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Prolactin protects against mercury-induced toxicity in the Amazonian fish *Geophagus brasiliensis* (Perciformes, Cichlidae)

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ABSTRACT. Most riverside communities consume large quantities of fish as a part of their diet, increasing the risk of mercury exposure in polluted areas. The potential protective role of prolactin (PRL) against the toxic action of methylmercury (MeHg) was investigated in the fish *Geophagus brasiliensis* using the micronucleus test and analyzing lipid peroxidation, catalase (CAT) and glutathione peroxidase (GPx) levels. *G. brasiliensis* is endemic to Brazil, very resistant, important as an aquarium fish, and also used as human food. Fish were acquired commercially and divided into three groups of six animals each: 1) NC: Negative Control; 2) MeHg: exposure to 0.5 mg/L MeHg via water; and 3) MeHg/PRL: exposure to 0.5 mg/L MeHg and treated with 250 µg/kg PRL every 12h. The treatments lasted 5 days. There was a highly significant increase in the formation of micronuclei (MN) and erythrocyte nuclear abnormalities (ENA) in the MeHg group compared to the NC group, but when PRL

was administered (MeHg/PRL group), the numbers of MN and ENA were reduced. The TBARS results did not differ significantly between the tested groups. CAT levels were significantly reduced in the treated groups compared to the NC group. GPx levels showed a very significant increase in the MeHg group in relation to the NC group, and in the MeHg/PRL group, there was a significant decrease, although it did not reach the same values as those in the NC group. Although the role of prolactin in protecting against oxidative stress has not been elucidated, the protective effect of prolactin against the mutagenic effects of mercury has been very well demonstrated. To the best of our knowledge, this is the first demonstration of the protective effect of prolactin against mercury-induced mutagenicity in fish.

Key words: *Geophagus brasiliensis*; Mercury; Micronuclei; Oxidative stress; Prolactin

INTRODUCTION

Pollution of water resources is one of the major obstacles to the realization of sustainable development goals, especially in developing countries (Onen et al., 2023). Toxic elements such as mercury, chromium, lead and arsenic migrate along trophic chains and are transferred to humans through the ingestion of contaminated food, particularly fish (Rahman et al., 2020). The mutagenic and carcinogenic potential of heavy metals has already been demonstrated, as well as the ability of some of them to induce oxidative stress with the production of reactive oxygen species (ROS), which can cause DNA damage and cell death. Effectively, environmental contamination through the bioaccumulation and biomagnification of compounds containing heavy metals, such as mercury, is a potential cause of damage to genetic material (Siraj et al., 2018).

In the Amazon Basin, mercury waste from gold mining operations and leaching from soil after deforestation constitutes the greatest contribution to contamination in the aquatic environment (Guzmán-Uria et al., 2020). Fish have great socioeconomic, cultural, and nutritional importance for Amazonian populations, and fishing is considered the main source of income and food for riverside communities in the Amazon (Da Silva and De Oliveira Lima, 2020).

Mercury impairs tubulin polymerization, thereby causing the contraction of metaphase chromosomes, a delay in centromere division, and slower chromosome movement during anaphase (Cunha et al., 2022), which can result in chromosomal abnormalities and micronucleus formation. Analyses on mercury genotoxicity have already been conducted in several aquatic organisms, with an emphasis on fish (Table 1). Most of these analyses have been performed using the comet assay and micronucleus test. The comet assay detects DNA strand primary damage (breaks) and alkali-labile sites by measuring the migration of DNA from immobilized nuclear DNA. The micronucleus test is considered to be one of the most useful methods for evaluating genotoxicity in aquatic systems. Micronuclei are formed by chromosome fragments or whole chromosomes that lag at cell division due to mitotic spindle defects or direct damage to DNA (Vicari et al., 2012).

Erythrocytes are the most frequently chosen cell type to evaluate the formation of micronuclei (MNs) in fish. It is interesting to note that the same test slides are also used to screen for the presence of of erythrocyte nuclear abnormalities (ENAs), as shown in Table 1.

Table 1. Genotoxicity studies in fish exposed to mercury compounds.

