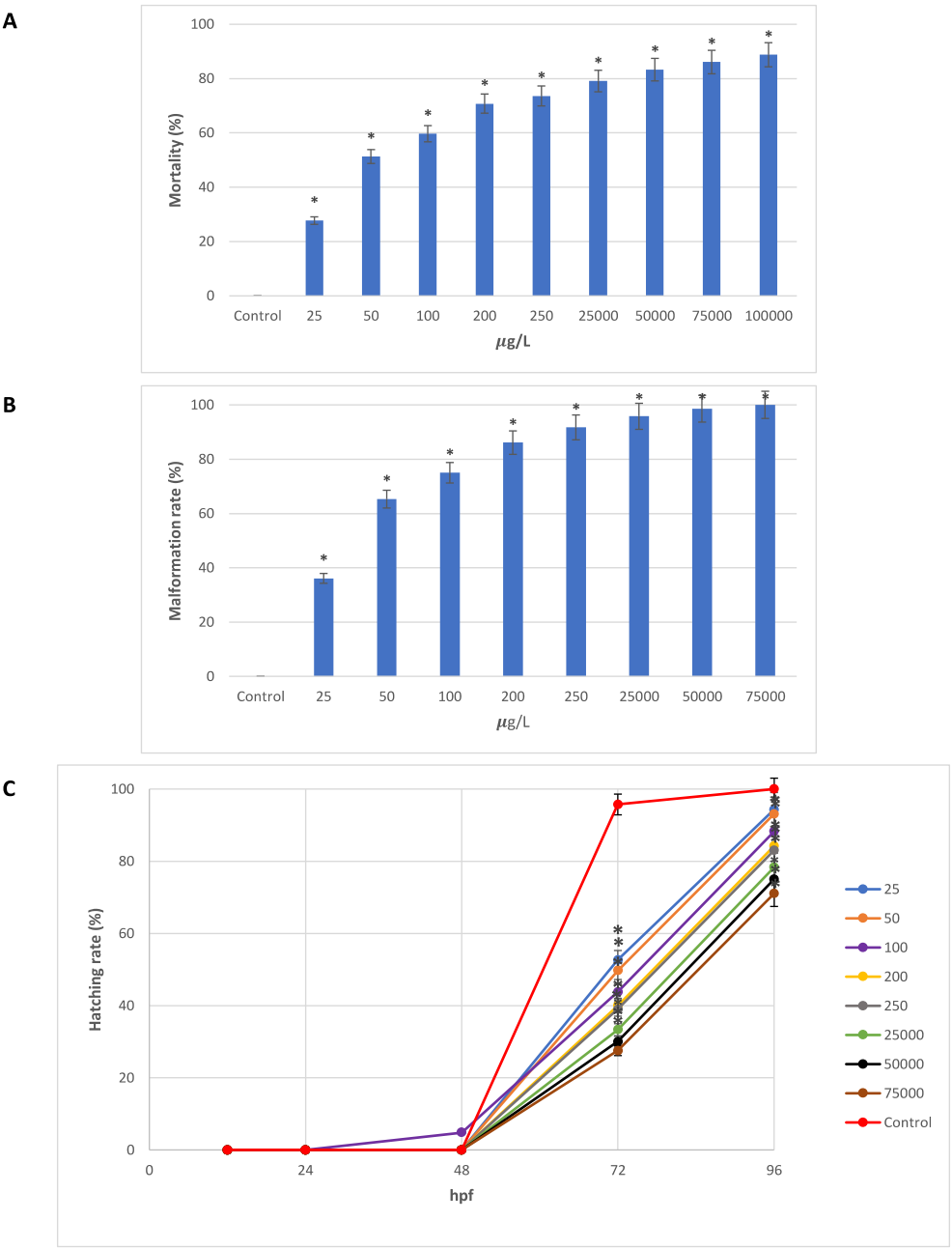

Fig. 1. Mortality, malformations and hatching rate in *Danio rerio* embryos exposed to GUA (µg/L). A represents the cumulative mortality rate of zebrafish embryos exposed to each GUA concentration at 96 hpf. B represents the cumulative malformations rate of zebrafish embryos exposed to each GUA concentration at 96 hpf. C represents the cumulative hatching rate of zebrafish embryos exposed to each GUA concentration at 12, 24, 48, 72 and 96 hpf. Data are expressed as means ± standard deviation (SD) from three independent experiments. * indicates a significant difference in relation to the control group $p < 0.05$. hpf: hours post fertilization.

