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IN VIVO EVALUATION OF THE POTENTIAL ANTIOXIDANT EFFECT OF THE HORMONE PROLACTIN AGAINST THE TOXIC EFFECTS OF METHYLMERCURY

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Abstract: Mercury is a xenobiont and is one of the most harmful organ-specific contaminants. One of the well-known effects of mercury is the production of free radicals, which induces a general breakdown of antioxidant mechanisms in the cell, favoring lipid peroxidation, followed by loss of membrane integrity and, finally, cell necrosis. Prolactin is a protein hormone that has more than 300 biological activities and many studies have demonstrated the protective functions that this hormone has in various tissues and experimental situations, including antioxidant action. To evaluate the possible antioxidant effect of prolactin, mice were subjected to sub-chronic exposure to methylmercury and treated with the prolactin hormone. After the treatment period, lipid peroxidation and the action of the enzymes Catalase and Superoxide Dismutase were evaluated in liver and kidney samples from these animals. The antioxidant action of prolactin was better observed in the evaluation of the action of the Superoxide Dismutase enzyme.
Keywords: Methylmercury; Prolactin; Antioxidant; Lipid Peroxidation; Catalase; Superoxide Dismutase

INTRODUCTION

Aquatic ecosystems are generally the temporary or final depositories of a wide variety and quantity of pollutants and contaminants (CAMPANELLI, 2012). The main source of exposure to heavy metal contaminants occurs through the consumption of food, mainly fish, containing non-essential toxic elements such as arsenic, cadmium, lead, chromium and mercury (IKEM & EGIEBOR, 2005). Mercury is a xenobiotic and is one of the most widespread and harmful organ-specific contaminants (BJØRKLUND *et al*., 2017). The International Agency for Research on Cancer (IARC: International Agency for Research on Cancer) has classified methylmercury in group 2B since 1993, indicating it as a possible carcinogen for humans (IARC, 2021).

The effects of mercury in general are detrimental to tubulin polymerization, which leads to chromosome contraction in metaphase, delayed anaphase movement and centromeric division (THEIR *et al*., 2003) and can also lead to chromosomal abnormalities such as polyploidy (SILVA-PEREIRA *et al*., 2005). Added to these effects is the production of free radicals, which can cause permanent damage to DNA (EHRENSTEIN *et al*., 2002; MANZOLLI *et al*., 2015). Mercury compounds induce a general breakdown of antioxidant mechanisms in the cell by binding to sulfhydryl groups of glutathione peroxidase, a major selenoenzyme with antioxidant properties. The results of this collapse favor lipid peroxidation, followed by loss of membrane integrity and, finally, cell necrosis, which can be indicated by a decrease in the mitotic index (NASCIMENTO *et al*., 2008).

Because metals such as mercury are not biodegradable, they accumulate in organisms throughout their lives (bioaccumulation) and along food chains (biomagnification or trophic magnification), and can reach high concentrations when they reach humans (ROCHA, 2009). The main way in which humans are exposed to mercury is still through eating fish (CRESPO-LOPEZ *et al.* 2021). In Brazil, studies have shown that several species of carnivorous fish in the Amazon have high levels of methylmercury (MeHg) (MALM, 1998; PINHEIRO *et al.,* 2003). Studies carried out in the Tapajós River basin, using fish, found values ranging from 0.02 to 2.75 mg/kg in mining areas, and for fish from unexposed rivers, the values ranged from below the limit to 0.10 mg/kg (SANTOS *et al*., 2000; BISINOTI & JARDIM, 2004).

Prolactin (PRL) is a protein hormone from the same family as growth hormone and placental lactogens (SOARES JR & GADERLHA, 2004). In addition to stimulating milk production by the mammary

glands (lactogenesis), PRL has more than 300 other biological activities, including acting on homeostasis by regulating the immune system, osmotic balance and angiogenesis; it also has an effect on cell growth and proliferation, and as a neurotransmitter (IGNACAK *et al*., 2012 MARANO & BEN-JONATHAN, 2014). All these functions performed by

PRL and the signaling pathways activated may be related to the protective effects of this hormone in various tissues and experimental situations. An antioxidant action of PRL has already been identified in many studies. Terra *et al*. (2019), observed a reduction in protein degradation, related to the increased action of peroxide radical dismutation enzymes, when the hormone was present.