Fish species	Cell type	Biomarkers	References
Aequidens tetramerus	Erythrocytes	MN, ENA and Comet assay	Rocha et al (2011).
Hoplias malabaricus	Erythrocytes	ENA	Da Silva et al (2012).
Hoplias malabaricus	Erythrocytes and kidney cells	MN and Comet assay	Vicari et al (2012).
Astronotus ocellatus	Erythrocytes	MN	Rodríguez et al (2017).
Colossoma macropomum	Erythrocytes	MN and ENA	Pinheiro-Sousa et al (2019).
Andinoacara rivulatus	Erythrocytes	MN and ENA	Nirchio et al (2019).
Anabas testudineus	Erythrocytes	MN	Swetha and Kiran (2021).
Channa punctatus	Erythrocytes	MN and ENA	Trivedi et al (2022).

Bioaccumulation of mercury in different tissues and organs can trigger oxidative stress due to the high production of reactive oxygen species. Furthermore, mercury itself can act as a reactive species, as it has a high affinity for thiol (R-SH) and selenol (R-SeH) groups of enzymes and other proteins, which may impair their functions (Farina et al., 2009). The free diffusion of these ROS can lead to reactions, such as protein oxidation and lipid peroxidation, causing damage to the structure of biological membranes and oxidative damage to DNA (Haidari et al., 2021).

Oxidative stress has been described as a key mechanism of mercury toxicity in fish (Cappello et al., 2016). Fish, like all other aerobic organisms, are susceptible to attack by ROS and have consequently developed antioxidant defenses, particularly in the form of adapted enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (García-Medina et al., 2017). Table 2 displays some recent publications on the use of antioxidant enzymes as biomarkers of oxidative stress in fish exposed to mercury.

Table 2. Studies with biomarkers of oxidative stress in fish exposed to mercury compounds.

Fish species	Tissues and organs	Biomarkers	References
Hoplias malabaricus	Liver	CAT e GST.	Da Silva et al (2012).
Liza aurata	Gills	CAT, GPx, SOD, GST, GSH e GR.	Cappello et al (2016).
Cyprinus carpio	Blood, liver, brain and gills	CAT, GPx e SOD.	García-Medina et al (2017).
Dicentrarchus labrax	Liver and gills	CAT, GPx, SOD, GST e GR.	Barboza et al (2018).
Plagioscion squamosissimus and *Colossoma macropomum*	Liver and kidney	CAT, GPx, SOD e GST.	Bittarello et al (2020).
Channa punctatus	Blood, liver and kidney	CAT, SOD, GSH e GR.	Trivedi et al (2022).
Pinirampus pirinampu	Liver and muscle	CAT, GPx e SOD.	Vieira et al (2022).
Cichla sp., *Brachyplatystoma filamentosum* and *Semaprochilodus* sp.	Liver and muscle	CAT, GPx e SOD.	Vieira et al (2023).

CAT = catalase; GPx = glutathione peroxidase; SOD= superoxide dismutase; GST = glutathione S-transferase; GR = glutathione reductase; GSH = glutathione.

Many studies have been conducted to elucidate the varied toxic effects of mercury on biological systems, and more recently, some protective agents against the action of mercurial compounds have been tested, including plant extracts (Pamplona et al., 2015), selenium (Bjørklund, 2015) and the hormone prolactin (Silva-Pereira et al., 2014; Cunha et al., 2022).

The hormone prolactin (PRL) is a protein produced and secreted mainly by the pituitary gland. Although PRL is best known for its function in lactation, it has more than 300 biological activities. In fish, PRL plays an important role in osmoregulation, regulation of calcium secretion, migration, parental behavior, reproduction, and development along with other hormones (Saha et al., 2021).

In vitro studies on human cell lines exposed to cytotoxic and mutagenic effects of mercury have shown that prolactin has potential cytoprotective properties and may act as a comitogenic factor and inhibitor of apoptosis (Silva-Pereira et al., 2014). More recently, our research group also demonstrated *in vivo* that prolactin significantly reduced micronucleus formation induced by methylmercury exposure in mice (Cunha et al., 2022). Considering that more than one species must be used to confirm the response to pollutants under experimental conditions (Zhelev et al., 2018), it can be inferred that confirmation of the protective action against the xenobiotic also requires more than one bioindicator species and that the choice of a fish seems appropriate to continue these analyses, considering that they are animals frequently exposed to mercurial contamination.

Geophagus brasiliensis (Cichlidae), commonly known as pearl cichlid (known as acará in Amazonian), was chosen as a model. This Cichlidae species is territorial and very resistant (Queiroz et al., 2019), endemic to Brazil and well-adapted to the tropical climate, inhabiting freshwater ecosystems. With an omnivorous feeding pattern, it has a broad trophic niche (detritus, plants, zoobenthos, insects and algae) (Honorato et al., 2021); can also serve as food for some larger fish. *G. brasiliensis* has already been identified as an excellent indicator of environmental impacts on water bodies (Morais et al., 2016) and some cichlids have already been used as bioindicators of mercury toxicity, as in studies by Rocha et al. (2011) and Nirchio et al. (2019), with *Aequidens tetramerus* and *Andinoacara rivulatus*, respectively.

