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Daily intake of lead in Wistar rats at different ages: Biochemical, genotoxic and physiological effects

Cássia Regina Bruno Nascimento, Claudia Bueno dos Reis Martinez*

Department of Physiological Sciences, State University of Londrina, Londrina, PR, Brazil

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ABSTRACT

The effects of the daily intake of feed containing lead (2.0 mg Pb kg^{-1}) were evaluated in 45 (Pb45) and 90 (Pb90)-day-old Wistar female rats. Compared to the respective control groups, Pb45 rats consumed more feed and showed greater weight gain, but these parameters returned to control values in Pb90 rats. Higher blood glucose levels were observed in both Pb groups, whereas plasma insulin concentrations were higher in Pb45 but lower in the Pb90 group. Liver glycogen content was lower only in the Pb90 rats. There were no changes in plasma cortisol and acetylcholinesterase activity in the brain. Hematological alterations were observed only in Pb90 rats, which showed lower hemoglobin levels. In the liver, Pb45 rats showed decreased catalase and glutathione peroxidase activities and increased glutathione reductase activity, but in the Pb90 group, glutathione levels were higher. Increased hepatic lipid peroxidation and DNA damage in the lymphocytes were observed in both Pb groups. These results indicate that the daily intake of Pb for different periods results in metabolic changes and in the establishment of oxidative and genotoxic damage in female rats.

1. Introduction

Lead (Pb) is a cumulative toxic metal that can affect various functions at relatively low levels, leading to neurological, hematological, gastrointestinal, cardiovascular and renal disorders (Fewtrell et al., 2003; Ashry et al., 2010). Its widespread use has caused environmental pollution problems in several regions of the world (Eades et al., 2002; Mielke et al., 2013; Paoliello and Capitini, 2005). There are several reports of Pb-contaminated water bodies, resulting in fish that are susceptible to this contaminant (Leung et al., 2014; Rahman et al., 2012). Pb accumulation in the flesh of multiple fish species has been observed in several countries, including the United States, Egypt, Spain, Slovakia and Croatia (Castro-González and Méndez-Armenta, 2008). In Brazil, Pb has been detected in the flesh of fish from different trophic levels, ranging from 0.01 to 7.98 mg kg^{-1} (Terra et al., 2008).

Fish consumption is an important route of exposure for Pb, considering that fish accumulate significant amounts of the metal in their tissues, particularly in muscle, which represents the main source of Pb in the diet of human populations (Castro-González and Méndez-Armenta, 2008). In Brazil, fish consumption averaged 17.3 kg *per capita* in 2013 (MPA, 2013), whereas in Europe, fish consumption was 22 kg *per capita* in 2010 but will be an estimated 24 kg *per capita* in the year 2030 (FAO, 2007).

In mammals, Pb absorption occurs in the duodenum through the divalent metal transporter 1 (DMT1), which can transport up to 8 different metals, including Fe^{2+} and Pb^{2+} (Garrick et al., 2003). After being absorbed, Pb is rapidly distributed into plasma, erythrocytes, soft tissues and bone. The concentrations of Pb in soft tissues are higher in the liver and kidneys and lower in the brain. Although the Pb concentration in plasma is only up 0.2–0.3% of the concentration in whole blood, plasma contains the Pb that is available for distribution within and excretion from the organism (IARC, 2006). Adults absorb 5–15% of Pb from their diets and retain 5% in their bodies, 94% of which is retained in the bones; in contrast, children absorb 42% and retain approximately 32%, only 73% of which is retained in the bones (Liu et al., 2008). Children absorb 4–5 times more Pb than adults, and infants and small children are more susceptible to the adverse effects of this metal. These differences occur because the absorption of Pb from the gastrointestinal tract is increased and the blood brain barrier is not fully developed in younger individuals (WHO, 2010).

Pb exposure accounts for 0.6% of disease occurrence in the world, the highest incidence of which occurs in developing regions (UNEP, 2008). The effects of Pb on human health have been known for centuries, but a wealth of information has emerged in recent

* Corresponding author at: Department of Physiological Sciences, State University of Londrina, Postal Box 10011, 86057-970, Londrina, PR, Brazil.
Tel.: +55 43 3371 4650; fax: +55 43 3371 4467.
E-mail address: cbueno@uel.br (C.B.d.R. Martinez).

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decades on the damaging effects of relatively low levels of exposure (Allouche et al., 2011). Adverse effects caused by this metal include changes in glucose profiles (Feldman et al., 2011) and hematological parameters (Hegazy et al., 2010), occurrence of oxidative stress and lipid peroxidation in different organs (Patra et al., 2001; Harishekar and Kiran, 2011) and DNA damage in blood cells (Danadevi et al., 2003).

The impacts on the health of those who eat contaminated animal-based foods should be constantly monitored (Arnold et al., 2005); thus, there is a need for studies on the actions of Pb on different body functions at different ages. Therefore, the aim of this study was to evaluate the effects of Pb intake using biomarkers that represent different levels of biological organization, such as biochemical, physiological and genotoxic markers in female rats at different ages that were supplied daily with Pb-contaminated feed at a concentration of 2.0 mg Pb kg^{-1} feed until they were 45 and 90 days old.

2. Material and methods

2.1. Animals

Female Wistar rats (*Rattus norvegicus*) supplied by the central animal house of the State University of Londrina were used in this study. Shortly after weaning (22 days), animals weighing 37 ± 5.58 g ($n = 40$) were transferred to the animal house of the Physiological Sciences Department, where they were housed individually in cages under standard conditions and 12:12-h light/dark cycle. Food and water were freely available. The weight of the animals and their feed intake were monitored three times a week. Feed intake was calculated by subtracting the feed remaining (from inside the cage and feed residue that fell into the tray below the cage) from the feed supplied. This work was approved by the ethics committee for animal experiments of the State University of Londrina, Brazil (CEUA/UEL – Process: 34715.2011.16).

2.2. Feed composition

Nuvilab® commercial feed for rats was ground, and Pb was added in the form of lead nitrate ($Pb(NO_3)_2$) at a ratio of 2.0 mg Pb per kg feed (dissolved in 800 mL water). New pellets were formed and dried at 60 °C for 24 h. The feed for the control group underwent the same process but without the addition of $Pb(NO_3)_2$. The Pb concentration of 2.0 mg kg^{-1} was defined based on the maximum Pb concentration for fish meat established by the Brazilian Health Surveillance Agency (ANVISA/685, 1998).

2.3. Pb determination in feed

Dry feed samples were digested in a solution of 5 N nitric acid (HNO_3 – Suprapur, Merck) at 60 °C for 48 h. After digestion, the material was centrifuged, and the supernatant was analyzed by graphite furnace atomic absorption spectrometry (AAnalyst 700, PerkinElmer, USA).

2.4. Experimental design

Four groups of female rats were used ($n = 10$ each), including 2 groups of 45-day-old animals (CTR45 and Pb45) and 2 groups of 90-day-old animals (CTR90 and Pb90). The animals were supplied manipulated feed shortly after weaning at 22 days of age until they were 45 (CTR45 and Pb45) or 90 days of age (CTR90 and Pb90). The CTR groups were supplied feed without added Pb, and the Pb groups were supplied feed contaminated with $Pb(NO_3)_2$. For female rats, 45 days of age corresponds to their pubescent period, and 90 days corresponds to adulthood, at which point they are able to mate (Anderson et al., 2004).

2.5. Sampling

The animals were weighed and euthanized by exsanguination under general anesthesia (40 mg kg^{-1} sodium thiopental) without prior fasting. Blood was collected from the inferior vena cava, and an aliquot was used to analyze the hematological parameters and to perform the alkaline comet assay. Another aliquot of whole blood was centrifuged ($1870 \times g$, 15 min, Hsiangtai centrifuge, MCD-2000 model), and plasma was used to determine glucose and insulin concentrations. The brain was removed to analyze acetylcholinesterase (AChE) enzymatic activity. The liver was removed to analyze the glutathione (GSH) content and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) enzymatic activities, along with the occurrence of lipid peroxidation and protein carbonylation (PCO).