The antioxidant effect of PRL on β cells was observed by Marmentini (2019), who used the INS-1E cell line of rat insulinoma and found an increase in the survival of these cells and in the production of the enzymes superoxide dismutase 2 and catalase, induced by the hormone. The authors suggest that PRL activates peroxisome proliferator-activated receptors, which are involved in the transcription of antioxidant enzymes.

Antioxidant and antiapoptotic actions of PRL were evaluated in the retinal pigment epithelium by García *et al*. (2016) and Arnold *et al*. (2020), in rats of different ages. The PRL receptor was identified and this hormone was characterized as a trophic factor for the cells of this tissue, their results demonstrating the need for PRL for the proper functioning of photoreceptor cells and the possible therapeutic value of this hormone against age-related retinal disorders. García *et al*. (2016), also used the human cell line ARPE-19 (retinal pigment epithelium cells) and the presence of PRL reduced the damage caused by hydrogen peroxide and the levels of reactive oxygen species in these cells, this effect was related to the action of PRL inhibiting the increase in Ca^{2+} induced by the deacetylase SIRT2, mediated by the TRPM2 receptor, these two factors are considered targets of the antioxidant action of this hormone.

Considering the protective actions already observed, including against the effects of MeHg, and the fact that PRL is not related to the development of breast cancer, as has been thought (GOODMAN & BERCOVICH, 2008), this study evaluated prolactin as an alternative antioxidant protection against the effects of methylmercury.

METHODOLOGY

60 healthy, young adult mice (*Mus musculus*) of both sexes were obtained from the vivarium of the Evandro Chagas Institute and kept in the vivarium of the State University of Pará - UEPA, in an environment with a temperature of 22ºC (± 3ºC), in a 12h light/12h dark cycle, receiving water and feed (OECD, 2016).

TREATMENTS

The animals were divided into six groups of 10 animals each, 5 of each sex, according to the doses of methylmercury and prolactin. After the acclimatization period, the animals were treated with CH3HgCl (Sigma-Aldrich®) and PRL (Sigma Aldrich®), both diluted in distilled water (mother solution) and 0.9% saline solution (use solution), at concentrations of 30 μg/kg/day of MeHg and 25 and 250 μg/kg/12h of PRL, for a period of 45 days (PARK *et al*., 2011; RIZZETTI, 2012; MANZOLLI, *et al*., 2015).

In the animals that received both treatments, prolactin was administered simultaneously with the methylmercury treatment and 12 hours later. The CN group (negative control group) received a subcutaneous injection of 0.9% saline solution during the same treatment period as the other groups (12h/12h). At the end of the treatments, all the animals were

euthanized and liver and kidney samples were collected for enzymatic bioassays

The study followed the precepts of national legislation for the use and breeding of animals for experimentation (Federal Law No. 11,794 of 2008) and the Ethical Principles of the Brazilian College of Animal Experimentation (COBEA), and was carried out in accordance with the Brazilian guideline for the care and use of animals for scientific and didactic purposes - DBCA (CONCEA, 2013), and was submitted for approval by UEPA's research ethics committee on the use of animals (CEUA/UEPA), under protocol No. 16/2017 and with a favorable opinion.

ENZYMATIC BIOASSAYS

The liver and kidney samples from each animal were collected, weighed and homogenized in a ratio of 1:10 (0.1g of sample to 1000 µL of 0.9% saline solution), using a homogenizer. The homogenates were transferred to microtubes and centrifuged. Samples of the supernatant were aliquoted into microtubes and stored at -80º C for later biochemical analysis. Aliquots were kept for peroxidation analysis and for the Catalase (CAT) and Superoxide Dismutase (SOD) enzyme activity tests.

Protein quantification - Brasford method

The Bradford method was used to estimate protein concentration by spectrophotometric analysis. The method is based on the colorimetric reaction between the protein inside the sample and the Bradford solution (Coomassie Blue 0.01%, ethanol 4.75%, phosphoric acid 8.5%) in dark and cold environments. The results are expressed in mg/mL. Protein quantification is taken into account when calculating lipid peroxidation and the activities of the enzymes analyzed.

Lipid Peroxidation

To determine the concentration of thiobarbituric acid reactive substances (TBARS), which are the products resulting from lipid peroxidation, the method of Winterbourn *et al.* (1985) modified for spectrophotometric analysis was used.

The method is based on the change in color of the sample (plasma) when it is placed to react with TBARS at 1% at a temperature of 90 to 100 °C and in an acidic medium.

