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Original Article

Tissue Damage, Mutagenic Effect, and Alteration in Antioxidant Defense in *Danio rerio* (*Cypriniformes: Cyprinidae*) after Chronic Exposure to Metformin Hydrochloride.

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Abstract

Basic sanitation systems are not fully effective in removing all the contaminants, promoting contamination to rivers and supply reservoirs. The Metformin hydrochloride (MTF) is one of the most pharmaceutical contaminant found in rivers. The aim of this study was to test the potential effects of MTF sublethal concentrations on the antioxidant system, and mutagenic and tissue damages in *Danio rerio*. The animals were acclimatized and separated into six groups and exposed to different concentrations of MTF (0, 250, 500, 750, 1250 mg L^{-1}) over 96 hours to determine the Lethal Concentration of 50% of the population (LC50). In another experiment, five groups of ten animals were separated: four groups for evaluation of the chronic effect of 450 g L^{-1} of MTF (15, 30, 45 and 60 days) and a negative control group (NC). The antioxidant system (SOD, CAT, GST, LPO) and the tissue damage (AST, ALT, CK, CK-MB) were analyzed in the muscle samples, and the mutagenicity assessment (MN and nucleus abnormalities) were performed in the blood samples. Univariate statistical analyzes were performed, as well as integrative analyzes of the Antioxidant System, Tissue Damage and Mutagenicity domains were performed using Principal Component Analysis. The results showed evidence of oxidative stress with changes in lipid peroxidation, mutagenicity with a significant increase in the frequency of micronucleus, and activation of the antioxidant system up to 30 days of treatment. There were also intense tissue damage and the emergence of apoptotic cells at 60 days. This evidence of the toxic effects promoted by sublethal concentrations of MTF can lead to irreversible metabolic damage which reduces the ability of non-target animals to survive.

Keywords: Environmental contamination; Mutagenicity; Micronucleus; Oxidative stress; Xenobiotics; Zebrafish.

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INTRODUCTION

With the increasing use of synthetic pharmaceuticals, there has been a considerable growth in the worldwide concern with the consequent increase of these compounds in the environment (Quadra *et al*., 2021). Many pharmaceuticals reach surface waters because they are not efficiently removed in wastewater treatment plants (WWTPs) and there are no global legal maximum environmental concentrations for pharmaceutically active compounds (Patel *et al*., 2019; Quadra *et al*., 2021). It is important to understand differences in the effects of drugs according to their administration, individually or in combination, chronic or acute. However, such effects on flora and fauna, and even humans, are not well understood. In addition, studies have shown that, even at low concentrations, drugs can adversely affect organisms that are not the target of their therapeutic actions (Patel *et al*., 2019; Ambrosio-Albuquerque *et al*., 2021).

The main cause of contamination of natural environments is the incorrect disposal of medicines. Only 40% of prescription drugs in the United States of America are taken to treat the condition, and the rest is discarded (Patil *et al*. 2017). Buffington et al. (2019) showed that a large part of expired medicines is discarded directly into the sewer, with the population not having the habit of giving these products the correct destination. Because of this fact, it is noteworthy that in a study carried out in 59 streams in the USA, the antidiabetic drug metformin (MTF) was detected in 89% of the samples and 97% of the sites, being among the ten most commonly found drugs in the environment (Bradley *et al.*, 2016).

Another form of environmental contamination with MTF is elimination through urine and feces. In the urban sewage system, through a bioremediation process, this drug is transformed into guanylurea. Both MTF and guanylurea are stable in water and cannot be remedied by techniques such as UV light irradiation, ozonation and/or activated carbon. Thus, even greater amounts of these two compounds are being found in aquatic environments (Trautwein *et al*., 2014).

MTF was among the ten most common medicaments found in aquatic environments and, it has been detected in global aquatic systems in over 91 countries (Oosterhuis *et al*., 2013; Bradley *et al*., 2016; He *et al*., 2022). It is a biguanide drug usually indicated as the first therapeutic option in the treatment of patients with diabetes mellitus type 2. Brazil, with the sixth-highest number of diabetics in the world, has no monitoring data on metformin concentration in the environment (IDF, 2021). It has been detected in urban wastewater, on the surface of both freshwater and seawater, in drinking water, in hospital effluents, and in solid matrices (Briones *et al.*, 2016; Ambrosio-Albuquerque *et al.*, 2021). This is a result of the excretion of a large, unaltered portion of metformin by the human body (Niemuth *et al*., 2014).

The presence of metformin in the aquatic environment has a negative influence on the survivability and reproduction of different fish species (Niemuth; Klaper, 2015). Fish are bioindicators in ecotoxicology research due to their economic and aquatic ecosystems importance, comprehensive basic knowledge of toxicology, physiology, and histology, ease of culture, and representation of vertebrates in general (Chovanec *et al*., 2003). *Danio rerio*, Zebrafish, is a small tropical fish that has become one of the most popular model organisms in developmental genetics and toxicology (Scholz *et al*., 2008). The Zebrafish has emerged as an excellent model organism for studies of vertebrate biology. External development and optical clarity during embryogenesis allow for visual analyses of early developmental processes, and high fecundity and short generation times facilitate genetic analyses, and furthermore, they undergo a rapid development and provide easy maintenance in the laboratory (Dooley and Zon, 2000). The International Organization for Standardization (ISO) first published the Zebrafish toxicity test in 1984. Thereafter, multiple countries promulgated their own toxicity test standards by using Zebrafish according to ISO 7346-1996 (Dai *et al*., 2013). The Zebrafish is also a suitable model for screening drugs for potential use to treat human diseases based on phylogenetic analysis of fish and human genomes, which show similar morphology and physiology of the nervous, cardiovascular, and digestive systems (Mohammadi *et al*., 2020), and study designs ranging from short-term acute toxicity tests to long-term multigenerational chronic reproduction assays (Padilla and Glaberman, 2020).