2.6. Hematological parameters: hematocrit, hemoglobin and RBC

To determine hematocrit (Hct) blood from each animal was placed in a capillary tube and centrifuged ($1200 \times g$, 5 min) in a micro-capillary centrifuge (Luguimac – S.R.C., LC-5 model, Argentina) to separate cells from plasma, and readings were performed using a standardized chart. The number of erythrocytes per mm^3 blood (RBC) was counted by adding formaldehyde citrate buffer to blood and analyzing it in a Neubauer chamber under a light microscope. The hemoglobin content (Hb) was estimated using the cyanomethemoglobin colorimetric method (commercial kit, Labtest Diagnóstica, Brazil); readings were performed at 540 nm using a spectrophotometer (Libra S32, Biochrom, United Kingdom).

2.7. Plasma analysis: glucose, insulin and cortisol

Plasma glucose levels were determined using the glucose oxidase method with a commercial kit (Doles Reagentes Ltda, Brazil) and a microplate spectrophotometer (Victor 3, PerkinElmer, USA) at 505 nm. Insulin was measured using a radioimmunoassay (RIA) (Scott et al., 1981) in a gamma particle emission counter (Wizard^2 Automatic Gamma Counter™-2470, PerkinElmer®, Shelton, CT, USA), using rat insulin, anti-rat insulin antibody (Sigma–Aldrich®, St. Louis, MO, USA) and recombinant human insulin labeled with I^{125} (PerkinElmer®, Shelton, CT, USA) as standards. The hormone cortisol was measured using a commercial enzyme immunoassay kit (DRG International, Inc., USA) based on the principle of competitive binding. Absorbance was measured using a microplate reader (450 nm, ELX 800, BioTek, USA).

2.8. Biochemical analyses of the brain

Brain tissue samples were homogenized in potassium phosphate buffer (0.1 M; pH 8.0; 1:10 w/v) and centrifuged ($10{,}000 \times g$, 20 min, 4 °C), and the supernatant was used to measure AChE activity. AChE activity was measured as described by Ellman et al. (1961) and adapted for a microplate by Alves Costa et al. (2007), based on the reaction of acetylcholine iodide (9 mM) with the color reagent containing 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.5 mM) and read in a spectrophotometer at 415 nm. AChE activity was expressed in nmol DTNB min^{-1} mg $protein^{-1}$. Protein concentration in the homogenate was determined spectrophotometrically at 595 nm according to Bradford (1976), using bovine serum albumin (BSA) as the standard.

2.9. Biochemical analyses of the liver

Glycogen measurements were performed as described by Bidinotto et al. (1997), in which liver samples were incubated with 6.0 N KOH at 100 °C for 5 min. After tissue dissolution, ethanol and K_2SO_4 (10%) were added, the tissues were centrifuged and the pellet was resuspended in distilled water. The content of total reducing sugars in the dissolution was analyzed using the hydrolytic acid method described by Dubois et al. (1956). This analysis consists of adding 4.1% phenol and concentrated sulfuric acid to the tissue sample. The absorbance was determined at 480 nm (spectrophotometer, Libra S32, Biochrom, United Kingdom).

Liver samples were weighed, homogenized (1:10, w/v) in potassium phosphate buffer (0.1 M, pH 7.0) and centrifuged (10,000 × *g*, 20 min, 4 °C). The supernatant was used to analyze biochemical biomarkers. The protein concentration in the homogenate was determined spectrophotometrically at 595 nm according to Bradford (1976), using bovine serum albumin (BSA) as the standard.

The concentration of GSH in the liver sample was determined as described by Beutler et al. (1963), in which GSH reacts with the substrate DTNB to form the thiol (TNB), which was quantified at 412 nm.

SOD activity was measured by the inhibition of the rate of reduction of cytochrome c by superoxide (resulting from the oxidation of xanthine by xanthine oxidase) at 550 nm and is expressed as U SOD mg protein^{-1}, where U is the levels of SOD required to inhibit 50% of the rate of reduction of cytochrome c (McCord and Fridovich, 1969). CAT activity was evaluated based on the decomposition rate of hydrogen peroxide (H_2O_2) measured as a decrease in absorbance at 240 nm (Beutler, 1975). Enzyme activity was expressed in μmol H_2O_2 metabolized min^{-1} mg protein^{-1}. GPx activity was evaluated as described by Hopkins and Tudhope (1973) using the oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. Enzyme activity was expressed in nmol NADPH oxidized min^{-1} mg protein^{-1}. GR activity was estimated indirectly, by the reduction of NADPH by glutathione disulfide (GSSG), at 340 nm and expressed in nmol min^{-1} mg protein^{-1} (Carlberg and Mannervil, 1975). GST activity was determined spectrophotometrically by monitoring the conjugates formed from reduced GSH and the substrate CDNB at 340 nm, as described by Keen et al. (1976). Enzyme activity was expressed in nmol CDNB conjugated min^{-1} mg protein^{-1}.

Lipid peroxidation was estimated from the production of malondialdehyde (MDA), one of the products of lipid peroxidation. MDA concentrations were determined using the TBARS (thiobarbituric acid reactive substances) assay, which measures the levels of substances that react with thiobarbituric acid (TBA) at 60 °C based on fluorescence readings (ex/em: 535 mm/590 nm), as described by Federici et al. (2007).

To evaluate protein oxidative damage, the methods described by Levini et al. (1994) were used, which are based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyls (PCO) to form dinitrophenylhydrazones that can be detected at 348–370 nm.

2.10. Comet assay

The alkaline comet assay was performed with lymphocytes as described by Singh et al. (1988), with some modifications as described by Ramsdorf et al. (2009). Only blood samples that had more than 80% cell viability, as determined by the trypan blue exclusion method, were used in the comet assay. After sampling, an aliquot of blood mixed with fetal calf serum (1:10) was added to low-melt agarose. This mixture was placed in a glass slide that had previously been covered with normal agarose, and the slides were subjected to the following steps: (a) lysis: 1 h at 4 °C, protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA denaturation: 40 min, in the dark, in electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; (d) neutralization: three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). Slides were fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses.

The slides stained with GelRed (Uniscience®) were analyzed under a Leica microscope (DM 2500) adapted for fluorescence/epifluorescence, fitted with a blue excitation filter (450–490 nm) and a 515 nm barrier filter with a magnification of 1000×. All slides were blind-reviewed and 100 nucleotides were evaluated per animal. The extent of DNA damage was quantified by the length of the tail formed by the migration of the DNA fragments. Comets were classified by visual inspection, typically into five categories: 0 representing undamaged cells (comets with no or barely detectable tails) and 1–4 representing increasing relative tail intensities, according to Collins et al. (2008). DNA damage scores were obtained by multiplying the number of cells in each comet class by the class value.

2.11. Statistical analysis

Comparisons of the parameters evaluated in the female rats in each age category (45 and 90 days) were made between the CTR and Pb groups using Student's *t* test or the Mann–Whitney test, according to distribution of data (normality and homogeneity of variance). Values of $P < 0.05$ were considered significant. The results are expressed as mean ± standard deviations.

3. Results

3.1. Pb concentration in the feed

The concentrations of Pb were 2.09 ± 0.3 mg Pb kg^{-1} feed (mean ± SD, $n = 15$) in the feed contaminated with $Pb(NO_3)_2$ and 0.03 ± 0.02 (mean ± SD, $n = 6$) in feed of the CTR group.

3.2. Weight gain, feed intake and growth curves

The weight gain (Fig. 1A) and feed intake (Fig. 1B) were significantly greater in the animals in the Pb45 group compared to the respective CTR group. After 90 days, no significant differences were observed for these parameters between the Pb and respective CTR groups. An analysis of growth curves indicated that the weight of the animals in the Pb45 group increased more during weeks 2, 3 and 4 compared with the CTR45 group (Fig. 2A). The rats in the Pb90 group also gained more weight until week 3 (Fig. 2B), but after this period, there was no difference in weight fluctuations between the Pb and CTR groups.

3.3. Glucose, insulin and plasma cortisol

The plasma glucose values (Fig. 3A) were significantly higher in the animals in the Pb45 ($P = 0.015$) and Pb90 ($P = 0.029$) groups compared to their respective CTR groups. The plasma insulin levels (Fig. 3B) were significantly higher in the Pb45 group ($P = 0.008$) relative to the respective CTR group but were significantly lower in the group Pb90 relative to the respective CTR group ($P = 0.009$).