The amplification of peroxidation during the assay was prevented by the addition of an antioxidant (BHT) (BROWN &KELLY, 1996). The concentration of TBARS was determined using the molar extinction coefficient of MDA (e = 1.56 x 10-5. M mL^{-1-1}). TBARS concentration = Absorbance/1.56 x dilutions (WINTERBOURN *et al.*, 1985).

Catalase (CAT) activity

The activity of the enzyme catalase was determined using the method of Aebi (1984), which consists of measuring the activity of this enzyme through the decomposition of exogenous hydrogen peroxide, generating oxygen and water, using spectrophotometry. The rate of hydrogen peroxide decomposition is measured at 240 nm for 20 seconds. A solution of 10 mM hydrogen peroxide (H2O2) in mM phosphate buffer pH 7.0, prepared and titrated on the day of analysis, is used. To do this, 2 mL of this solution was added to the cuvette, with the addition of 20µL of the sample, then the drop in absorbance is read. The values are expressed in $mmol.min^{-1}$ .mL^{-1}

Superoxide Dismutase (SOD) activity

SOD activity was measured spectrophotometrically at 550 nm, according to the method adapted from Flohé (1987). This enzyme catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. The values are expressed in $USOD.mL^{-1}$.

RESULTS

LIPID PEROXIDATION

No statistically significant difference was observed in the liver of the males between any of the treatment groups; in the females, only the group that received MeHg together with PRL at the lowest concentration showed a significant increase compared to the other groups (Figure 1). With regard to the analysis carried out on samples of the animals' kidneys, in males, only the group that received MeHg together with PRL at the lowest concentration showed a significant reduction compared to the control group; while in females, there was no statistical difference (Figure 2).

In these experimental conditions, we were unable to prove a cytotoxic effect of MeHg, compared to the control, nor an action of PRL against this damage.

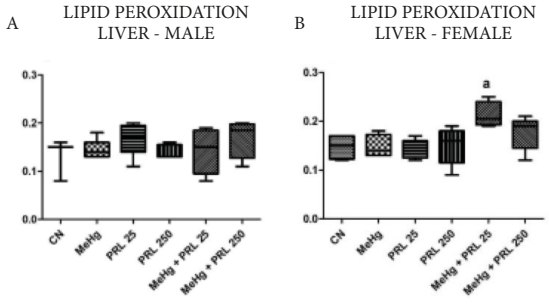

Figure 1: Comparison between lipid peroxidation levels in the liver of males (image A) and females (image B). **a**: statistically significant difference in the MeHg + PRL25 group compared to the other treatments (females). **a** ($p<0.05$), ANOVA with Tukey post-test.

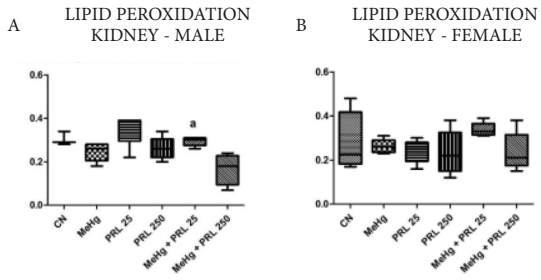

Figure 2: Comparison between lipid peroxidation levels in the kidney of males (image A) and females (image B). **a**: statistically significant difference in the MeHg + PRL250 group compared to the control group (males). **a** ($p<0.05$), ANOVA with Tukey post-test.

CATALASE ACTIVITY (CAT)

The evaluation of catalase enzyme activity showed no statistically significant difference in any treatment group, in either gender or in any of the organs analyzed (liver and kidney) (Figures 3 and 4).

Under these experimental conditions, we were unable to define an effect of MeHg and PRL on the action of the enzyme catalase

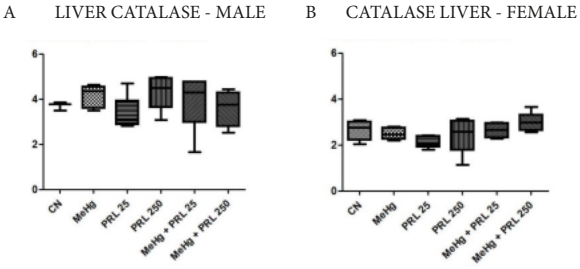

Figure 3: Comparison of Catalase activity in the liver of males (image A) and females (image B) from each treatment group.