The present study was designed to investigate the potential protective effects of prolactin against mutagenic damage caused by mercury in young adult fish of *Geophagus brasiliensis* using a bioassay under controlled laboratory conditions. In addition, antioxidant enzymes were analyzed to evaluate the protective action of prolactin against the cytotoxic activity of mercury.

MATERIAL AND METHODS

Fish Maintenance

Specimens of *G. brasiliensis* (n = 18), with an average length of 9.4 ± 1.90 cm and an average weight of 19.61 ± 13.42 g, were acquired commercially and acclimated for 15 days. Experiments were conducted in 30 L aquariums (one fish per aquarium) under constant aeration, with a 12-h light/dark photoperiod and chlorine-free water at pH 6.8 ± 0.29, dissolved oxygen at 5.9 ± 0.67 mg/L and a temperature of 26 ± 1.3 °C. The procedures

adopted in this study were approved by the UFPA Animal Research Ethics Committee (Process CEUA/UFPA 9070260819).

Chemical exposure and sampling

For five days, fish were divided into three groups of six animals each: negative control (NC), nontreated; MeHg group (methylmercury at 0.5 mg/L); and MeHg/PRL group (methylmercury at 0.5 mg/L and prolactin at 250 µg/kg, 12 h/12 h). Treatments were performed with CH_3HgCl (Sigma–Aldrich®) dissolved in water (Swetha and Kiran, 2021; Yulianto et al., 2023) and with PRL (Sigma–Aldrich®) administered by intraperitoneal injection (Park et al., 2011; Cunha et al., 2022). At the end of the experiment, fish were euthanized (immersed in benzocaine at 250 mg/L for 10 minutes) to obtain whole blood samples for the micronucleus test and liver samples for oxidative stress analyses. The mercury concentrations in kidney and liver samples were determined as described by Yasutake et al (2005), in which the tissue samples were first homogenized in distilled water and the concentrations of mercury in the homogenates (100 µL) were defined by the oxygen combustion-gold amalgam method using an MD-A atomic mercury absorption analyzer (https://mercuryanalyzer.com/index.html).

Micronucleus test

The experimental protocol was modified from Rocha et al (2011). Peripheral blood smears were fixed in absolute ethanol for 10 minutes and stained with 5% Giemsa in phosphate buffer, pH 6.8, for 10 minutes. In addition to erythrocytes with micronuclei (MN), three erythrocyte nuclear abnormalities (ENA) were considered: buds, lobes, and invaginations. Slides for MN and ENA scores were analyzed (by a single observer) under a microscope (Coleman Anti Mold X57 – N107) at 1,000x magnification in 2,000 erythrocytes per animal.

Oxidative stress biomarkers

Liver samples were weighed, homogenized in saline solution,centrifuged at 3,000 rpm for 10 min, transferred to Eppendorf tubes and stored at -80 °C until biochemical analysis, which was performed in an Ultrospec™ 8000/8000PC spectrophotometer.

The Bradford method (Oliveira et al., 2020) was used to estimate the protein concentration. The method is based on a colorimetric reaction between protein in the sample and the Bradford solution (Coomassie Blue 0.01%, ethanol 4.75%, phosphoric acid 8.5%) that occurs after incubation in a dark, cold room. The protein concentration of the liver sample was estimated by albumin equivalence (absorbance 0.66 to 280 nm). The results are expressed in mg/mL.

The determination of thiobarbituric acid reactive substances (TBARS) was based on Winterbourn (Winterbourn, 1985). Liver samples (200 µL) were added to 20 µL BHT (2% ethanolic solution), 200 µL 25% HCl, and 200 µL 1% thiobarbituric acid solution (dissolved in 0.05 N NaOH solution). Then, the samples were incubated in a dry bath (100 °C) for 15 min, and 618 µL of butanol was added, followed by centrifugation at 1,200 rpm for 3 min.

The supernatant was collected in duplicate for analysis in the spectrophotometer at 532 nm. The TBARS concentration was expressed as nM/mg protein.