HPx and POx at 96 hpf. The levels of all oxidative damage biomarkers increased in a concentration dependent manner, reaching the highest peak at a dose of 75,000 µg/L of MET.

Overall, results from fish embryo toxicity tests, as well as from oxidative stress test got a C.V. < 10%.

3.6. Guanylurea determination

For both test, GUA concentrations in water samples was in accordance with the nominal GUA concentrations (Table 2). As we can see in this table, concentrations of GUA in both experiments decreased in time dependent manner. Furthermore, concentrations of this TP in the control group remained below of the quantification limit. Since the concentration of GUA was maintained above 80% for all samples, analyses of the results were based on nominal values. In both experiments, the final concentration of GUA (96 hpf) was similar, showing comparable trends of uptake and/or degradation.

3.7. IBR

IBR values increased as GUA in a concentration dependent manner, indicating that high concentrations of this drug induced more oxidative stress effects in the embryos (Fig. 5). 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 increased, the HPx and LPx biomarkers got more influence over the embryo. Regarding exposure time, none concentration

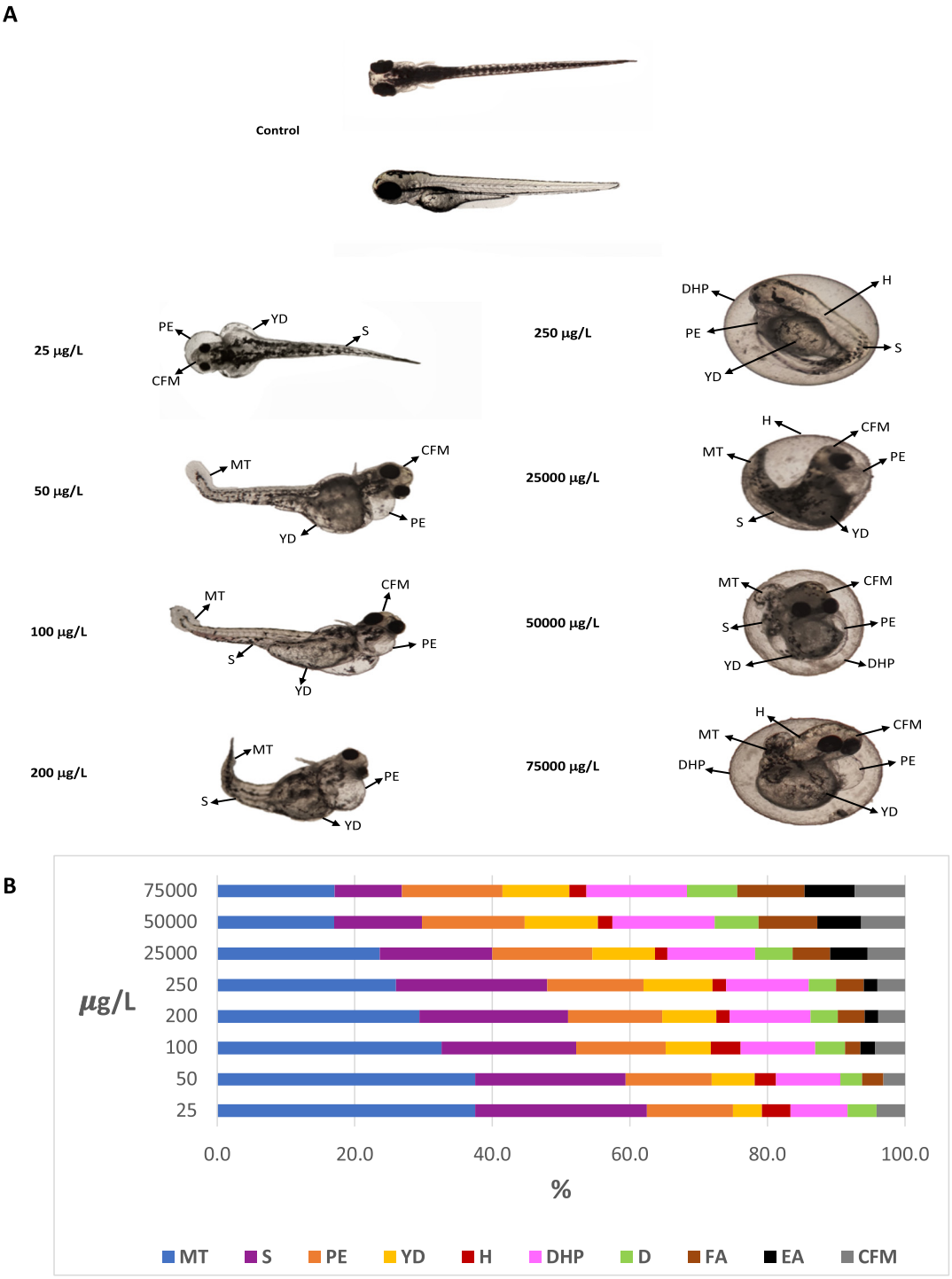

Fig. 2. Main malformations induced by each concentration of GUA in *Danio rerio* embryos. A Representative photos of morphological abnormalities in zebrafish embryos exposed to GUA at 96 hpf. Arrows indicate all malformations found on each embryo. B Cumulative incidence of each malformation found in zebrafish embryos exposed to GUA, expressed in percentage. Data are expressed as means ± standard deviation (SD) 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.

showed a significant increase in the mean IBR values compared to 72 hpf.

4. Discussion

The present study aimed to evaluate the toxic effects of GUA on the embryonic development of zebrafish. Our findings demonstrated that environmentally relevant concentrations of this TP (25, 50, 100, 200 and 250 µg/L) may increase the mortality and malformation rate in the embryos of this fish. In disagreement with these results, Jacob et al. (2019) established that 10, 100, and 1000 µg/L of GUA did not induce any lethal effects on larvae and juvenile of brown trout. One possible factor that may explain the differences between studies could be the dissimilar stages of embryonic development at which organisms where exposed. In our study for instance, zebrafish embryos were exposed to GUA at the sphere stage (4 hpf), while brown trout embryos were exposed to this TP at the eyed stage (48 dpf). This is noteworthy, because Braunbeck et al. (2005) and Yang et al. (2020) have pointed out that as