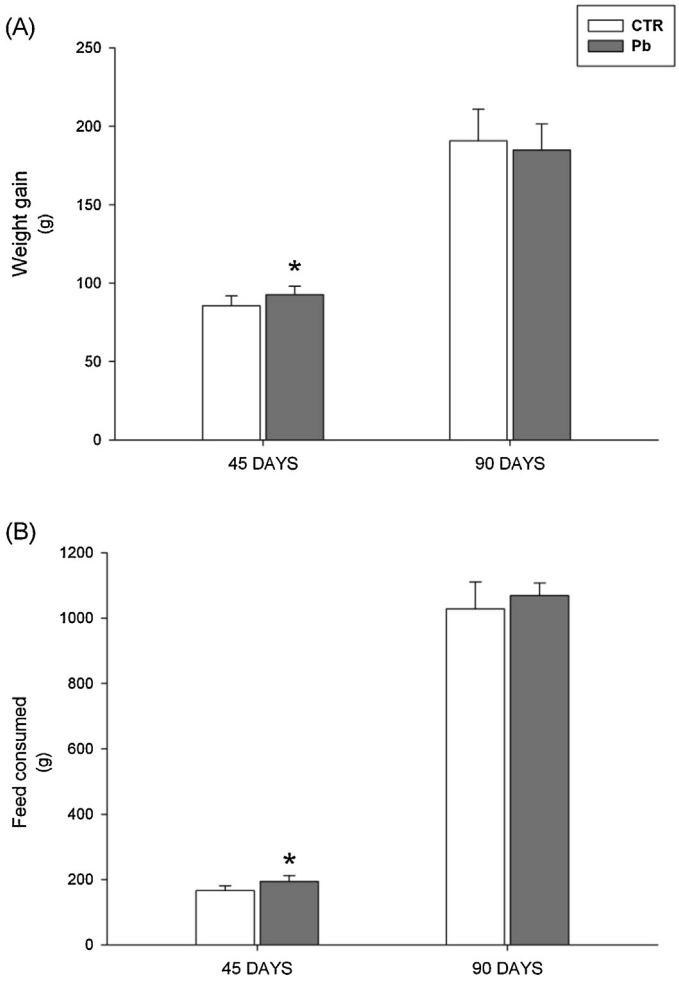

Fig. 1. Weight gain (A) and feed intake (B) in female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 8–10). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

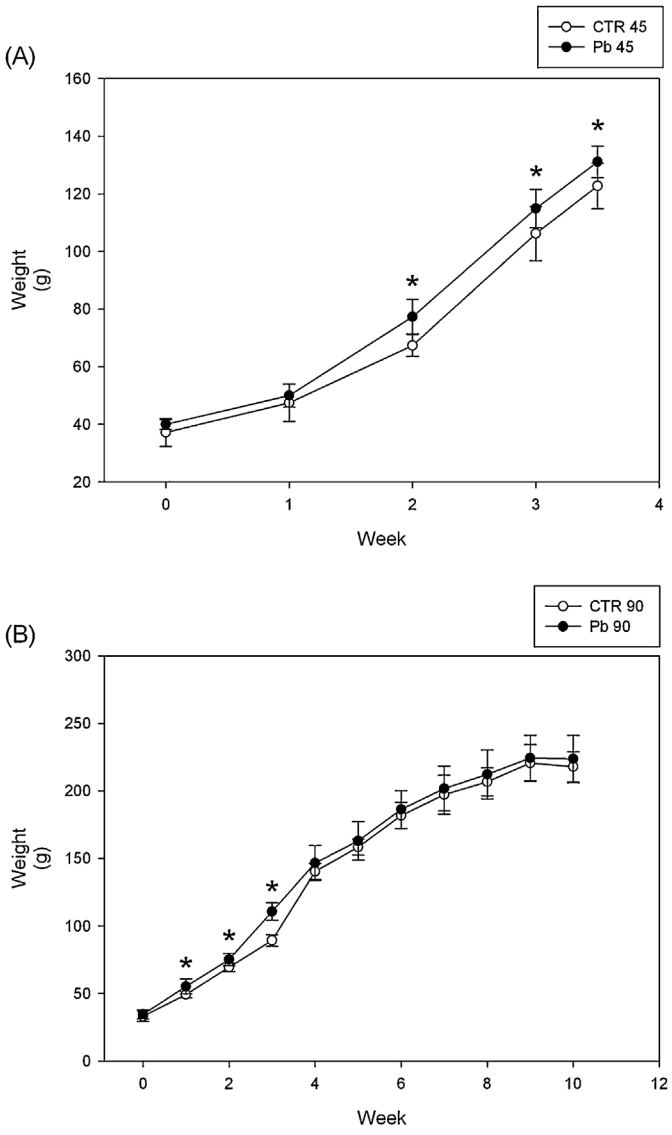

Fig. 2. Growth curve in female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 (A) and 90 days old (B). The values are presented as mean ± standard deviation (N = 8–10). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

There were no significant differences in the cortisol values (Fig. 3C) for either group compared to their respective CTR group.

3.4. Glycogen content in the liver

The glycogen content (Fig. 4) was lower in the Pb90 group (P < 0.001) compared to its CTR group.

3.5. Htc, Hb and RBC

For the hematological parameters Htc (Fig. 5A), Hb (Fig. 5B) and RBC (Fig. 5C), the results indicated that the animals in the Pb45 group did not show significant differences in Htc, Hb or RBC values compared to their respective CTR group. However, the 90-day-old rats in the Pb group showed significantly lower Hb values compared to their respective CTR group (P = 0.047), whereas the other parameters (Htc and RBC) remained unchanged.

3.6. AChE enzyme activity in the brain

The AChE enzyme activity levels (Fig. 6) in the brain of female rats in the Pb45 and Pb90 groups were not significantly different compared to their respective CTR groups.

3.7. Antioxidants, lipid peroxidation and protein carbonyls in the liver

In the liver, for the primary antioxidant enzymes SOD (Fig. 7A), CAT (Fig. 7B) and GPx (Fig. 7C), only the CAT (P = 0.022) and GPx (P = 0.020) activity levels were significantly lower in the animals in the Pb45 group compared to their respective CTR group. In contrast, the SOD values in the Pb45 and Pb90 groups and the CAT and GPX values in the Pb90 group remained unchanged compared to their respective CTR groups. The GSH content (Fig. 8A) was significantly higher in the Pb90 group compared to the CTR90 group (P = 0.013). However, the animals in the Pb45 group showed no significant changes in GSH content but did show significantly higher GR activity (Fig. 8B) (P = 0.030) compared to their respective CTR group. GR activity was not significantly different between groups at 90 days. GST activity (Fig. 8C) remained unchanged in both the Pb45 and Pb90 groups compared to their respective CTR groups. The TBARS assay (Fig. 9A) indicated that there was significantly more