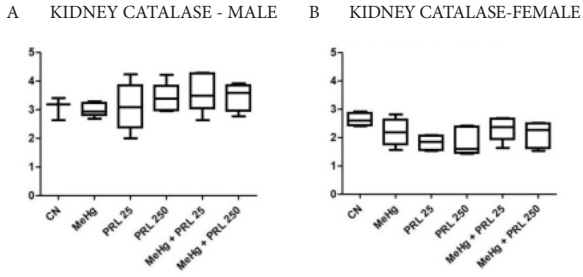

Figure 4: Comparison of Catalase activity in the kidney of males (image A) and females (image B) from each treatment group.

SUPEROXIDE DISMUTASE (SOD) ACTIVITY

In the liver samples, in both sexes, we observed an increase in the activity of the SOD enzyme in all the treated groups when compared to the control group, but only in the group that received the conjugate treatment of MeHg and PRL at the highest concentration was this increase statistically significant ($p<0.01$). In males, the significant difference was between this group (MeHg + PRL250) and the control and MeHg-only groups. In females, SOD activity was statistically higher when compared to the control group, the group that received only MeHg and the group that received only PRL at the same concentration (Figures 5).

The evaluation of SOD activity in the kidney proved to be more sensitive, showing greater variation between the groups, with the groups that received the conjugate treatment (MeHg and PRL in both concentrations) showing a statistically significant difference ($p<0.01$) when compared to the other groups, in both genders. In females, we also observed a statistical difference between the control group and the group that received only MeHg (Figures 6).

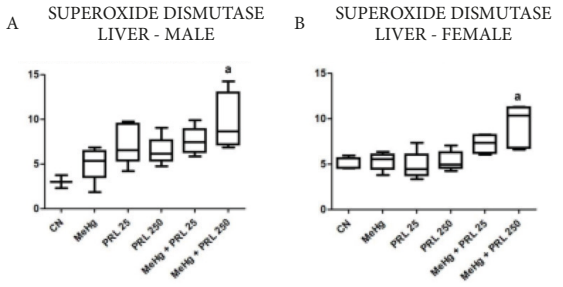

Figure 5: Comparison of SOD activity between treatment groups in the liver of males (image A) and females (image B). In image A, **a**: statistically significant difference in the MeHg + PRL250 group, compared to the control group and the group that received only MeHg; in image B, **a**: statistically significant difference in the MeHg + PRL250 group, compared to the control group, the group that received only MeHg and the group that received only PRL at the same concentration (in females). **a** ($p<0.01$), ANOVA with Tukey post-test.

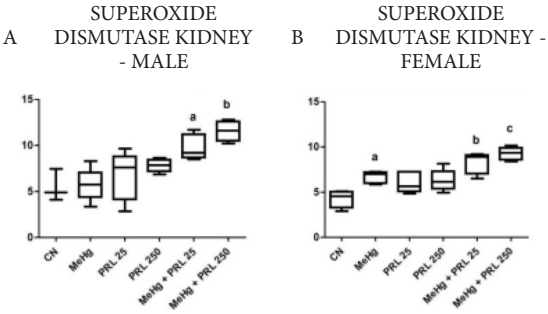

Figure 6: Comparison of SOD activity between treatment groups in the kidneys of males (image A) and females (image B). In image A, **a**: statistically significant difference in the MeHg + PRL25 group, compared to the control group and the group that received only MeHg; **b**: statistically significant difference in the MeHg + PRL250 group, compared to the control group, the group that received only MeHg and the group that received only PRL at the same concentration. In image B, **a**: statistically significant difference in the MeHg group compared to the control group; **b**: statistically significant difference in the MeHg + PRL25 group, compared to the control group and the group that received only PRL in the same concentration; **c**: statistically significant difference in the MeHg + PRL250 group, compared to the control group, the group that received only MeHg and the group that received only PRL in the same concentration. **a**, **b** and **c** ($p<0.01$), ANOVA with Tukey post-test.

DISCUSSION

In the present study, it was not possible to characterize the cytotoxicity of MeHg based on lipid peroxidation or the effect of this metal on the activity of the enzyme catalase, as well as the possible antioxidant effect of PRL based on these parameters. However, the results observed in the analysis of the action of the SOD enzyme show some differences in the activity of this enzyme when comparing the treatment groups. Several studies have already evaluated the effects of mercury based on the oxidative stress caused by this metal, using the same parameters (lipid peroxidation and antioxidant enzyme activity) that we evaluated.

Costa-Malaquias *et al*. (2014) analyzed oxidative damage induced by methylmercury in the C6 rat glioma cell line and, as in our findings, observed no significant difference between the control group and the MeHg-treated group with regard to lipid peroxidation. For these authors, this lack of difference may be related to a high level of lipid peroxidation in the control group, due to cell culture conditions.