Numerous studies also use *D. rerio* to evaluate the toxicological effects of xenobiotics, since they can be a consequence of the imbalance of the antioxidant system and generation of oxidative stress reactions and, consequently, disruption of cellular homeostasis (Samet and Wages, 2018). Oxidized molecules from oxidative stress can promote changes in protein metabolism (Scherz-Shouval and Elazar, 2007) and increase DNA damage (Czarny *et al*., 2018), leading to genomic instability, which facilitates mutations and carcinogenic processes (Fayh *et al*., 2018).

Given the potential of *D. rerio* as a bioindicator in ecotoxicology studies, it is considered that this model can be used for the evaluation of the chronic effects of metformin in non-target organisms. There is still little information about the chronic effects of metformin hydrochloride, and it is important to carry out studies within this scope. The present study aimed to evaluate the potential chronic effects of metformin hydrochloride drug on the antioxidant system, as well as mutagenic effects and tissue damages using *D. rerio* bioindicator evaluations.

METHODOLOGY

This study was approved by the Animal Research Ethics Committee (CEUA – n° 53-17) of the Universidade Estadual do Oeste do Paraná (UNIOESTE).

Danio rerio

Danio rerio (n=120) was obtained from the commercial supply. The fish were maintained in dechlorinated water, pH 7.89, at 24 ± 0.5 °C, with constant aeration and under a 12:12 h light-dark photoperiod. The organisms were fed with commercial fish food with 45% crude protein, twice a day, receiving 5 % of the total aquarium biomass.

The evaluations of water quality were performed twice a week, evaluating the pH and the dissolved oxygen ($mg.mL^{-1}$) using a pH-meter and an oximeter, respectively. Ammonia and nitrite concentrations were evaluated using specific kits (ALFAKIT®) adapted for microplates and readings by spectrophotometer at 620 nm and 490 nm wavelengths, respectively.

Experimental design

To verify the LC50 (50% lethal concentration) 60 animals were exposed, respecting the density of 1 g of $fish.L^{-1}$ of water. The following concentrations of metformin hydrochloride (CAS 1115-70-4, content 99.58%) were administered to the aquariums for 96 h: A – control (only dechlorinated water); B - 250 mg L^{-1}; C - 500 mg L^{-1}; D - 750 mg L^{-1}; E - 1250 mg L^{-1}. Mortality rates per treatment were recorded daily, and the LC50 was estimated nonlinearly by probit regression using the Quasi-Newton estimation method. The prediction of LC50 was defined by multiple regression analysis, based on predicted probabilities (U.S. EPA, 2011).

After the LC50 definition, other animal groups (n=60) were exposed to a chronic effect evaluation experiment. These animals were stored in four 10 L aquariums (n=10 per aquarium) after the initial measurement of total and standard lengths and weights. In the aquariums, the sublethal concentration of 450 mg L^{-1} metformin hydrochloride was administered and subdivided according to the time of drug exposure: T15: exposure for 15 days; - T30: exposure for 30 days; - T45: exposure for 45 days; - T60: exposure for 60 days; Control group: kept in dechlorinated water for 60 days.

Total aquarium water was changed weekly in all containers, including control, and a new sublethal concentration of 450 g L^{-1} metformin hydrochloride was administered to each treatment's aquarium, characterized by pressure experiments (Gotelli and Ellison, 2011). According to Tincani et al. (2017), ecotoxicology experiments that evaluate biomarkers and that do not show true replication (i.e., several animals per aquarium) show equivalent results and with the same type I and type II error values as an experiment with replication (i.e., a animal per aquarium).

After the experimental periods described above, the animals were anaesthetized in water containing 30 mg of eugenol per liter, according to the methodology suggested by Honczaryk and Inoue (2009). The total and standard length and weight of the animals were measured. The blood was collected for subsequent micronucleus test (MN) through the caudal fin section. The tail muscle was also removed with the aid of a scalpel for the analysis of the antioxidant system.

Laboratory Tests

Sample Preparation

The muscle of each animal was homogenized for 5 minutes in 1 mL of Tris-HCl Buffer pH 7.4 and centrifuged at 13,680 G at 4 °C for 10 minutes. Protein quantitation of the samples was determined by the Bradford method using bovine serum albumine as standard (Bradford, 1976). The samples were analyzed by microplate spectrophotometry using 595 nm wavelength. All samples were subsequently normalized to 1 mg of protein. mL^{-1}.

Antioxidant System Analysis

The dosage of the superoxide dismutase (SOD) enzyme followed the protocol proposed by Crouch *et al.* (1981), whose principle is to quantify tetrazolium blue (NBT). 100 µL of 100 % ethanol was added to 300 µL of the samples and centrifuged at 13,680 G at 4 °C for 10 minutes. In a 96-well microplate, 30 µl of the triplicate sample supernatant was pipetted, to the blank 30 µl of 25 % ethanol in 200 mM Tris-HCl buffer and 100 µL of the hydroxylamine solution were pipetted. Absorbance measurements were performed at 560 nm for 90 minutes at 1-minute intervals. SOD unit expression was given to enzyme activity capable of inhibiting by 50 % the oxidation of $NBT.mg\ of\ protein^{-1}$.