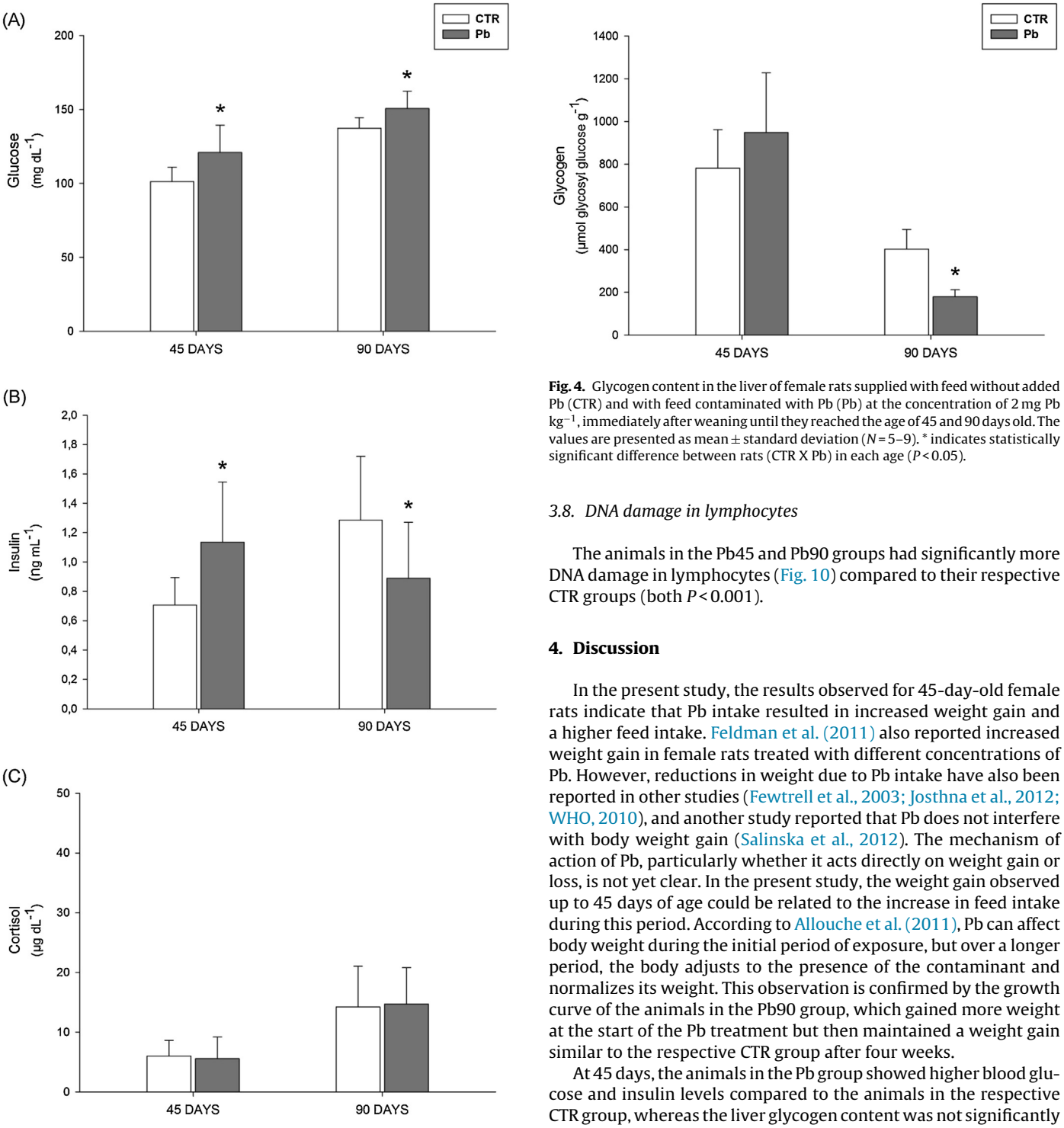

Fig. 3. Plasma glucose (A), insulin (B) and cortisol (C) in female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as means ± standard deviation (N = 6–10). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

lipid peroxidation in the livers of rats in the Pb45 (P = 0.002) and Pb90 (P = 0.010) groups compared to their respective CTR groups. However, there were no significant differences in the levels of protein carbonyls (Fig. 9B) in the liver of rats in both the P45 and P90 groups compared to their respective CTR groups.

Fig. 4. Glycogen content in the liver of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 5–9). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

3.8. DNA damage in lymphocytes

The animals in the Pb45 and Pb90 groups had significantly more DNA damage in lymphocytes (Fig. 10) compared to their respective CTR groups (both P < 0.001).

4. Discussion

In the present study, the results observed for 45-day-old female rats indicate that Pb intake resulted in increased weight gain and a higher feed intake. Feldman et al. (2011) also reported increased weight gain in female rats treated with different concentrations of Pb. However, reductions in weight due to Pb intake have also been reported in other studies (Fewtrell et al., 2003; Josthna et al., 2012; WHO, 2010), and another study reported that Pb does not interfere with body weight gain (Salinska et al., 2012). The mechanism of action of Pb, particularly whether it acts directly on weight gain or loss, is not yet clear. In the present study, the weight gain observed up to 45 days of age could be related to the increase in feed intake during this period. According to Allouche et al. (2011), Pb can affect body weight during the initial period of exposure, but over a longer period, the body adjusts to the presence of the contaminant and normalizes its weight. This observation is confirmed by the growth curve of the animals in the Pb90 group, which gained more weight at the start of the Pb treatment but then maintained a weight gain similar to the respective CTR group after four weeks.

At 45 days, the animals in the Pb group showed higher blood glucose and insulin levels compared to the animals in the respective CTR group, whereas the liver glycogen content was not significantly affected. These animals had higher feed intake during this period, which could have increased their blood glucose and insulin levels. Glucose and insulin are factors that lead to the deposition of hepatic glycogen (Bollen et al., 1998) and excess nutrients are converted into triacylglycerol in the liver and stored in adipose tissue (Nolan and Prentki, 2008), which may explain why these animals maintained their hepatic glycogen content, even with the consequent weight gain. The Pb seems to elevate blood glucose levels in both acute and chronic exposure cases. Krishna and Ramachandran (2009) evaluated the biochemical parameters induced by acute Pb exposure and observed that the group exposed only to Pb had high blood glucose concentrations. However, the 90-day-old animals in the Pb group had high blood glucose levels, but their insulin

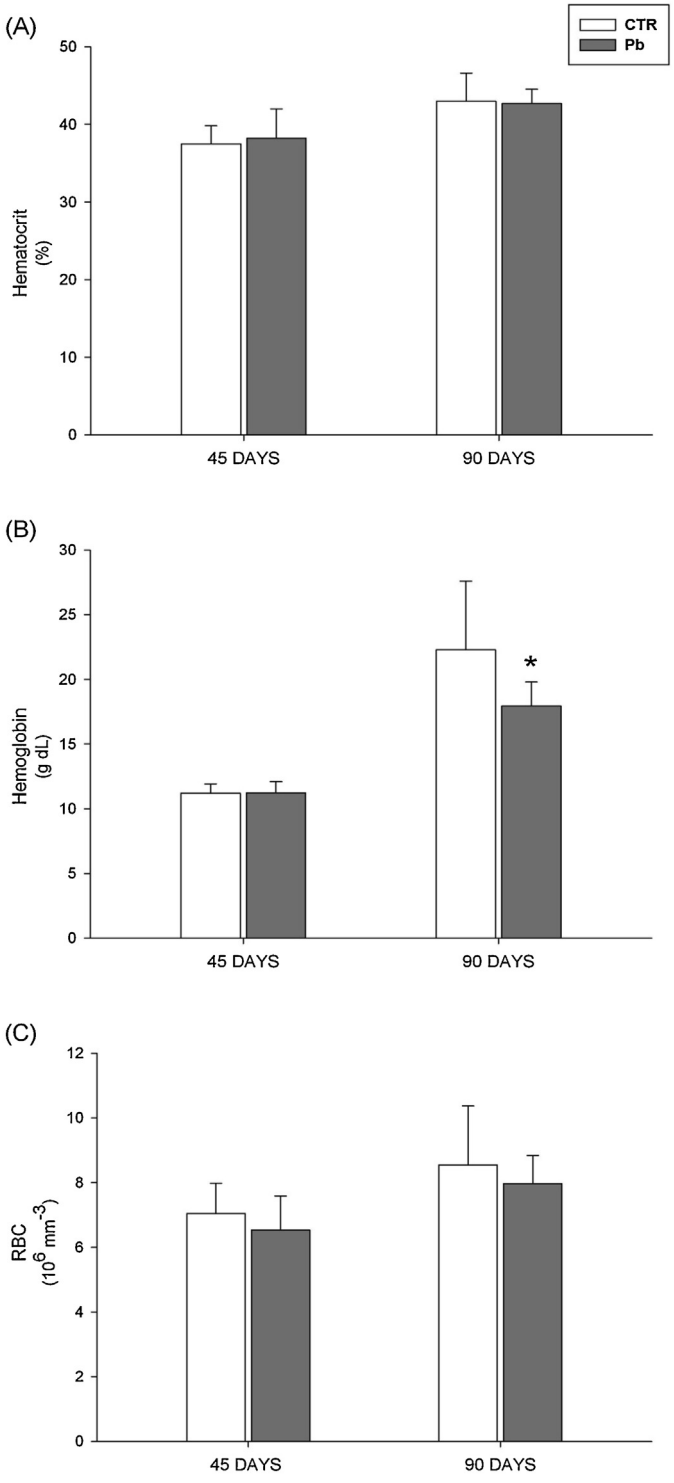

Fig. 5. Hematocrit (A), hemoglobin concentration (B) and red blood cells count – RBC (C) in female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as means ± standard deviation (N = 8–10). * indicates statistically significant difference between rats (CTR X Pb) in each age ($P < 0.05$).