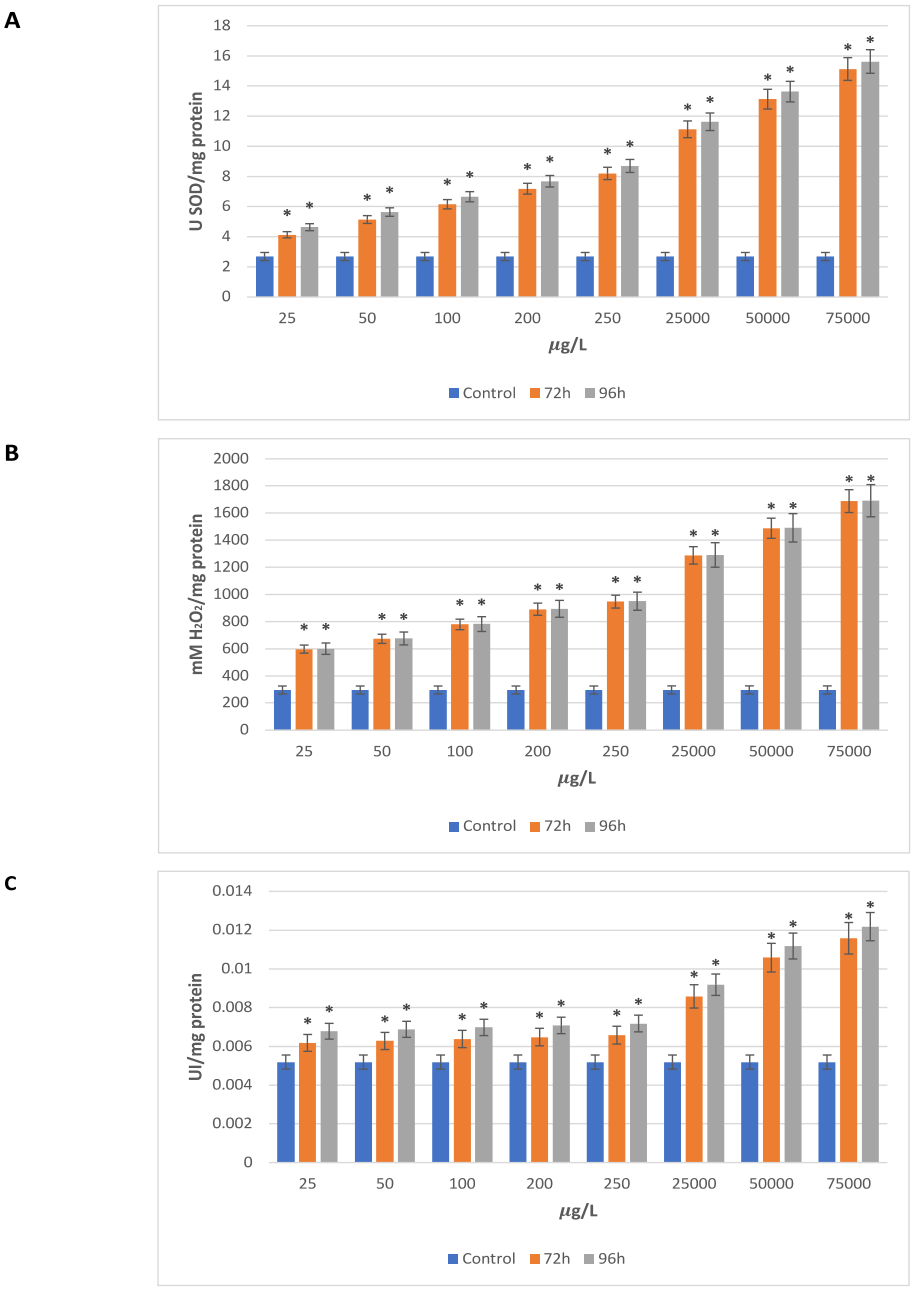

Fig. 3. Activity of SOD A, CAT B and GPx C, in *Danio rerio* embryos exposed to GUA at 72 hpf and 96 hpf. Statistics are expressed as the mean of three replicates ± standard deviation (SD). * indicates a significant difference in relation to the control group $p < 0.05$.

time passes the structure of chorion change and the toughness of the embryo membrane begins to raise, leading to a low permeability. This impermeability was evident in the study of Jacob et al. (2019), who indicated that GUA was only measurable in brown trout larvae exposed to the highest concentration of this TP (1000 µg/L). Nonetheless, larvae only reached a maximum internal concentration of 10.65 µg/L. In general, the chemical properties and structure of GUA are similar to those of its parent compound, MET (Scheurer et al., 2012), suggesting a comparable environmental behavior and parallel toxic responses (Escher and Fenner, 2011). Markiewicz et al. (2017) for instance, exposed several *D. magna* neonates to five concentrations of this TP (5–150 mg/L) for 48 h. At the end of exposure, they counted the number of immobilized or dead organisms. According to their results, GUA reached an EC_{50} of immobilization of 40 mg/L. Furthermore, as EC_{50} of immobilization of MET was reported to be 64 mg/L (Cleuvers, 2003), they pointed out that both, parent compound (MET) and its metabolite (GUA) have comparable toxicities. Similarly, Ussery et al. (2019) exposed embryos of Japanese medaka to five concentrations (1, 3.2, 10, 32 and 100 ng/L) of GUA. After 28 days of exposure, the mean body weight and the mean fish length were significantly decreased in all GUA exposed fish. Moreover, as they previously described the effects of MET on the growth of Japanese medaka (Ussery et al., 2018), they concluded that GUA induces similar growth effects to those of Japanese medaka exposed to its parent compound. This is important to remark, as Ussery et al. (2018) demonstrated that Japanese medaka embryos exposed to 10 µg/L of ^{14}C-MET prior to hardening (<6 hpf) took up more ^{14}C-MET from the medium relative to embryos exposed to this compound post-chorion hardening (24 hpf). Therefore, the chorion plays an important role in the uptake of MET and possibly also in the uptake of GUA.

Hatching rate of the embryos was also affected by GUA. According to our results, this TP retarded the hatching process on zebrafish embryos.