The catalase (CAT) enzyme dosing process was carried out according to the protocol proposed by Aebi (1984), which has as its principle the dismutation of peroxide, whose molar extinction coefficient is 40 $M^{-1}.cm^{-1}$. 3 µL of the samples were pipetted into microplates for reading in ultraviolet spectrophotometry and were added to 297 µL of the reaction system (2.5 mL 1.0 M Tris-HCl Buffer, 5.0 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 47.32 ml of deionized water and 70 µL Hidrogen Peroxide (H_2O_2)). Subsequently, the absorbance was measured at 240 nm for 2 minutes at 10-second intervals. The unit was expressed in mmol of degraded $H_2O_2.min^{-1}.mg\ of\ protein^{-1}$.

The principle of Glutathione S-Transferase (GST) activity analysis is that the enzyme catalyzes the conjugation of reduced glutathione (GSH) with the synthetic substrate 1-Chloro-2,4-dinitrobenzene (CDNB) (Habig, *et al.*, 1974). 20 µL of the normalized/blank samples were pipetted in triplicate, and 20 µL of potassium phosphate buffer and 180 µL of the reaction system (0.94 mM CDNB/GSH) were added at room temperature (25 °C). Absorbance measurements were performed at a wavelength of 340 nm for 5 minutes at 30-second intervals. The unit was expressed in nmol of $formed\ thioether.min^{-1}.mg\ of\ protein^{-1}$.

In the indirect quantification of lipid peroxides, the protocol followed was that of Buege and Aust (1978). For sample preparation, a medium containing 0.33 mg mL^{-1} rate of protein was added to a medium containing 6.7 % trichloroacetic acid (TCA) with a final volume of 180 µL and vortexed. The material was then placed in an ice bath for 5 minutes and centrifuged for 5 minutes at 13,680 G at 4 °C. 40 µL of the supernatant and standard Malonaldehyde (MDA) concentrations were pipetted in triplicate into a reaction medium containing 21.42 mM of 2-Thiobarbituric acid (TBA), 17.86 mM of NaOH, 0.73 M of TCA, 0.032 mM of 3,5-Di-tert-4-butylhydroxytoluene (BHT), 3 % ethanol in Phosphate buffered saline (PBS). The reaction was read at 22 °C, after 60 minutes of incubation at 60 °C, at an absorbance of 535 nm. Lipid peroxidation (LPO) was estimated from standard MDA concentrations and results were expressed as nmol of MDA. mg of $protein^{-1}$.

Tissue damages

In tissue homogenates of each animal, the concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and the muscular fraction of creatine kinase (CK-MB) enzymes were measured by commercial kits assays (Bioclin®). The absorbances of ALT and AST were read on a microplate spectrophotometer at 343 nanometers and, CK and CK-MB at 510 nanometers.

Mutagenicity Assessment

For the micronucleus test, we followed the protocol according to the one proposed by Hooftman and Raat (1982) with adaptations. The blood taken from each specimen was dripped onto a slide and smeared, one per animal. The slides were kept dry for 24 h, then fixed in absolute methanol for 10 minutes and an additional 24 h of drying at room temperature. They were then stained for 10 minutes with Giemsa solution diluted with 0.06M (pH 6.8) phosphate buffer in the ratio of 1 mL dye to 20 mL buffer, washed in distilled water and brought to dryness again. One thousand red blood cells per slide were analyzed under a light microscope at 1000x magnification for the micronucleus test (MN) count and to analyze blebbed cells, notched cells, bud, necrosis, binucleated cells, apoptosis, and lobed nucleus, always statistically compared to the negative (low mutagenic potential).

Statistical analysis

The assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene Test) were tested for all variables. The groups were compared by the One-way ANOVA or by the Kruskal Wallis test and, in case of statistical significance ($p<0.05$), the post-hoc test was performed using the Tukey-HSD test or the Dunn test, respectively.

After univariate analysis, principal component analysis of variables related to matrices was performed, including the Antioxidant System, Mutagenic Effects and Tissue Damage. For each matrix, data quality was assessed using the Kaiser-Meyer-Olkin criterion (overall $k>0.5$). The first main component of each analysis was considered as the summary of linear relationships, and therefore, represents a latent variable from each set of observed variables. Latent variables were compared between experimental groups using single factor ANOVA, followed by the Tukey-HSD post-hoc test. All inferential analyses were performed using the R program (R Development Core Team, 2019), assuming a significance level of 0.05.

RESULTS

50% lethal concentration (LC50)

It was found that the control (8%) and groups exposed to 250 mg L^{-1} (0%), 500 mg L^{-1} (8%) did not show statistically significant differences regarding mortality ($p>0.05$), observing differences from the concentration of 750 mg L^{-1} (13%) and 1250 mg L^{-1} (70%) ($p<0.05$).

From the observed data, application of the estimation method and expression of the results in a second-order polynomial model, it was possible to estimate that the lethal concentration of 50% of the individuals (LC50) was 1105 mg L^{-1} [95%CI 986 – 1225 mg L^{-1}] in 96 h. The same LC50 calculation was also performed for 48 and 72 h, with values of 2546 [95%CI 2069 – 3022] and 1260 [95%CI 1094 – 1427] mg L^{-1}, respectively. These data were evaluated by regression analysis, defining a negative exponential model (Fig. 1B). From the present model, it was possible to infer that the LC50 value for seven days (168 h) of exposure is 510 mg L^{-1} of metformin hydrochloride. The calculation of this value was necessary since it would have to be reset weekly in the chronic exposure experiment, and a non-lethal concentration of 450 mg L^{-1} of metformin hydrochloride was defined.