Catalase (CAT) activity was determined by the Aebi method (Aebi, 1984), which quantifies the rate of decomposition of hydrogen peroxide (H_2O_2) at 240 nm for 20 seconds by the enzyme present in the sample. A 10 mM H_2O_2 solution in mM sodium phosphate buffer (pH 7) prepared and titrated on the day of analysis was used. To do this, 2 mL of H_2O_2 solution was used with the addition of 20 µL of the sample, and then the absorbance drop was read. Values were expressed as U/mg of protein.

The glutathione peroxidase (GPx) activity assay was performed according to the Flohé and Günzler method (Flohé and Günzler, 1984), with adaptations. The reaction is based on the formation of oxidized glutathione (GSSG), catalyzed by glutathione peroxidase. Thus, the measurement consists of the oxidation (verified through the drop in absorbance) of nicotinamide adenine nucleotide phosphate (NADPH) measured at 340 nm, since NADPH is used in the regeneration of GSH by the enzyme glutathione reductase (GR). Therefore, the rate of oxidation of NADPH is proportional to the rate of production of GSH, catalyzed by the GPx present in the sample analyzed. The technical procedure was carried out by adding the following solutions to the cuvette: 10 µL of sodium azide (NaN_3 0.25 M); 10 µL of GSSG 0.25 U/ml; 50 µL of 20 mM GSH; 380 µL of the sample diluted in buffer (50 µL of sample + 330 µL of phosphate buffer); and 500 µL of 0.24 mM NADPH. There was incubation for 5 minutes and the addition of 50 µL of TBHP (t-butyl hydroperoxide) 10.43 M. The reading was carried out for 8 minutes, and the values were expressed in U/mg of protein.

Statistical analysis

Data were tested for normality of distribution (Kolmogorov–Smirnov test) and homogeneity of variances (Levene test). Differences between mean values were compared by one-way ANOVA and the Tukey–Kramer test. As the TBARS data did not meet the normality assumption, a nonparametric analysis of variance was adopted, the Kruskal–Wallis test. Analyses were run using the statistical package BioEstat 5.0 and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Table 3 presents the mercury levels found in kidney and liver samples from the *G. brasiliensis* specimens used in this study, both those from the control group and those exposed to the xenobiotic. All of the data are expressed as the mean ± standard deviation (SD).

Table 3. Mercury levels in control and MeHg-treated fish tissues.

Tissues	Control group(µg Hg/g tissue)	Treated group(µg Hg/g tissue)
Kidney	0.0345 ± 0.0085	11.9 ± 1.92
Liver	0.0128 ± 0.0052	2.3 ± 0.27

Micronucleus test

The micronuclei (MN) and erythrocyte nuclear abnormality (ENA) frequencies were significantly increased ($p < 0.001$) in the erythrocytes from fish treated with MeHg compared to the negative control fish. In the MeHg/PRL group, however, the frequency of MNs was reduced to a frequency similar to that in the control group, while the frequency of ENAs, although not the same as the control, was also significantly reduced (Figures 1, 2). Thus, the present study demonstrated a clear protective effect of prolactin (PRL) against the mutagenic impact induced by MeHg.

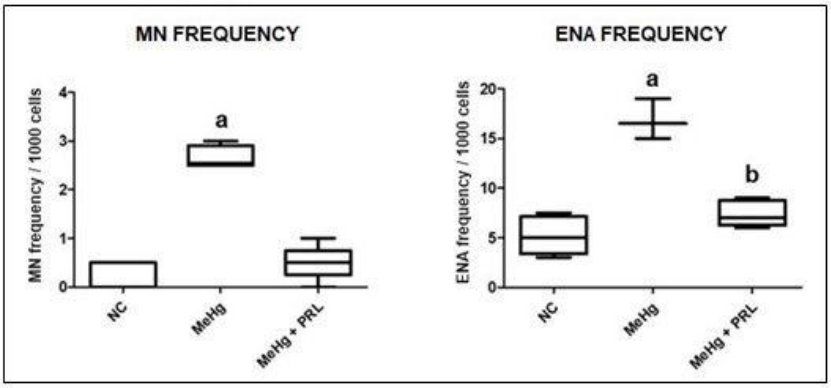

Figure 1. Micronuclei (MN) and erythrocyte nuclear abnormality (ENA) frequencies per 1,000 erythrocytes in the pearl cichlid *Geophagus brasiliensis*. [a]$p < 0.001$ compared to other groups; [b]$p < 0.05$ compared to the NC group (ANOVA with Tukey–Kramer's posttest).