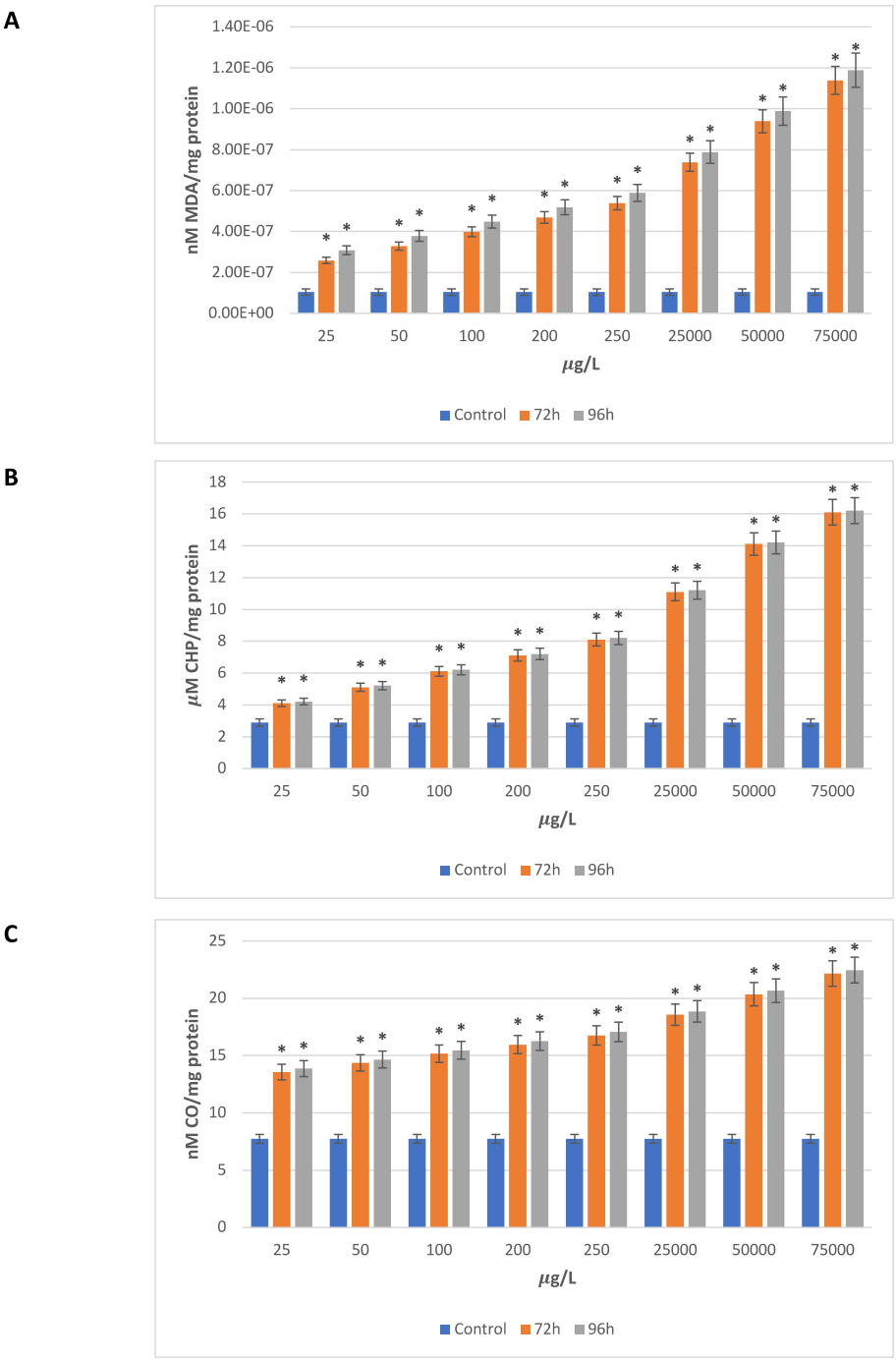

Fig. 4. Levels LPX A, HPx B and POx C, in *Danio rerio* embryos exposed to GUA at 72 hpf and 96 hpf. Statistics are expressed as the mean of three replicates ± standard deviation (SD). * indicates a significant difference in relation to the control group $p < 0.05$.

Nonetheless, these results are not in agreement with those found by Jacob et al. (2019) and Ussery et al. (2019), who demonstrated that none of the concentrations they tested altered the hatching time on brown trout embryos and on ELS of Japanese medaka, respectively. On the other hand, regarding its parent compound, Flores et al. (2020) reported that several concentrations of MET (10 µg/L–2000 mg/L) altered the hatching process on zebrafish embryos in a concentration dependent manner. Alterations in hatching process can be induced by several factors, including toxic agents, oxygen availability, central nervous system chemical modulators and hormonal levels (De la Paz et al., 2017). Since MET has been tagged as an endocrine disruptor (Niemuth and Klaper, 2015; Lee, 2017; Monshi, 2017; Niemuth and Klaper, 2018; Lee et al., 2019) it could be suggested that the hormonal disturbances on fish might be involved in the alterations of the hatching process. Furthermore, as MET and GUA have shown similar effects before, this could be the mechanism by which both compounds alter the hatching process. Nonetheless, more studies are needed, first to investigate whether this TP may disrupt the hormone levels, and second if this disruption is related to the hatching process.

All along the exposure period, GUA (25 µg/L–75,000 µg/L) 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. Once again, our results are in disagreement with those

Table 2
Measured concentrations of GUA during zebrafish embryo toxicity test and oxidative stress test.