Chronic exposure to metformin hydrochloride

Water quality and biometry of animals

Regarding water quality, it was possible to observe that throughout the experiment there were no significant statistical differences in pH (General mean = 7.89 ± 0.06; $F=0.989$; $p=0.435$) and dissolved oxygen (OD) (General mean = 7.92 ± 0.42; $H=0.534$; $p=0.749$) between treatments, remaining at values considered adequate for the conditioning of *D. rerio*. However, a statistically significant difference was found in the evaluation of ammonia, indicating a significant increase in values at 15, 30, 45 and 60 days of exposure to metformin

hydrochloride when compared to the control (H=12.43; p<0.0001; Tab. 1). On the other hand, for nitrite, no significant results can be observed (H=0.31; p=0.863; Tab. 1), indicating the similarity of the treatments with the control.

When observing the total length and standard length of the animals, it was observed that there was no statistical difference between treatments (p=0.202; p=0.182, respectively). Regarding the weight variable, when comparing the initial and final weight of all animals, a significant weight loss can be noticed (Difference mean=-0.07±0.04 g; V=909.5; w=0.008).

Antioxidant system

A significant increase in SOD activity values (F=6.90; p=0.0003) was observed among animals exposed to metformin hydrochloride for 30 days concerning any other period (Fig. 1A). No statistically significant difference was observed regarding CAT activity when comparing groups (F=1.36; p=0.265; Fig. 1B). Regarding GST, a significant activity reduction was found at 15 and 30 days of drug exposure when compared to the control and a significant increase in 45 days (F=101.05; p<0.0001; Fig. 1C). In this analysis it was observed that the Lipid peroxidation (LPO) reaction remained high in the first three treatment periods (15, 30 and 45 days) (F=2.51; p=0.058; Fig. 1D).

Mutagenic action

There was a significant increase in the frequency of micronucleus (MN; H=16.228; p= 0.002; Tab. 2; Fig. 1E) and the number of necrotic cells (H=20.468; p=0.000; Tab. 2; Fig. 1F) over 15 days (T15) compared to other treatments, these being statistically different from T0. Binucleated cells (H=4.397; p=0.355; Tab. 2; Fig. 1G) and cells with bud (H=5.727; p=0.220; Tab. 2; Fig. 1H) showed no statistically significant differences. At 60 days (T60), an increase in the frequency of cellular apoptosis could be seen compared to the other groups, a statistical difference also could be seen when comparing the periods of 15 (T15) and 30 days (T30), in which these two showed lower levels of apoptosis than other treatments (H=33.913; p < 0.0001; Tab. 2; Fig. 1I). In turn, Blebbed (H=10.3865; p=0.034; Tab. 2) and Lobed Nucleus (H=10.7937; p=0.029; Tab. 2), also exhibited differences in the cellular frequencies, and specifically for Blebbed, T15 and T45 were different statistically (Tab. 2).

Table 1 - Medians, interquartile and interpercentric ranges of water quality variables (mg L^{-1}) in *D. rerio* exposed to 0.45 g L^{-1} of metformin hydrochloride and negative control (NC) for 60 days (T60).

	Md (1°Q - 3°Q)	
Treatments	**Ammonia (mg L^{-1})**	**Nitrite (mg L^{-1})**
NC	0.01 (0.01-0.04)*	0.07 (0.06-0.14)
T15	0.13 (0.11-0.14)	0.16 (0.09-0.21)
T30	0.10 (0.08-0.11)	0.08 (0.06-0.13)
T45	0.09 (0.08-0.11)	0.21 (0.14-0.44)
T60	0.09 (0.08-0.14)	0.11 (0.07-0.20)
p-value	**< 0.0001**	0.863

Table 2 – Medians and interquartile ranges of the variables analyzed in 1000 *D. rerio* erythrocytes exposed to 0.45 g L^{-1} of metformin hydrochloride at different times: negative control (NC); 15 days (T15); 30 days (T30); 45 days (T45); 60 days (T60).

Treatments	NC	T15	T30	T45	T60	p-value
MN	0 (0 - 0)	2 (1 - 2)*	0 (0 - 1)	1 (0 - 1)	0 (0 - 0)	**0.002**
Necrosis	0 (0 - 0)	7 (6 - 8)*	0 (0 - 2)	3 (1 - 7)	0 (0 - 0)	**0.000**
Binucleated	0.0 (0 - 0)	0 (0 - 0)	0 (0 - 0)	0 (0 - 0.25)	0 (0 - 0)	0.354
Bud	0 (0 - 0)	0 (0 - 0.25)	0 (0 - 1)	0 (0 - 0.25)	0 (0 - 0)	0.220
Apoptosis	157 (135 - 169)	13 (1 - 21)*	8 (1 - 10)*	147 (75 - 168)	222 (149 - 290)*	**> 0.0001**
Blebbed	0 (0 - 1)	0 (0 - 1)*	0 (0 - 0)	0 (0.0 - 1)*	1 (0.7 - 1.2)	**0.034**
Lobed Nucleus	0 (0 - 0)	0 (0 - 0)	0 (0 - 1)	0 (0 - 0)	0 (0 - 0)	**0.029**