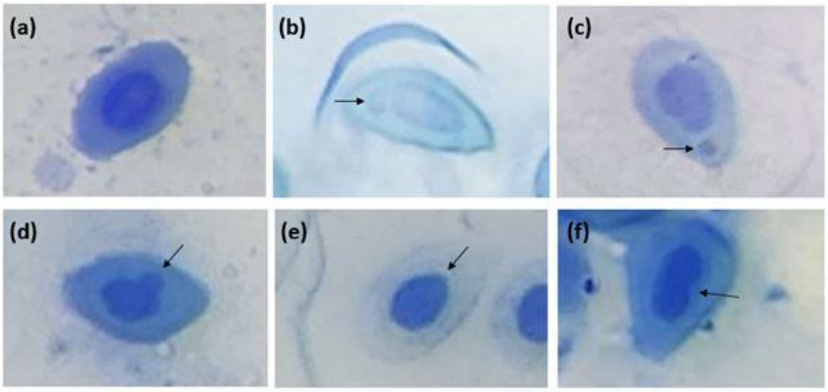

Figure 2. Photomicrographs of fish erythrocytes. (a) Normal; (b) and (c) Micronucleus; (d) Blebbed nucleus, (e) Lobed nucleus; and (f) Nucleus with invagination.

Oxidative stress biomarkers

The results for lipid peroxidation and for CAT and GPx enzymes are illustrated in Figure 3. The mean concentration of TBARS in the liver of *G. brasiliensis* was lower in the

NC group; however, there was no significant difference ($p_{Kruskal\text{-}Wallis} = 0.456$) between the NC group and the treatment groups (MeHg and MeHg/PRL). The CAT activity was significantly reduced in the MeHg and MeHg/PRL groups compared to the NC group. Regarding the activity of the GPx enzyme, there was a very significant increase in the group treated with MeHg compared with the NC group; in the MeHg/PRL group, there was a significant reduction, although it did not reach the same values as the NC group.

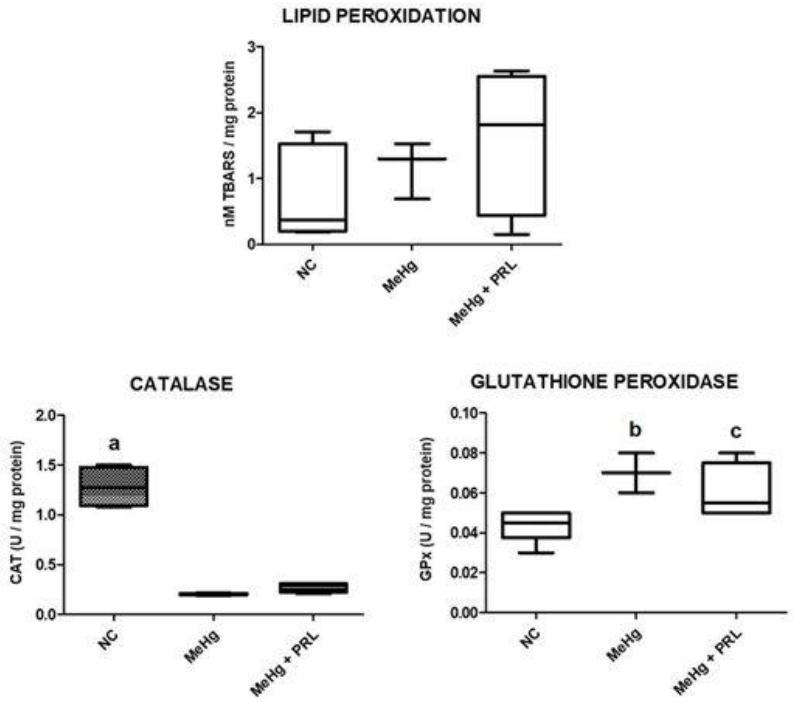

Figure 3. Lipid peroxidation, LPX; catalase, CAT; and glutathione peroxidase, GPx, in the liver of the pearlcichlid *Geophagus brasiliensis*. [a]$p < 0.001$ compared to other groups; [b]$p < 0.01$ compared to the NC group; [c]$p < 0.05$ compared to the NC group (ANOVA with Tukey–Kramer's posttest.

DISCUSSION

The bioaccumulation of mercury in the kidney and liver of exposed fish was clearly demonstrated. The most significant accumulation occurred in the kidney, which, in fish, is responsible for filtration and the production of erythrocytes. Exposure to mercury (0.5 mg MeHg/L) for five days was sufficient to induce the formation of MNs and ENAs in erythrocytes, in addition to changes in antioxidant enzyme activity in the liver.