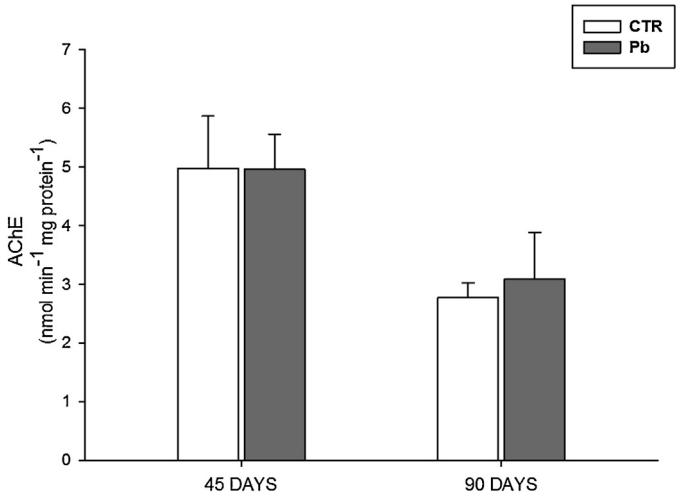

Fig. 6. AChE activity in the brain of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 5).

levels and liver glycogen contents were lower than the corresponding levels in the CTR group. Pb appears to elevate blood glucose levels, although the exact mechanism by which it mediates this effect is not yet clear. Feldman et al. (2011) observed increased blood glucose levels in rats treated with Pb for 2 and 3 months and cited Pb as a factor capable of altering various biochemical parameters, including blood glucose levels. This finding may be related to the fact that Pb suppresses the release of insulin from the pancreas. A reduction in the insulinogenic index (insulin-to-blood glucose ratio) in rats, after subacute and chronic lead treatment, was already observed by Stevenson et al. (1976), who suggested that the suppression of pancreatic function may be responsible for the disorders induced by Pb on glucose homeostasis. The suppression of the release of insulin by the pancreas may explain the increased glucose levels and decreased plasma insulin levels in the 90-day-old animals. First, Pb can promote lipid peroxidation as a consequence of oxidative stress in the pancreas (Ambali et al., 2011). Second, Pb can promote hyperglycemia (Feldman et al., 2011), and the prolonged exposure to high glucose concentrations can result in pancreatic β cell dysfunction (McKenzie et al., 2010). As for oxidative stress compared to other organs, the pancreas has a lower antioxidant defense capacity, given that SOD expression is only 30% of that found in the liver, and CAT and GPx are only at 5%. Thus, pancreatic β cells exhibit lower levels of antioxidant enzymes, and under oxidative stress conditions, they are unable to adapt by significantly upregulating the expression of the genes for these enzymes (Tiedge et al., 1997). Hyperglycemia results when the uptake of glucose into peripheral tissues is hindered by the decrease in insulin levels. However, at this point, the liver activates pathways to provide glucose to the blood. One of the important functions of the liver is to supply glucose to the bloodstream from gluconeogenesis and glycogen stored after meals (Felber and Golay, 1995), which might be the reason why these animals showed a significant decrease in liver glycogen content.

Pb is known to cause changes in the hematologic system, such as decreases in hemoglobin content and subsequent anemia (ATSDR, 2007). This effect of Pb was only observed in the 90-day-old female rats, which had lower hemoglobin levels. Pb causes anemia by blocking the synthesis of heme (the prosthetic group that is a component of hemoglobin) through the inhibition of two key enzymes: ALAD (delta-aminolevulinic acid dehydratase) and ferrochelatase (Flora et al., 2008; Patrick, 2006).

Pb poisoning can cause oxidative stress via two distinct but related pathways: (1) the direct action of Pb in the generation of ROS (reactive oxygen species), and (2) direct action in the reduction of antioxidant defenses (Patrick, 2006). The results of the present study on the antioxidant defense system mediated by enzymes SOD, CAT, GPx and GR and non-enzymatic pathways (GSH) indicate

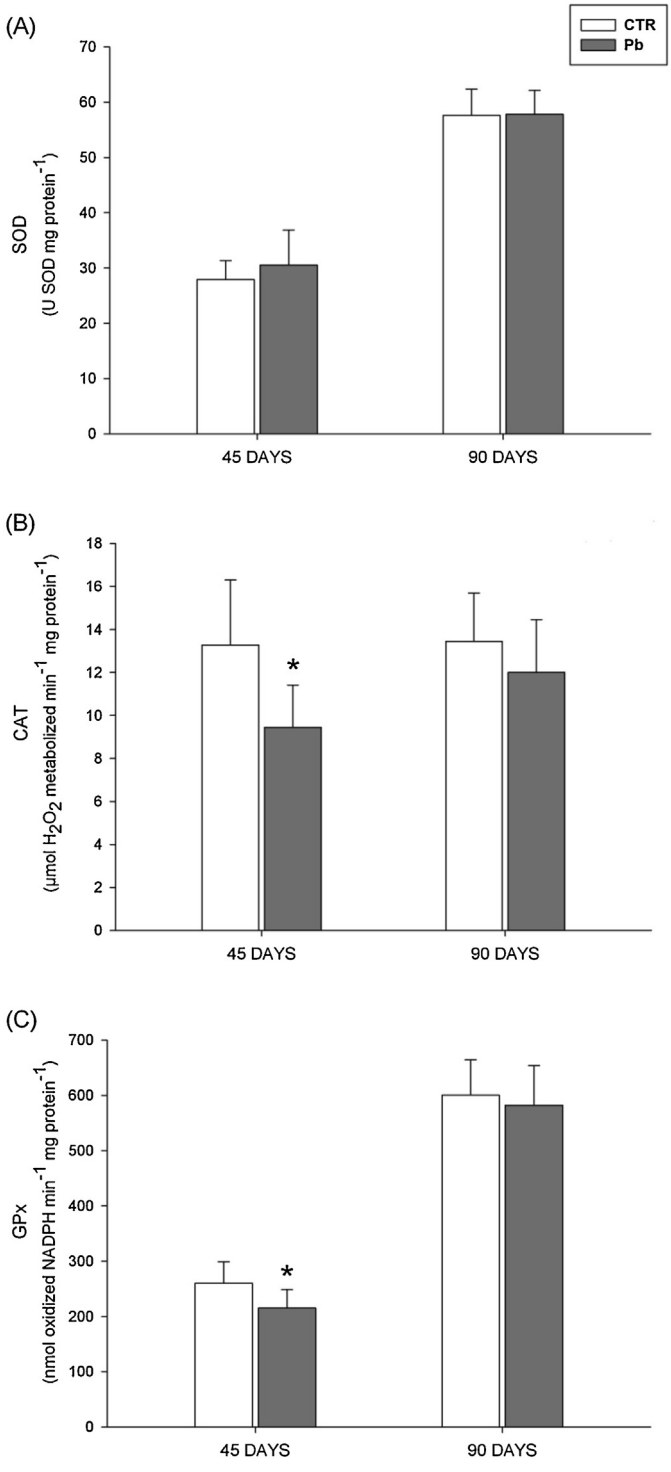

Fig. 7. Activity of SOD (A), CAT (B) and GPx (C) in the liver of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 7–10). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