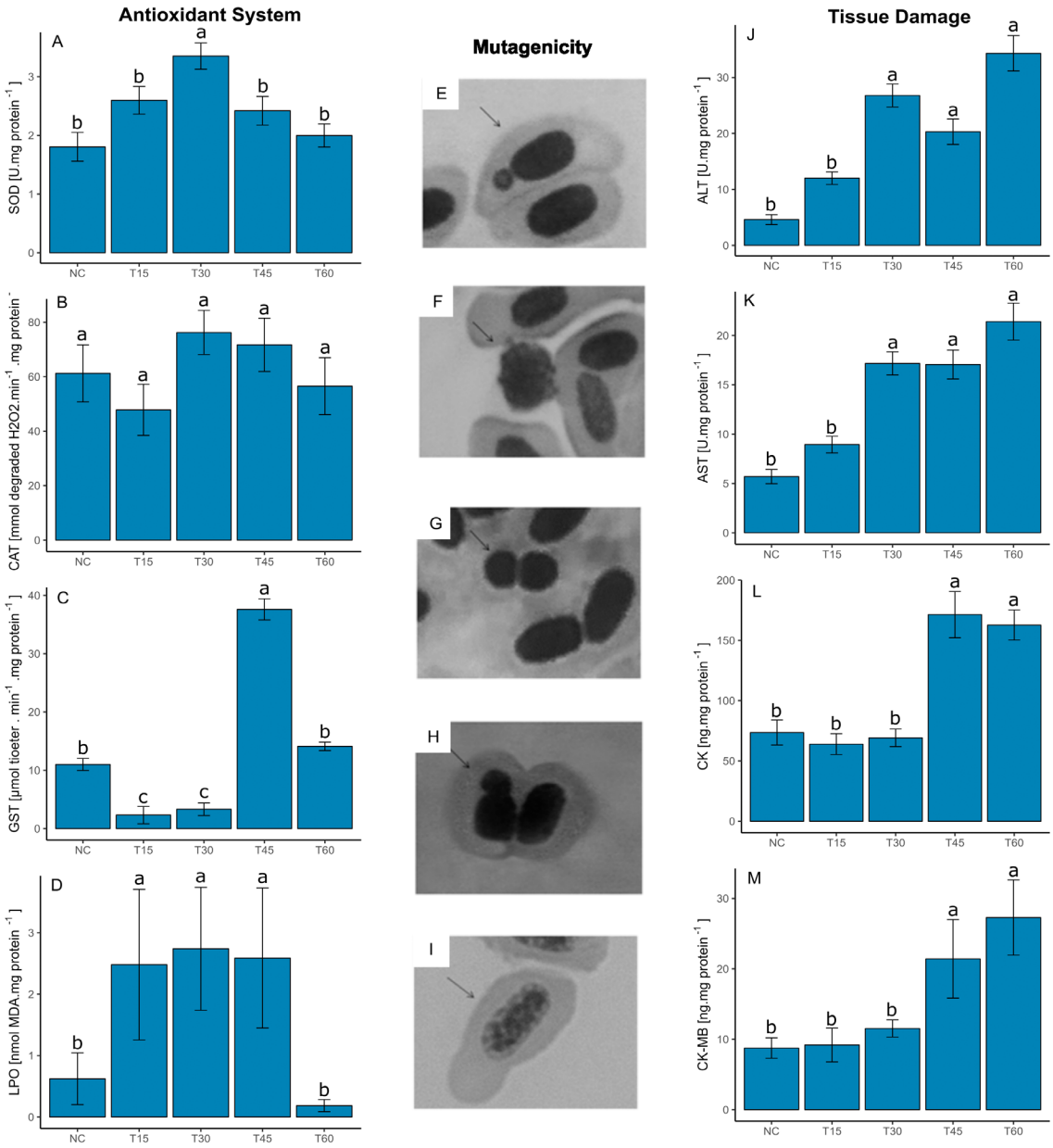

Figure 1 - Means and standard error of the antioxidant system variables and tissue damages, and types of mutagenicity cells analyzed in *D. rerio* muscle, exposed to 0.45 g L^{-1} of metformin hydrochloride at different treatments: 15 days (T15); 30 days (T30); 45 days (T45); 60 days (T60); and the Negative Control (NC). The different letters above the error bars represent statistical differences. A) SOD; B) CAT; C) GST; D) LPO; E) MN; F) Necrosis; G) Binucleated; H) Bud; I) Apoptosis; J) ALT; K) AST; L) CK and M) CK-MB.

Tissue damages

When evaluating the variables that reflect tissue damage, we found that ALT (k=34.901; p<0.0001; Fig. 1J), AST (k=33.062; p<0.0001; Fig. 1K), CK (k=28.656; p<0.0001; Fig. 1L) and CK-MB (k=10.738; p=0.030; Fig. 1M) showed significant differences among groups. It was possible to verify that after 30 days of exposure to metformin hydrochloride there was a significant increase in the values of ALT and AST, and after 45 days there was an increase in CK and CK-MB.