Nominal concentrations of GUA	Measured GUA concentrations at different exposure times (μg/L)					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Zebrafish embryo toxicity test						
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
25 μg/L	25.03 ± 0.42	23.87 ± 0.39	23.02 ± 0.43	23.02 ± 0.43	22.76 ± 0.47	22.25 ± 0.49
50 μg/L	50.09 ± 0.62	49.03 ± 0.60	48.21 ± 0.57	47.55 ± 0.61	46.91 ± 0.67	46.07 ± 0.65
100 μg/L	100.01 ± 0.82	97.19 ± 0.74	96.43 ± 0.78	95.31 ± 0.83	94.87 ± 0.79	94.18 ± 0.85
200 μg/L	200.06 ± 0.92	196.45 ± 1.03	194.32 ± 0.99	193.74 ± 0.94	192.45 ± 1.01	191.05 ± 0.94
250 μg/L	250.04 ± 0.94	244.78 ± 0.98	242.04 ± 1.10	241.33 ± 0.99	240.83 ± 0.97	239.45 ± 1.08
25,000 μg/L	25,000.88 ± 1.52	24,615.90 ± 1.31	24,021.03 ± 1.48	23,821.31 ± 1.53	23,104.21 ± 1.87	22,949.88 ± 1.83
50,000 μg/L	50,000.53 ± 2.14	49,431.21 ± 2.49	48,945.73 ± 2.21	48,145.73 ± 2.54	47,841.54 ± 2.38	47,489.95 ± 2.27
75,000 μg/L	75,000.64 ± 3.47	74,234.79 ± 3.12	73,834.21 ± 3.56	73,434.21 ± 3.51	73,178.32 ± 3.23	72,947.85 ± 3.81
Oxidative stress test						
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
25 μg/L	24.98 ± 0.12	23.77 ± 0.15	23.03 ± 0.12	22.97 ± 0.11	22.57 ± 0.15	22.03 ± 0.16
50 μg/L	50.01 ± 0.17	48.98 ± 0.20	48.13 ± 0.19	47.34 ± 0.16	46.95 ± 0.18	46.45 ± 0.20
100 μg/L	99.95 ± 0.24	98.05 ± 0.22	97.15 ± 0.26	96.33 ± 0.29	95.40 ± 0.29	94.24 ± 0.31
200 μg/L	199.89 ± 0.38	197.09 ± 0.40	195.92 ± 0.47	194.42 ± 0.43	193.64 ± 0.39	192.43 ± 0.42
250 μg/L	249.94 ± 0.40	245.11 ± 0.43	244.23 ± 0.46	242.91 ± 0.40	241.47 ± 0.45	240.67 ± 0.46
25,000 μg/L	25,000.52 ± 0.91	24,100.32 ± 0.98	23,870.89 ± 0.95	23,370.89 ± 0.95	22,751.43 ± 0.94	22,857.87 ± 0.93
50,000 μg/L	50,000.32 ± 1.02	49,534.78 ± 1.11	48,712.56 ± 1.08	48,025.63 ± 1.03	47,971.74 ± 1.05	47,376.34 ± 1.10
75,000 μg/L	75,000.19 ± 1.43	74,373.35 ± 1.39	73,729.67 ± 1.47	73,229.19 ± 1.41	73,022.94 ± 1.38	72,839.73 ± 1.49

Values represent mean (N = 18) ± standard deviation (SD) of each concentration. LoQ: limit of quantification (500 ng/L). LoD: limit of detection (400 ng/L).

found by Jacob et al. (2019), who indicated that GUA (10 μg/L–1000 μg/L) did not induce any malformations on brown trout embryos. However, in a more recent study, Ussery et al. (2021) demonstrated that 1 ng/L of this TP dysregulated the skeletal muscle ryanodine receptor 1b (Ryr1) and two important polyamides putrescine and o-phosphocolamine in Japanese medaka larvae. This is of paramount importance as O-phosphocolamine is involved in cell signaling pathways that regulate cellular proliferation, differentiation, necrosis and apoptosis, as well as oxidative stress response (Toda et al., 2017; Bridges et al., 2018). Therefore, it is suggested that dysregulation of these signaling pathways may led to developmental abnormalities. Furthermore, concerning MET, Flores et al. (2020) pointed out that this drug may cause microcephaly and decreased tail length in zebrafish embryos exposed to multiple concentrations (10 μg/L–2,000,000 μg/L) of this drug.