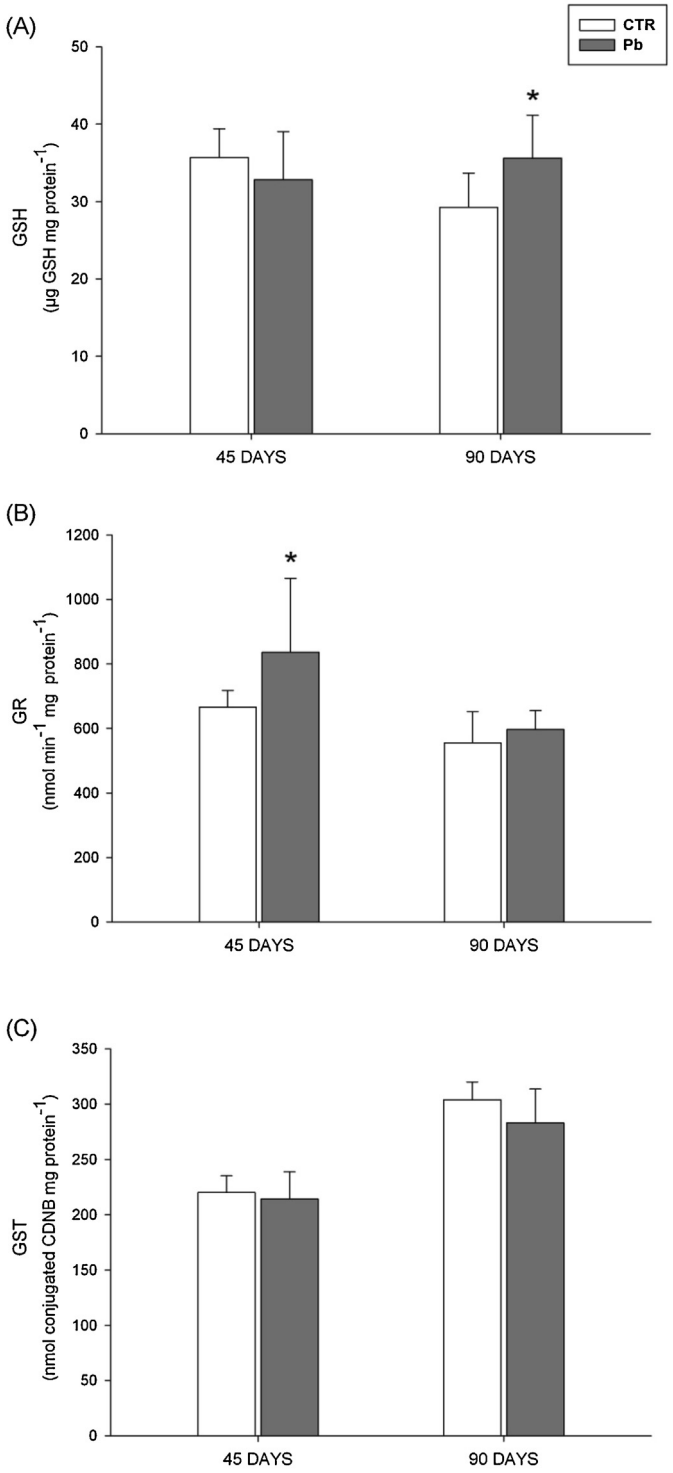

Fig. 8. GSH content (A) and activity of GR (B) and GST (C) in the liver of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 8–10). * indicates statistically significant difference between rats (CTR X Pb) in each age (P < 0.05).

that Pb interferes with the antioxidant defenses of the liver in both 45- and 90-day-old female rats. In the 45-day-old group, the three primary defenses for neutralizing ROS were in imbalance (SOD, CAT and GPx). SOD activity, which neutralizes superoxide anions, was normal, whereas CAT activity, which neutralizes hydrogen peroxide, and GPx activity, which neutralizes hydrogen peroxide and other peroxides, were both reduced. Although the increase in GR activity, which reduces GSSH to GSH, may have contributed so that the GSH values were not significantly lower in the livers of these rats, there was an imbalance between the production and neutralization of ROS caused by Pb, which can result in oxidative stress. Thus, with the decreases in GPx and CAT activity, the actions of Pb

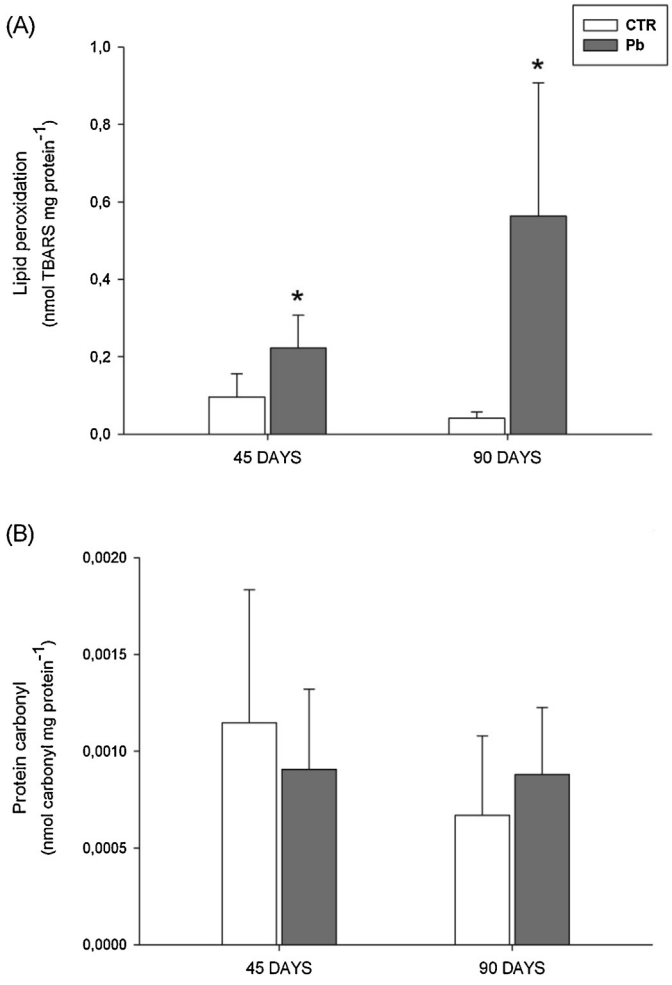

Fig. 9. Lipid peroxidation (A) and protein carbonyl (B) in liver of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 5–9). * indicates statistically significant difference between rats (CTR X Pb) in each age ($P < 0.05$).

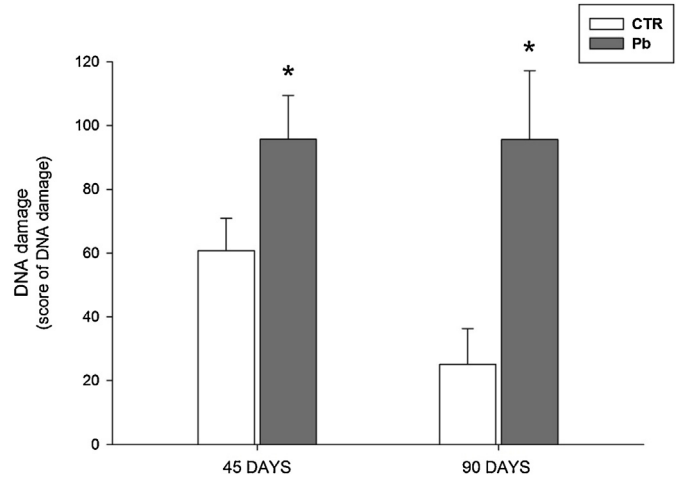

Fig. 10. Scores of DNA damage in lymphocytes of female rats supplied with feed without added Pb (CTR) and with feed contaminated with Pb (Pb) at the concentration of 2 mg Pb kg^{-1}, immediately after weaning until they reached the age of 45 and 90 days old. The values are presented as mean ± standard deviation (N = 7–8). * indicates statistically significant difference between rats (CTR X Pb) in each age ($P < 0.05$).

resulting in the decrease in antioxidant defenses was more evident. Pb can act directly on GPx and form a complex with the selenium ion that is essential for its activity, thus decreasing its activity (Flora et al., 2008). Pb can also act on CAT, which contains heme in its structure, given that Pb is known to inhibit heme synthesis (Flora et al., 2008).

Although the animals exposed to Pb for 45 days had decreased antioxidant defenses, such was not the case for the 90-day-old animals, which had increased GSH content and increased activity levels of defense enzymes similar to those of the respective CTR group. Stohs and Bagchi (1995) reported that as with some transition metals, such as cadmium, Pb produces an increase in GSH levels in tissues, including the liver, kidneys and erythrocytes. This increase in GSH appears to be a compensatory response aimed at attenuating Pb toxicity; however, this response was not sufficient to prevent the oxidative damage observed in the present study.