Integrative analysis

Since organisms present their biological systems integrated, analysis and interpretations must be carried out in an integrated manner. Therefore, we carried out evaluations in three systems: Antioxidant System, Mutagenic Effects and Tissue Damage. This evaluation was carried out with the Principal Component Analysis (PCA), evaluating only the first dimension of the analysis, since it is the component that accumulates the greatest variation in linear relationships. When performing the analysis for the variables related to the Antioxidant System, relationships between SOD, LPO and

GST can be observed in the first dimension, the latter being inversely related to the others (Dimension 1 - Antioxidant System; Eigenvalue = 1.52; Variance = 38.04%; Fig. 2A). In this interpretation, the activation of the Antioxidant System, increased oxidative stress (evidenced by increased lipid peroxidation) and reduced detoxification capacity can be verified due to reduction activity of GST, especially in 30 days of exposure to Metformin Hydrochloride (F=9.05; p<0.0001; Fig. 2B).

As for the integrated assessment of variables related to DNA damage, it is possible to see that the first dimension refers to the linear relationships among binucleate cells, bud formation, blebbed and apoptosis, the latter being inversely related to the others (Dimension 1 - Mutagenicity; Eigenvalue = 1.92; Variance = 27.44%; Fig. 2C). We verified that in 15, 30 and 45 days there is an increase in binucleate cells, sprouts and blebbed, while in 60 days there is more occurrence of apoptosis (F=6.309; p=0.0005; Fig. 2D). Finally, regarding Tissue Damage, we found that the first dimension presents strong linear relationships between CK, ALT and AST, all of them directly related (Dimension 1 – Tissue Damage; Eigenvalue = 2.66; Variance = 66.57%; Fig. 2E). It was possible to verify the gradual increase in Tissue Damage, with greater intensity of damage being observed after 60 days of exposure to metformin hydrochloride (F=35.791; p<0.0001; Fig. 2F).

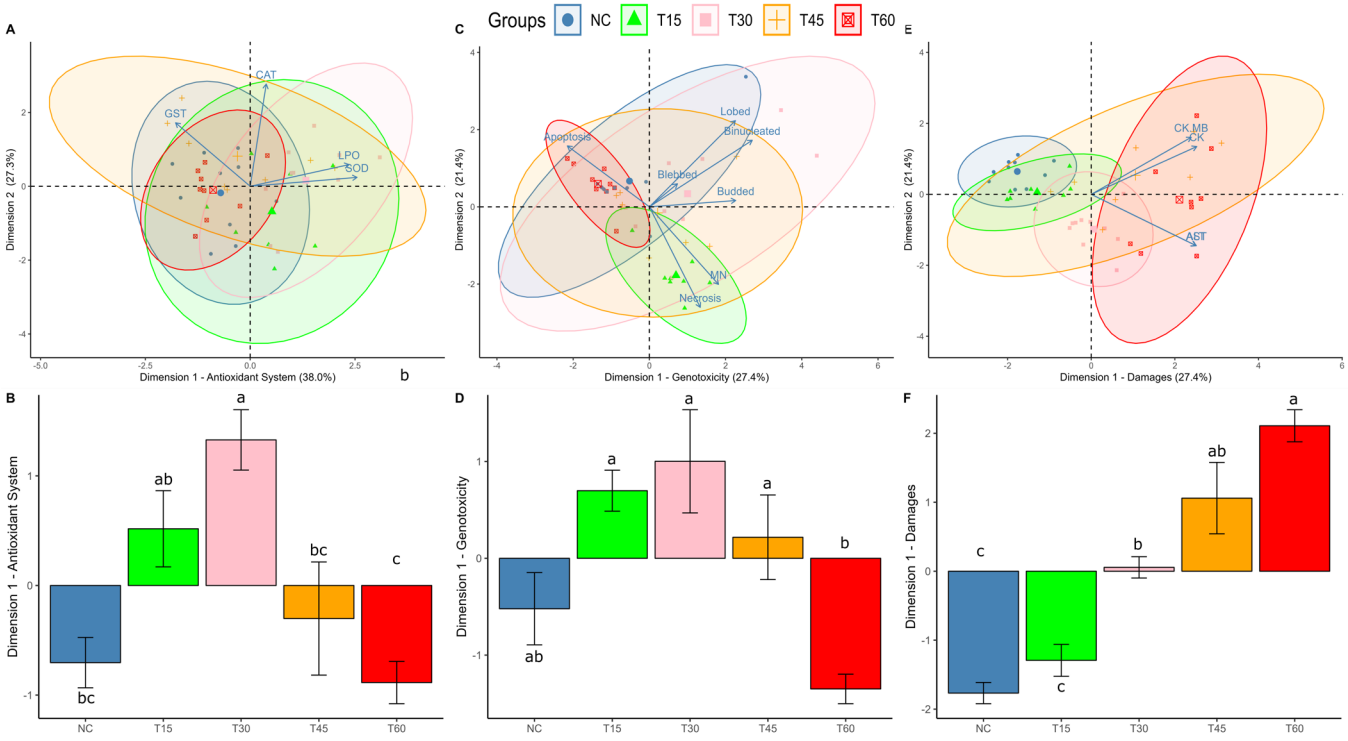

Figure 2 – Ordering diagram of the principal component analysis and average + standard error barplot of the different systems analyzed in *D. rerio* exposed to 450 mg L^{-1} of metformin hydrochloride at different times: negative control (NC - blue); 15 days (T15 - green); 30 days (T30 - pink); 45 days (T45 - orange); 60 days (T60 - red). A and B) Antioxidant System; C and D) DNA Damage; E and F) Tissue damages.