Micronucleus test

To the best of our knowledge, this is the first demonstration of the protective effect of prolactin against mercury-induced mutagenicity in fish. The increase in MN and ENA frequencies as a result of exposure to mercury compounds has already been demonstrated in numerous studies, both in fish (Rocha et al., 2011; García-Medina et al., 2017) and in other

organisms (Cunha et al., 2022). Mercury has an aneugenic effect, as it induces the formation of MNs by acting directly on microtubules, impairing the formation of the mitotic spindle and normal chromosomal segregation. However, more recently, studies have indicated that PRL can prevent or reverse these mutagenic effects of mercury *in vitro* with human cell lines, as demonstrated by Silva-Pereira et al (2014), and *in vivo* with models, as demonstrated by Cunha et al (2022). PRL acts as a comitogenic factor, regulating proliferation by modulating the expression of genes that are essential for cell cycle progression, in addition to contributing to the organization of the cytoskeleton (Silva-Pereira et al., 2014).

Oxidative stress biomarkers

The results of liver TBARS levels (lipid peroxidation) showed no significant difference between the study groups. Similar results were reported by García-Medina et al (2017) when addressing mercury-induced oxidative stress in common carp (*Cyprinus carpio*). It is possible that the activities of antioxidant enzymes were sufficient to neutralize the action of ROS caused by Hg, which could explain the lack of significantly different values between the NC group and the treated groups. In the present analysis, there was no evidence of an effect of PRL on lipid peroxidation in the liver.

The results of analyses on the effect of exposure of fish to mercury in relation to CAT enzyme activity have been contradictory. Monteiro et al (2010), for example, observed an increase in enzyme activity in the exposed group; however, Shang et al (2021) obtained results similar to ours, that is, a reduction in enzyme activity in the group exposed to mercury. According to these last authors, the inhibition of CAT activity is most likely due to the depletion or inactivation of H_2O_2 by enzyme consumption under mercury stress conditions. Although the transcription levels of CAT increased, due to the accumulation of H_2O_2 over a long period of time, oxidative stress is induced, resulting in the inhibition of CAT activity. In the present analysis, there was no evidence of an effect of PRL on the activity of the CAT enzyme.

Additionally, in the case of the GPx enzyme in fish exposed to mercury compounds, the literature presents divergent results. Our result is in line with, for example, that observed by Barboza et al (2018) in the European seabass *Dicentrarchus labrax* but differs from that reported by García-Medina et al (2017) in their experiment with *C. carpio*. Possibly, different species show differences in the degree of enzyme response; different concentrations of mercury compounds, as well as the exposure time, may also exert some influence on the results. Moreover, in the present study, while exposure to MeHg very significantly increased GPx values in the liver of treated fish compared to the NC group ($p < 0.01$), in the MeHg/PRL group, the enzyme activity exhibited a decrease that, although did not reach the levels of the group not exposed to MeHg, may suggest some contribution of PRL toward the establishment of normality.

The protective role of PRL may be related to the reciprocal influence of PRL and glutathione levels, and PRL acts by activating the detoxification enzyme glutathione-S-transferase (GST) (LaPensee and Ben-Jonathan, 2010; Silva-Pereira et al., 2014), which plays a role in the detoxification of several xenobiotics (Haidari et al., 2021), catalyzing conjugation reactions of reduced glutathione (GSH) with metabolites and increasing their water solubility, thus facilitating their excretion.

CONCLUSION

The mutagenic and cytotoxic effects of MeHg, as well as the antimutagenic action of prolactin, have been clearly demonstrated. In conclusion, although the role of prolactin in protecting against oxidative stress have not been elucidated, the significant results obtained in mutagenicity and antimutagenicity analysis of the present study allow us to suggest the continuity of the research for the potential future use of prolactin as an alternative to prevent the damage caused by mercury, especially in human populations that are more exposed.

AUTHOR CONTRIBUTIONS

JANM, CAMR, and RMRB conceived and designed the study; CAMR, DDFAA, and RMRB contributed to obtaining resources; JANM, LAC, RCSC, and SSCL performed the mutagenicity analyses; RCS and JJSF performed the oxidative stress analyses with contribution from JANM and LAC; DDFAA and RMRB performed the statistical analyses; JANM, DDFAA, and RMRB wrote the manuscript; CAMR made a critical review, adding improvements and rewriting sentences. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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