Pb intake resulted in lipid peroxidation in the liver of both the 45- and 90-day-old female rats, which is indicative of oxidative stress. Pb can promote lipid peroxidation either by increasing the number of hydroxyl radicals and their subsequent action on cellular membrane polyunsaturated fatty acids or by the capacity of Pb to stimulate the ferrous ions (Fe^{2+}) to initiate lipid peroxidation in membranes. There is a relationship between the membrane damage induced by Pb and the natural changes that occur in the composition of membrane fatty acids. The elongation and unsaturation of linoleic acid to form arachidonic acid is associated with the susceptibility of the membrane to peroxidation, as it increases the number of double bonds (Lawton and Donaldson, 1991). Ahamed and Siddiqui (2007) reported that after incubating linoleic acid, linolenic acid and arachidonic acid with Pb, which increased the number of double bonds, there was an increase in the final product of MDA. Pb intake appears to increase arachidonic acid levels in tissues in a dose-dependent manner, and it is believed that many of the biological effects observed in Pb toxicity are the result of changes in the composition of fatty acids in cellular membranes (Donaldson and Knowles, 1993). These changes could be a possible cause for the results observed in the female rats in the Pb90 group, in which lipid peroxidation increased over 140% in hepatocytes.

Pb exposure is also associated with DNA damage. This fact was also confirmed in the present study, which showed an increase in DNA damage in lymphocytes of the female rats in both the Pb45 and Pb90 groups. ATSDR (2007) describes a significant correlation between DNA strand breaks, increased ROS production and decreased antioxidant defenses in lymphocytes, indicating that the oxidative stress caused by Pb is one possible cause for this damage. In addition, the review by García-Lestón et al. (2010) notes that studies, including studies on lymphocytes, indicate the mechanisms by which Pb causes genotoxicity are indirect, such as the production of free radicals, but that Pb can also act by inhibiting DNA repair.

In summary, the results of the present study indicate that the daily intake of Pb at concentrations of 2.0 mg Pb kg^{-1} results in significant changes in the body, which may vary with age. Although the increases in blood glucose and insulin levels in the animals in the Pb45 group can be accounted for by the increased feed intake resulting in more weight gain, in the animals in the Pb90 group, there was an increase of blood glucose levels with insulin suppression in addition to reduced hepatic glycogen content. This finding suggests that Pb intake at this concentration can result in increased food intake and weight gain and that over time, insulin suppression may occur with a consequent increase in blood glucose levels, with glycogen utilization further elevating blood glucose levels. Among the hematological parameters analyzed, only the Hb content was significantly reduced and only in the Pb90 group, that is, after a longer period of Pb consumption. At both 45 and 90 days of age, there was no significant change in AChE activity in the brain.

The antioxidant defenses were most affected by Pb in the 45-day-old female rats, which had less CAT and GPx activities and higher GR activity, without changes in the GSH content. However, in the 90-day-old female rats, all of the antioxidant enzymes were at levels similar to the control rats, although the GSH content was now higher. However, these increased levels were not able to prevent lipid peroxidation from occurring in the livers of these animals, as was observed in the animals in the Pb45 group. It was also noted that in both the Pb45 and Pb90 groups, there was increased incidence of DNA damage in lymphocytes, but none of the study groups presented increases in protein oxidation. Thus, these results indicate that Pb ingestion at concentrations of 2.0 mg Pb kg^1 is not safe for daily consumption because it can damage health at various levels of biological organization, with different responses depending on whether an individual is older or younger.

Conflict of interest

There is no conflict of interest.

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References

Arnold, S.M., Lynn, T.V., Verbrugge, L.A., Middaugh, J.P., 2005. Human biomonitoring to optimize fish consumption advice: reducing uncertainty when evaluating benefits and risks. Am. J. Public Health 95, 393–397, http://dx.doi.org/10.2105/AJPH.2004.042879.

Ahamed, M., Siddiqui, M.K.J., 2007. Low level lead exposure and oxidative stress: current opinions. Clin. Chim. Acta 383, 57–64, http://dx.doi.org/10.1016/j.cca.2007.04.024.

Allouche, L., Hamadouche, M., Touabti, A., Khennouf, S., 2011. Effect of long-term exposure to low or moderate lead concentrations on growth, lipid profile and liver function in albino rats. Adv. Biol. Res. 5, 339–347.

Alves Costa, J.R.M., Mela, M., Assis, H.C.S., Pelletier, E., Randi, M.A.F., Oliveira, C.R.A., 2007. Enzymatic inhibition and morphological changes in *Hoplias malabaricus* from dietary exposure to lead (II) or methylmercury. Ecotoxicol. Environ. Saf. 67, 82–88, http://dx.doi.org/10.1016/j.ecoenv.2006.03.013.

Ambali, S.F., Shuaib, K., Edeh, R., Orieji, B.C., Shittu, M., Akande, M., 2011. Hyperglycemia induced by subchronic co-administration of chlorpyrifos and lead in Wistar rats: role of pancreatic lipoperoxidation and alleviating effect of vitamin C. Biol. Med. 3, 6–14.

Anderson, M.L., D'Almeida, V., Ko, G.M., Kawakami, R., Martins, P.J.F., Magalhães, L.E., Tufik, S., 2004. Princípios éticos e práticos do uso de animais de experimentação. São Paulo: UNIFESP – Universidade Federal de São Paulo, São Paulo.

ANVISA (Agência Nacional de Vigilância Sanitária), 1998. Princípios Gerais para o Estabelecimento de Níveis Máximos de Contaminantes Químicos em Alimentos. D.O.U. – Diário Oficial da União; Poder Executivo, Brasil, de 28 de agosto de 1998.

Ashry, K.M., El-Sayed, Y.S., Khamiss, R.M., El-Ashmawy, I.M., 2010. Oxidative stress and immunotoxic effects of lead and their amelioration with myrrh (*Commiphora molmol*) emulsion. Food Chem. Toxicol. 48, 236–241.

ATSDR (Agency for Toxic Substances and Disease Registry), 2007. Toxicological Profile for Lead. U.S. Department of Health and Human Services, Public Health Services, Atlanta, GA.

Beutler, E., 1975. Red Cell Metabolism: A Manual of Biochemical Methods. Grune and Straton, New York.

Beutler, E., Durom, O., Kelly, B.M., 1963. Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61, 882–890.

Bidinotto, P.M., Moraes, G., Souza, R.H.S., 1997. Hepatic glycogen and glucose in eight tropical fresh water teleost fish: a procedure for field determinations of micro samples. Biol. Tec. CEPTA 10, 53–60.

Bollen, M., Keppens, S., Stalmans, W., 1998. Specific features of glycogen metabolism in the liver. Biochem. J. 336, 19–31, http://dx.doi.org/10.1042/bj3360019.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254, http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Carlberg, I., Mannervil, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Castro-González, M.I., Méndez-Armenta, M., 2008. Heavy metals: implications associated to fish consumption. Environ. Toxicol. Pharmacol. 26, 263–271, http://dx.doi.org/10.1016/j.etap.2008.06.001.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Štětina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151, http://dx.doi.org/10.1093/mutage/gem051.

Danadevi, K., Rozati, R., Banu, S.B., Rao, P.H., Grover, P., 2003. DNA damage in workers exposed to lead using comet assay. Toxicology 187, 183–193, http://dx.doi.org/10.1016/S0300-483X(03)00054-4.

Donaldson, W.E., Knowles, S.O., 1993. Is lead toxicosis a reflection of altered fatty acid composition of membranes? Comp. Biochem. Physiol. 104C, 377–379, http://dx.doi.org/10.1016/0742-8413(93)90003-4.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356, http://dx.doi.org/10.1021/ac60111a017.

Eades, L.J., Farmer, J.G., MacKenzie, A.B., Kirika, A., Bailey-Watts, A.E., 2002. Stable lead isotopic characterization of the historical record of environmental lead contamination in dated freshwater lake sediment cores from northern and central Scotland. Sci. Total Environ. 292, 55–67, http://dx.doi.org/10.1016/S0048-9697(02)00026-8.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95, http://dx.doi.org/10.1016/0006-2952(61)90145-9.

FAO (Food and Agriculture Organization of the United Nations), 2007. Future prospects for fish and fishery products. Fish Consumption in the European Union in 2015 and 2030, Part 1. European Overview. Rome.

Felber, J.P., Golay, A., 1995. Regulation of nutrient metabolism and energy expenditure. Metabolism 14, 4–9, http://dx.doi.org/10.1016/0026-0 495(95)90201-5.