DISCUSSION

In the present study we estimated that the LC50 was 1105 mg L^{-1} [95%CI 986-1225 mg L^{-1}] in 96 h, which is considered similar to the values estimated by Godoy et al. (2018) (LC50 = 1315.5 mg L^{-1} [95%CI 1278.3-1352.7]). From this ecotoxicity data, we defined the sublethal concentration of 450 mg of Metformin hydrochloride L^{-1} (MTF) to perform the chronic evaluations. This sublethal concentration was able to change protein metabolism, evidenced by the significant increase in ammonia excretion throughout the experimental period. The induction of defense response by the Antioxidant System and the DNA damage for up to 30 days was also evidenced. These evidences show how continuous exposure to sublethal concentrations of drugs inappropriately disposed of in the environment can cause damage to animal models.

The increase in ammonia excretion observed in *D. rerio* exposed to sublethal concentrations of MTF may have activated the antioxidant defense system, as already observed in other animal models. Zhang et al. (2020) observed the enhance of SOD activity in digestive gland of *Corbicula fluminea* exposed to 10 mg L^{-1} ammonia. Cheng et al. (2015) indicate that ammonia exposure (1.43 mg L^{-1}) induced oxidative stress in the *Takifugu obscurus*. Both researches also pointed the enhance in apoptosis after ammonia exposure. Therefore, environmental pollution caused by the presence of pharmaceutical contaminants such as MTF can induce cell detoxification in exposed non-target organisms (He *et al.*, 2022), which causes ROS production (Li *et al.*, 2018) and increased activity of antioxidant enzymes as a defense mechanism (Jiao *et al.*, 2020).

The present study also demonstrated that MTF activated the antioxidant system of *D. rerio*. Antioxidant activity changed in a time-dependent manner, as there was an increase in 15 days, followed by an activation peak in 30 days and a gradual inhibition at 45 and 60 days. The induction of an antioxidant response has also been evaluated in *D. rerio* embryos exposed to environmentally relevant concentrations of metformin with increased markers of oxidative damage (Elizalde-Velázquez *et al*., 2021). The authors show that this response may have been caused by an inhibition of the mitochondrial complex I of the electron transport chain and a consequent increase in superoxide production and triggering of apoptosis mechanisms.

Considering that SOD is the first enzyme of the antioxidant system (Jiao *et al*., 2020), we observed an increase in its activity until 30 days of exposure to MTF, considering an active cellular defense. However, this primary system began to be insufficient to defend against the constant production of ROS. The same response was identified in the CAT enzyme in order to eliminate the accumulation of hydrogen peroxide from metabolism reactions. Consequently, the change in the redox balance of cells activated the antioxidant defense of GST, as seen at 45 days of exposure to MTF, with its activation being significantly high, thus representing the second line of cellular defense against oxidative pressure to convert this xenobiotic and fulfil detoxification (Li *et al.*, 2018).

Despite the activation of the antioxidant system up to 30 days of exposure to the xenobiotic and consequent increase of ammonia excretion, the large period of the animals' exposure to MTF promoted an increase in cell damage and apoptosis markers. An important feature found in animals chronically exposed to MTF was the high activity of aminotransferases (ALT and AST), being both markers of changes in the energy metabolism of these animals (Khan *et al*., 2020; Meador *et al.*, 2018). Likewise, the high activity of creatinine kinase enzymes after 45 days of exposure may be associated with the failure of the repair system and apoptosis induction, as observed by other researchers (Song *et al*., 2018; Ucar *et al*., 2019; Wang *et al*., 2019). Due to the direct alteration of the excess of ROS and the dysfunction of the antioxidant system on the ways of obtaining energy in the muscle tissue, mainly reducing the activity of glycolysis, the cycle of tricarboxylic acids and the pentose-phosphate pathway, there is an increase in the activity of the protein metabolism to compensate for the lack of cellular energy (essentially shown by the increase in CK) (Sukhanov et al. 2011; Beyfuss and Hood, 2018). In addition, the damage caused to proteins by the direct action of ROS or the consumption of protein substrates in detoxification, as well as the ubiquitination of damaged sarcomeral proteins, increase the availability of amino acids that can be used for energy generation (Bhardwaj and Jun, 2020; Jiang and Liu, 2021) . Likewise, both AST and ALT participate in the elimination of ammonia and detoxification of nitrosated amino acids.

At the end of exposure for 60 days, the potential toxicity of MTF was also able to significantly induce the state of cellular apoptosis. Li et al. (2020) showed that the induction of the apoptotic process might be a result of the increase in the generation of ROS, activating a mitochondrial signaling pathway mediated by ROS. The same authors mention that this effect may be related to increased tissue damage in Zebrafish.

We also observed an increase in the levels of LPO between the 15th and the 45th days of treatment. According to Voulgaridou et al. (2011), LPO products can induce mutations in DNA strands, which over time can develop a carcinogenic process. With the peroxidation of fatty acids, the formation of lipid peroxyl occurs, which can be later converted into secondary aldehyde species and result in cell damage (Mustafa *et al.*, 2017). This type of cell damage was observed in the present study, with a simultaneous increase in the number of MN and Blebbed cells, which are indicators of mutagenicity within 15 days. Along with the induction of mutagenic processes, it is possible to visualize the high number of cells in a state of necrosis over the exposure to MTF, which demonstrates the possible cytotoxic potential of the drug, concomitantly with the increase in ROS. According to Malhi, Gores and Lemasters (2006), a common event that can trigger both the necrotic and the apoptotic processes is permeabilization and mitochondrial dysfunction.

The induction of protein metabolism evidenced by the increase in muscle phosphotransferases and transaminases, together with the subsequent loss of antioxidant defense and the increase in cellular apoptosis (after 45 days), can promote the loss of the animals' homeostatic regulation capacity. The synthesis of this discussion may be observed in the diagram in Figure 3.