Recently, Jacob et al. (2019) reported that LPX levels, in brown trout larvae exposed to GUA (10 μg/L–1000 μg/L), did not significantly change after 91 days of exposure. Moreover, they suggested that LPX levels in fish were not increased due to the enzymatic activity of GPx, SOD or CAT. Here, we demonstrated that after an acute exposure to GUA (25 μg/L–75,000 μg/L) on ELS of zebrafish, this TP may induce an oxidative stress response on the embryos. Even more important our IBR analyses indicated that oxidative damage biomarkers (LPX, HPx, and POx) got more influence over the embryos than antioxidant enzymes (SOD,

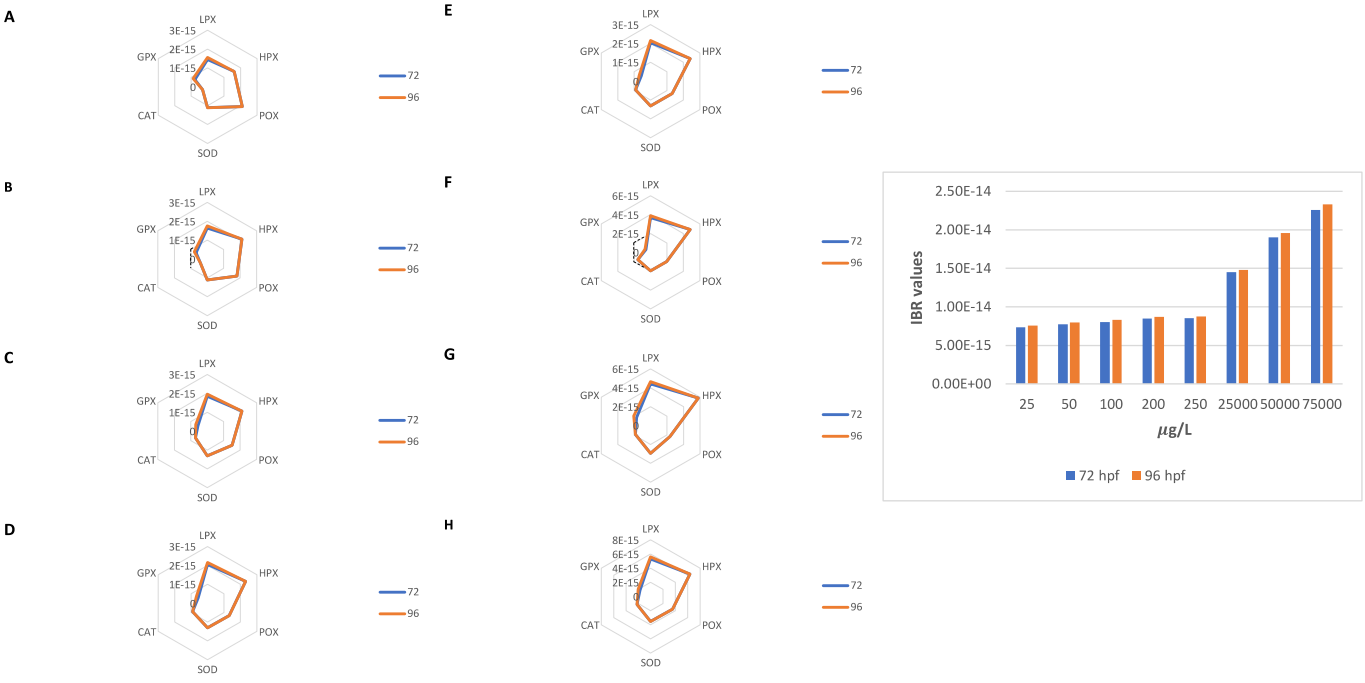

Fig. 5. IBR index values in *Danio rerio* embryos exposed to GUA. Star plots represent the IBR response of all treatment groups (color lines) compared to the control group (dashed line). Area above zero indicates the production of the biomarkers and under zero reflects the reduction of the biomarkers. Letters represent each of the GUA concentrations tested in this study, arranged from the lowest to the highest (A: 25 μg/L; B: 50 μg/L; C: 100 μg/L; D: 200 μg/L; E: 250 μg/L; F: 25000 μg/L; G: 50000 μg/L; H: 75000 μg/L). Bar graphs represent all the IBR index values calculated for each treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CAT, and GPx). In agreement with our results, Ussery et al. (2021) indicated that 1 ng/L of GUA may alter several pathways involved in the general health of Japanese medaka embryos, including detoxification of ROS. Nowadays, it is known that ROS play an important role in the immune system and are implicated in the activation of various cellular signaling pathways (Redza-Dutordoir and Averill-Bates, 2016). Nonetheless, excess cellular levels of ROS cause damage to biomolecules, membranes and organelles, which can lead to activation of cell death processes such as apoptosis (Rai et al., 2015; Kreuz and Fischle, 2016). Even though apoptosis is essential for the development and survival of multicellular organisms, cell death can severely impact formation and growth of cells during the embryonic development (Kupsco and Schlenk, 2015).