Similarly to what was observed in our study conditions, Belém filho (2015) did not identify an increase in lipid peroxidation in blood samples from Wistar rats exposed to a low concentration of MeHg. In addition to lipid peroxidation, this author assessed other oxidative biochemical parameters, such as CAT and SOD enzyme activity. Although the concentration used and the treatment time were similar to those in our study, the observations of the activities of these enzymes were the opposite of what we observed in our results; in the animals that received MeHg, the activity of the CAT enzyme was greatly increased compared to the control group, while in the evaluation of SOD, no change in enzyme activity was observed. The authors believe that they did not observe differences in SOD activity due to the fact that this enzyme acts in different ways in different tissues.

The evaluation of mercury's oxidizing activity still shows very divergent results; while in the studies cited above, as well as in our results, there was no increase in lipid peroxidation, in several others the increase in this oxidative damage is an important effect of the metal. Franco (2009), Wagner *et al*. (2010) and Jindal *et al*. (2010) identified a significant increase in lipid peroxidation *in vivo* lipid in samples from animals exposed to mercury, characterizing the metal's cytotoxicity. These authors also evaluated the activity of antioxidant enzymes; Franco (2009) associated the neurotoxicity observed in mice exposed to MeHg with the inhibition of the enzyme glutathione peroxidase; he also observed an increase in the activity of reduced glutathione in the offspring exposed through lactation.

Wagner *et al*. (2010) observed a significant reduction in glutathione peroxidase activity in the brain and kidney of Wistar rats exposed to MeHg; SOD activity was not altered. Jindal *et a.l* (2010) observed a reduction in the activity of the enzymes reduced glutathione and SOD; these authors relate the inhibition of enzymes by mercury, especially enzymes containing a sulfhydryl group, to the metal's ability to bind to this group.

Grotto *et al.* (2011) and Souza *et al*. (2016) used the same concentration of MeHg, 140 µg/Kg/day, for 100 and 60 days of treatment, respectively, and also observed different results in relation to the SOD enzyme; while for the former the activity of the enzyme remained unchanged, for Souza *et al*. (2016), treatment with MeHg increased the expression of this enzyme, indicating an increase in oxidative stress caused by the metal. This variation in results may be related to the tissue used for analysis. Grotto *et al*. (2011) evaluated blood samples, while Souza *et al*. (2016) used the brain. Grotto *et al.* (2011) also observed a significant reduction in CAT and glutathione peroxidase activities.

In humans, oxidative status was assessed in fish-consuming populations from different geographical regions of the state of Pará and individuals from the region closest to former mining areas showed the highest levels of total mercury in their blood, reaching levels higher than the tolerance limit established by the WHO. These individuals also showed a reduction in the quantification of glutathione, when compared to the analysis of samples from individuals in other regions. There was an increase in lipid peroxidation in these individuals, but not as evident as the reduction in enzyme activity (OLIVEIRA, 2014).

All this variety of results indicates that the evaluation of oxidative biochemistry parameters, although very important for characterizing mercury toxicity, must take other factors into account, such as the tissue analyzed, mercury type, dose and treatment time, among others.

Our results showed an increase in SOD activity, even in the groups that received PRL and significantly ($p<0.01$) in the groups that received the hormone together with the metal. This increase also characterizes an antioxidant action of PRL. Some studies have shown an increase in the expression of antioxidant enzymes stimulated by this hormone; Marmentini (2019), using a culture of INS-1E pancreatic β-cells, observed an increase in the protein content of antioxidant enzymes, including SOD and activation of the peroxisome proliferator-activated receptor (PPAR). Mansano (2018), using a murine insulinoma strain, demonstrated a differential expression of the HSPB1 protein, which also has its activation increased due to oxidative stress, being characterized as a mediator of the effect of PRL.

Even though the antioxidant effects of PRL are not very clear, the protective action of this hormone against the effects induced by MeHg has already been demonstrated by Cunha et al. (2022), who used *Mus musculus* mice and characterized the protection of this hormone against the mutagenic and histopathological effects of mercury; ratifying PRL as a target of interest to be investigated as an alternative prevention against the toxic effects of mercury.

CONCLUSION

Under the conditions of this study, the oxidative stress induced by MeHg was not very evident, which may be related to the low concentration of the metal used to treat the animals. The antioxidant action of PRL was better observed when evaluating the action of the SOD enzyme. Further studies are needed to better characterize these actions and demonstrate the protective potential of PRL against oxidative damage caused by mercury.

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