Federici, G., Shaw, B.J., Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. Aquat. Toxicol. 84, 415–430, http://dx.doi.org/10.1016/j.aquatox.2007.07.009.

Feldman, G., Chain, S., Soria, N., Bautista, N., Riera, N.M., 2011. Presencia de síndrome metabólico en ratas inducido por distintas concentraciones de plomo. Insuf. Card. 3, 112–116.

Fewtrell, L., Kaufmann, R., Prüss-Üstün, A., 2003. Lead: assessing the environmental burden of disease at national and local levels. Environmental Burden of Disease Series, No. 2, 2. World Health Organization, Geneva.

Flora, S.J.S., Mirtal, M., Mehta, A., 2008. Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian J. Med. Res. 128, 501–523.

García-Lestón, J., Méndez, J., Pásaro, E., Laffon, B., 2010. Genotoxic effects of lead: an updated review. Environ. Int. 36, 623–636, http://dx.doi.org/10.1016/j.envint.2010.04.011.

Garrick, M.D., Dolan, K.G., Horbinski, C., Ghio, A.J., Higgins, D., Porubcin, M., Moore, E.G., Hainsworth, L.N., Umbreit, J.N., Conrad, M.E., Feng, L., Lis, A., Roth, J.A., Singleton, S., Garrick, L.M., 2003. DMT1: a mammalian transporter for multiple metals. Biometals 16, 41–54, http://dx.doi.org/10.1023/A:1020702213099.

Harishekar, M.B., Kiran, B., 2011. Studies on lipid peroxidation induced by lead, alcohol on liver and amelioration by vitamin E. Asia J. Pharmacol. Clin. Res. 4, 164–167.

Hegazy, A.A.M., Zaher, M.M., Abdel-Hafez, M., Morsy, A.A., Saleh, R.A., 2010. Relation between anemia and blood levels of lead, copper, zinc and iron among children. BMC Research Notes 3, 133, http://dx.doi.org/10.1186/1756-0500-3-133.

Hopkins, J., Tudhope, G.R., 1973. Glutathione peroxidase in human red cells in health and disease. J. Hematol. 25, 563–575, http://dx.doi.org/10.1111/j.1365-2141.1973.tb01768.x.

IARC (Internacioanl Agency for Research on Cancer), 2006. World Health Organization: Summaries & evaluations: Inorganic and organic lead compounds. IARC Monographs for the Evaluation of Carcinogenic Risks to Humans, vol. 87. International Agency for Research on Cancer, Lyon.

Josthna, P., Geetharathan, T., Sujatha, P., Deepika, G., 2012. Accumulation of lead and cadmium in the organs and tissues of albino rat. Int. J. Pharm. Life Sci. 3, 2186–2189.

Keen, J.H., Habig, W.H., Jakobi, W.B., 1976. Mechanism for the several activities of the glutathione-S-transferases. J. Biol. Chem. 251, 6183–6188.

Krishna, H., Ramachandran, A.V., 2009. Biochemical alterations induced by the acute exposure to combination of chlorpyrifos and lead in Wistar rats. Biol. Med. 1, 1–6.

Lawton, L.J., Donaldson, W.E., 1991. Lead-induced tissue fatty acid alterations and lipid peroxidation. Biol. Trace Elem. Res. 28, 83–97, http://dx.doi.org/10.1007/BF02863075.

Leung, H.M., Leung, A.O.W., Wang, H.S., Ma, K.K., Liang, Y., Ho, K.C., Cheung, K.C., Tohidi, F., Yung, K.K.L., 2014. Assessment of heavy metals/metalloid (As, Pb, Cd, Ni, Zn, Cr, Cu, Mn) concentrations in edible fish species tissue in the Pearl River Delta (PRD), China. Mar. Pollut. Bull. 78, 235–245, http://dx.doi.org/10.1016/j.marpolbul.2013.10.028.

Levini, R.L., Williams, J.A., Stadman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Method. Enzymol. 233, 346–357.

Liu, J., Goyer, R.A., Waalkes, M.P., 2008. Toxic effects of metals. In: Klaassen, C., Doull's, J., Casarett, L.J. (Eds.), Toxicology: The Basic Science of Poisons. Mac Graw-Hill, New York, pp. 931–979.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: na enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
McKenzie, M.D., Jamieson, E., Jansen, E.S., Scott, C.L., Huang, D.C.S., Bouillet, P., Allison, J., Kay, T.W.H., Strasser, A., Thomas, H.E., 2010. Glucose induces pancreatic islet cell apoptosis that requires the BH3-only proteins Bim and Puma and multi-BH domain protein Bax. Diabetes J. 59, 644–652, http://dx.doi.org/10.2337/db09-1151.
Mielke, H.W., Gonzales, R.C., Powell, E.T., Mielke, P.W., 2013. Environmental and health disparities in residential communities of New Orleans: the need for soil lead intervention to advance primary prevention. Environ. Int. 51, 73–81, http://dx.doi.org/10.1016/j.envint.2012.10.013.
MPA (Ministério da Pesca e Aquicultura), 2013. Cartilha balanço 2013, pesca e aquicultura. Brasil.
Nolan, C.J., Prentki, M., 2008. The islet β-cell: fuel responsive and vulnerable. Trends Endocrinol. Metab. 19, 285–291, http://dx.doi.org/10.1016/j.tem.2008.07.006.
Paoliello, M.B., Capitini, E.M., 2005. Environmental contamination and human exposure to lead in Brazil. Rev. Environ. Contam. Toxicol. 184, 59–96, http://dx.doi.org/10.1007/0-387-27565-7_2.
Patra, R.C., Swarup, D., Dwivedi, S.K., 2001. Antioxidant effects of α tocoferol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology 162, 81–88, http://dx.doi.org/10.1016/S0300-483X(01)00345-6.
Patrick, L., 2006. Lead Toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Alternative Med. Rev. 11, 114–127.
Rahman, M.S., Molla, A.H., Saha, N., Rahman, A., 2012. Study on heavy metals levels and its risk assessment in some edible fishes from Bangshi River, Savar, Dhaka. Bangladesh. Food Chem. 134, 1847–1854, http://dx.doi.org/10.1016/j.foodchem.2012.03.099.
Ramsdorf, W.A., Guimarães, F.S.F., Ferraro, M.V.M., Gabardo, J., Trindade, E.S., Cestari, M.M., 2009. Establishment of experimental conditions for preserving samples of fish blood for analysis with both comet assay and flow cytometry. Mutat. Res. 673, 78–81, http://dx.doi.org/10.1016/j.mrgentox.2008.11.010.
Salinska, A., Wlostowski, T., Zambrzycka, E., 2012. Effect of dietary cadmium and/or lead on histopathological changes in the kidneys and liver of bank voles *Myodes glareolus* kept in different group densities. Ecotoxicology 21, 2235–2243, http://dx.doi.org/10.1007/s10646-012-0979-z.
Scott, A.M., Atwater, I., Rojas, E., 1981. A method for the simultaneous measurement of insulin release and β cell membrane potential in single mouse islet of Langerhans. Diabetologia 21, 470–475.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A single technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191, http://dx.doi.org/10.1016/0014-4827(88)90265-0.
Stevenson, A., Merali, Z., Kacew, S., Singhal, R.L., 1976. Effects of subacute and chronic lead treatment on glucose homeostasis and renal cyclic. Toxicology 6, 265–275.
Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Bio. Med. 18, 321–336, http://dx.doi.org/10.1016/0891-5849(94)00159-H.
Terra, B.F., Araújo, F.G., Calza, C.F., Lopes, R.T., Teixeira, T.P., 2008. Heavy metal in tissues of three fish species from different trophic levels in a tropical Brazilian river. Water Air Soil Pollut. 187, 275–284, http://dx.doi.org/10.1007/s11270-007-9515-9.
Tiedge, M., Lortz, S., Drinkgern, J., Lenzen, S., 1997. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 46, 1733–1942, http://dx.doi.org/10.2337/diab.46.11.1733.
UNEP (United Nations Environment Programme), 2008. Draft Final Review of Scientific Information on Lead. United Nations Environment Programme Chemicals Branch, Geneva.
WHO (Wold Health Organization), 2010. Exposure to Lead: A Major Public Health Concern. World Health Organization, Department of Public Health and Environment, Geneva.