During the early organogenesis of fish, embryos have relatively low levels of antioxidant activity, making them more susceptible to chemical agents that act as oxidants (Hansen, 2006). Chemicals can alter the redox state by either increasing ROS production or reducing antioxidant defenses (Goette et al., 2012). In this study, exposure to GUA causes oxidative stress due to increased ROS in zebrafish embryos. Oxidative stress caused by ROS disturbs the embryo homeostasis necessary for normal growth and function, resulting in dysmorphogenesis, teratogenesis, and congenital malformations (Fig. 2) (Impellizzeri et al., 2020). Skeletal alterations (scoliosis, malformation of tail, and craniofacial malformation) observed in this study, for instance, may be related to the presence of ROS that generate LPX. LPX inhibits the osteoblast and osteoclast differentiation through the increased expression of interleukin-6, which contributes to bone resorption, and consequently leading to skeletal alterations (Tseng et al., 2010; Boglione et al., 2013).

Concerning the parent compound, MET has been also associated with an increased production of ROS. Queiroz et al. (2014) for instance, 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. Analogously, Lee et al. (2019) evaluated the toxic effects of MET under a multigenerational exposure regimen, and found that several concentrations of this drug (40 μg/L–360 μg/L) increased the ROS content and decreased the GSH activity in F0 fish. Furthermore, they pointed out that the enzymatic activity of CAT was also significantly increased under MET treatment, suggesting that environmentally relevant concentrations of this pollutant may cause oxidative stress in Japanese medaka.

Mitochondria are an important source of ROS, and as we aforementioned these ROS are important in redox signaling inside cells. However, an imbalance between ROS and antioxidant defense, may contribute to induce oxidative damage to mitochondria, affecting their ability to synthesize ATP and to carry out their metabolic functions (Adam-Vizi and Chinopoulos, 2006). Until now, it is known that MET inhibit the complex I of the mitochondrial electron-transport chain (ETC), leading to different changes in the mitochondrial membrane and ATP production (Andrzejewski et al., 2014; Cameron et al., 2018). Taking into account the aforementioned, Lee et al. (2019) suggested that the inhibition of mitochondrial complex I by MET may disrupt the electron flow, and increase the production of superoxide by a flavin mononucleotide reduction. Although, none study has demonstrated GUA may inhibit the complex I of the mitochondrial ETC, recent studies have demonstrated that GUA exerts effects on ELS of fish in a similar manner than MET (Ussery et al., 2019; Ussery et al., 2021). Therefore, we suggest the increased production of ROS found in this study may be a consequence of the inhibition of complex I of the mitochondrial ETC. Nonetheless, more studies are needed to better understand the mechanism by which this transformation product increases the production of ROS in fish.

5. Conclusion

Zebrafish embryos exposed to GUA (25–75,000 μg/L) suffered a delay in the hatching process, making them to be more susceptible to other threats present in the environment such as predators and/or other toxic agents. GUA also disrupted the normal development of the embryos, producing them several body malformations that led them to their dead. The main malformations induced by this TP were malformation of tail, scoliosis, pericardial edema, craniofacial malformation and yolk deformation. Since in our study GUA impaired the equilibrium between ROS and antioxidant enzymes in zebrafish embryos, we suggest that the teratogenic effects that this TP induced over the embryos may be induced through an oxidative stress mechanism. Concerning the mechanism by which GUA increases the production of ROS, more studies are needed to elucidated whether this process is related to the inhibition of mitochondrial complex I and/or if other mechanisms are involved. Moreover, as currently there is a huge knowledge gap about the toxicity of this TP, more studies are needed to better understand the environmental impact of this drug. To our knowledge, this is the first study that provides evidence of oxidative damage in embryos of fish exposed to environmentally relevant concentrations of GUA (25, 50, 100, 200 and 250 μg/L).

CRediT authorship contribution statement

Gustavo Elizalde-Velázquez 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 Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from María Dolores Hernández-Navarro, 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.

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