Therefore, considering that xenobiotics such as Metformin Hydrochloride are not fully purified in city sewage treatment systems and are constantly dumped into rivers and streams, it can be said that aquatic animals are in constant exposure to such chemicals. However, this problem is not only due to the ineffectiveness of water purification by sewage treatment systems, but also to the incorrect disposal in the environment by the human population (Trautwein *et al*., 2014).

The present study demonstrated that chronic exposure to MTF promotes functional changes in the fish *D. rerio*, evaluated here regarding the Antioxidant Defense System, Mutagenicity and Tissue Damage. Such functional changes can reduce the health conditions and alter the probability of survival, reflecting negatively on the population. Therefore, there is a need for studies that develop systems for the removal of xenobiotics in the treatment of sewage in cities, as well as promoting educational campaigns regarding the correct disposal of expired pharmaceutical components. It is necessary to correctly guide the lay population with scientific evidence that demonstrates the deleterious effects that these chemicals cause on non-target aquatic fauna.

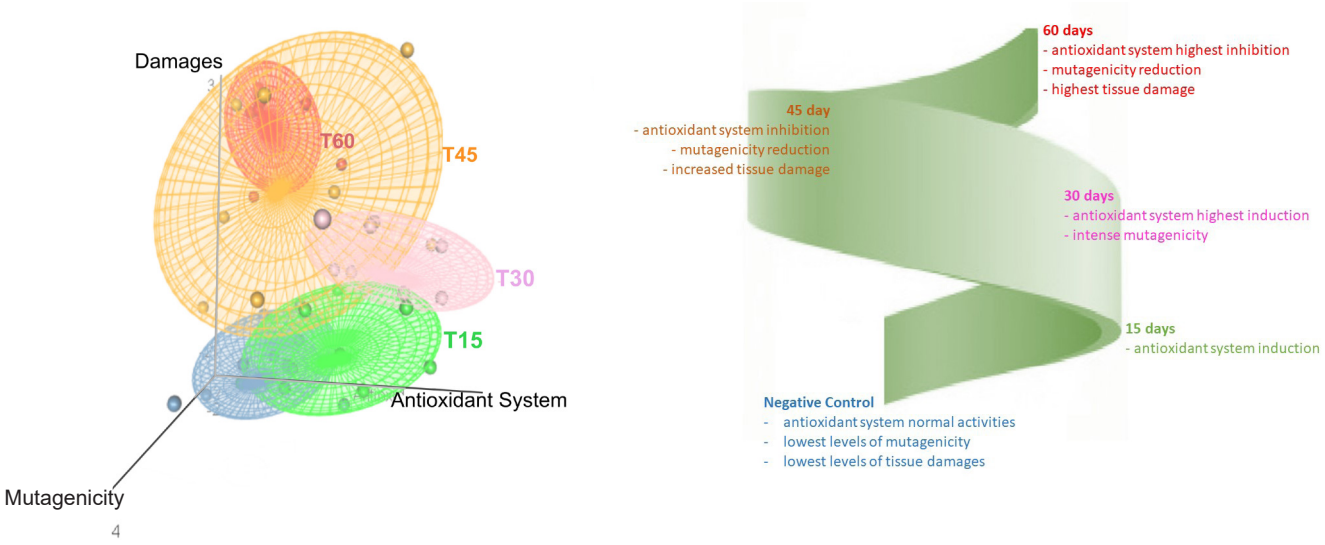

Figure 3 - Scheme of antioxidant system behavior and mutagenic potential in metformin hydrochloride chronic exposition. The graph on the left illustrates the first three axes of Principal Component Analysis (PCA) relating to the Antioxidant System, Tissue Damage and Mutagenicity domains. The ellipses in this graph indicate the variation of these three domains in an integrated manner at each of the exposure times to the sublethal concentration of Metformin (450 mg L^{-1}): Blue (Negative Control), Green (T15), Pink (T30), Orange (T45) and Red (T60). As this is a 3-dimensional diagram, it is possible to notice the integration of the domains in a spiral evolution, which was summarized in the diagram on the right. The spiral diagram shows the evolution of the main changes according to the domains evaluated throughout the study.

CONCLUSION

The present study demonstrated the toxic potential of MTF in chronically exposed animals (60 days). There was an induction of a response from the antioxidant system, followed by an inhibition of this system concomitant with the appearance of cellular and mutagenic damage. Toxic effects can reduce the ability of exposed animals to survive.

It is noteworthy that there are studies that have detected even lower MTF concentrations in the aquatic environment (Bradley et al. 2016). However, this study did not aim to evaluate such concentrations, since it is still necessary to understand the effects that sublethal concentrations derived from the LC50 can cause in non-target organisms.

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NRD: Data curation, Visualization, Investigation, Writing-original draft. **BZ**: Visualization, Writing-original draft. **MFZ**: Visualization, Writing-original draft. **TS**: Writing-review & editing. **LGDA**: Writing-review & editing. **ATBG**: Conceptualization, Data curation, Visualization, Writing-original draft, Methodology, Resource, Funding acquisition, Formal analysis, Project administration, Supervision. All authors read and approved the final manuscript. The authors declare that they have no competing interests.

ETHICS APPROVAL

The experiment was authorized by the Ethics Committee in Animal Experimentation of the Universidade Estadual do Oeste do Paraná (CEUA- n.53-17).

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