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Original Article

Comet assay to evaluate chromosomal changes in chickens (*Gallus gallus domesticus*) contaminated by lead in a city in Bahia

Teste cometa para avaliar alterações cromossômicas em frangos (*Gallus gallus domesticus*) contaminados por chumbo em uma cidade da Bahia

J. T. Braga-Neto[a], S. O. Tozetto[a], F. S. Oliveira[a], T. A. Conceição[a], W. P. C. Santos[b], M. S. Fernandes[c] and M. D. Baliza[a]*

[a]Universidade Federal do Recôncavo da Bahia – UFRB, Centro de Ciências da Saúde, Complexo Multidisciplinar de Estudos e Pesquisa em Saúde, Santo Antônio de Jesus, BA, Brasil

[b]Instituto Federal de Educação, Ciência e Tecnologia da Bahia – IFBA, Salvador, BA, Brasil

[c]Universidade Federal da Fronteira Sul – UFFS, Campus Passo Fundo, Passo Fundo, RS, Brasil

Abstract

Chicken (*Gallus gallus domesticus*) is one of the primary sources of animal protein for the Brazilian population. Thus, the safety of this food is highly relevant. This study was based on the evidence of severe contamination of these animals by metals such as lead in Santo Amaro, Bahia. This exploratory study aimed to evaluate associations between lead levels in blood of chicken exposed to a contaminated area with the occurrence of chromosomal alterations, evidencing genotoxic effects. Serum lead analysis was performed by GF-AAS after dilution with a matrix modifier solution (Triton X-100 0.2% v/v and HNO3 0.1% v/v), while chromosomal damage was evaluated using the comet assay. The results showed genotoxic effects (positive comet assay) only for the specimen sample with higher serum lead concentrations (33.9 µg dL^{-1}), suggesting the occurrence of toxic effects at this level of exposure. This work evaluated a relationship between the reduction of serum lead levels in chicken and increased distance from the primary polluting source – a lead processing plant (COBRAC). It also showed that lead is bioavailable in this territory, contaminating chicken and causing genotoxic effects in these animals, further expanding the concern with the local biota and the health of the residents of Santo Amaro.

Keywords: lead toxicity, comet assay, chromosomal damage, Santo Amaro-BA.

Resumo

O frango (*Gallus gallus domesticus*) é uma das principais fontes de proteína animal para população brasileira, sendo que a segurança deste alimento é extremamente relevante. Assim, evidências de severa contaminação dessas aves, por metais como chumbo no município de Santo Amaro – BA, estimularam a realização deste estudo. O objetivo deste estudo exploratório foi avaliar associações entre os níveis de chumbo no sangue de frangos expostos a área contaminada com a ocorrência de alterações cromossômicas, evidenciando efeitos genotóxicos. As análises de chumbo sérico foram realizadas por GF-AAS após diluição em uma solução modificadora de matriz (Triton X-100 0,2% v/v e HNO3 0,1% v/v), enquanto os danos cromossômicos foram avaliados empregando o teste cometa. Os resultados obtidos evidenciaram efeitos genotóxicos (teste cometa positivo) apenas para amostra do espécime que apresentou concentrações séricas de chumbo mais elevadas (33.9 µg dL^{-1}), sugerindo a ocorrência de efeitos tóxicos neste nível de exposição. Neste trabalho foi possível avaliar claramente uma relação entre a redução dos níveis séricos de chumbo no frango com o aumento da distância da principal fonte poluidora – uma fábrica de processamento de chumbo (COBRAC). O presente estudo evidenciou que neste território o chumbo está biodisponível, contaminando aves de criação e acarretando em efeitos genotóxicos nestes animais, ampliando ainda mais a preocupação com a biota local e com a saúde dos moradores de Santo Amaro.

Palavras-chave: contaminação por chumbo, teste cometa, dano cromossômico, Santo Amaro-BA.

1. Introduction

The city of Santo Amaro is located 72 km from Salvador (BA), has about 60.190 inhabitants (IBGE, 2021), and was where the lead ore beneficiation company, Companhia Brasileira de Chumbo (COBRAC), was established in the 1960s. From the beginning of its operation until its deactivation, the company caused, for more than 30 years, environmental contamination by toxic metals, such as lead, cadmium, and others (Machado et al., 2013).

*e-mail: marciliobaliza@ufrb.edu.br
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It is estimated that around 500 thousand tons of waste rich in toxic metals, with 1 to 3% lead, were dumped into the environment, dispersing contaminants throughout the city and region (Macedo et al., 2016). This case is widely described in the scientific literature since the first doctoral thesis works (Carvalho, 1978; Reis, 1975; Spínola, 1975), books with comprehensive literature reviews (Fernandes et al., 2012; Ferran, 2007; Oliveira et al., 2018) and scientific papers recognizing addressing studies on the site's contamination history (Andrade and Moraes, 2013; Carvalho et al., 2003, 2018; Costa et al., 2020; Macedo et al., 2016; Machado et al., 2013; Muñoz Magna et al., 2013, 2014).

Such contaminations can induce chromosomal alterations, leading to flaws in the proper separation of chromosomes during meiosis, resulting in variations in the chromosomal content of the gametes. Gametes have fragile sites, with regions susceptible to breaks, causing mutation and giving rise to qualitative or quantitative changes in the genetic material (Mitra et al., 2017).

Food intake is one of the primary routes of heavy metal contamination in humans. Thus, the population of Santo Amaro could be affected when the food consumed presents bioaccumulative contaminants in the food chain, especially lead (Pb). The results of previous studies conducted by our research group showed that, among the foods (of plant and animal origin) produced in Santo Amaro and most consumed by the population, the highest levels of cadmium and lead were found in samples of chicken (*Gallus gallus domesticus*) muscle tissue and in the cassava (*Manihot esculenta*) (Macedo et al., 2016).

In this sense, the concern arises regarding the possible increase in the mutagen load of the chicken species studied, mainly triggered by Pb. Knowing that all species genetically control the rate of their mutations through DNA repair processes, it is essential to understand the mutational processes and their causing factors since it allows for managing and minimizing unwanted effects (Lawal et al., 2021).

Although there are several studies on the contamination of biotic and abiotic elements by toxic metals in Santo Amaro (Carvalho et al., 1984; Costa et al., 2019; FUNASA, 2003; Hatje et al., 2006, 2010; Hatje and Andrade, 2009; Macedo et al., 2016; Muñoz Magna et al., 2013; Santos and Anjos, 2022), few tried to demonstrate potential biological effects caused by these contaminants (Batista et al., 2017; Carvalho et al., 1987; Pinheiro et al., 2023). Therefore, the present study aims to analyze possible chromosomal degradations in chickens (*Gallus gallus domesticus*) reared in an area potentially impacted by heavy metals and its associations with serum lead concentrations. The results of this study may reveal significant dietary risk factors for the local population, genetic implications in the production of these birds, and genotoxic impacts on humans. From our knowledge, it was the first time that this kind of study was carried out in this heavily contaminated region.

2. Material and Methods

2.1. Sampling

This study was conducted as an exploratory assessment of an area known to be contaminated by lead, with few local chicken producers and the specimens raised in open spaces. It also has a low production and a restrictive number of animals available, therefore the size and distribution of the sample was defined by convenience. One chicken specimen was obtained from four different small local producers of Santo Amaro (Bahia) distributed in different locations of the city. Figure 1 illustrates the sampling points and rearing properties used in this research. The location of the sample showed different distances from the main source of contamination (COBRAC): P1 = 7,22 Km, P2 = 7,76 Km, P3 = 6,35 Km and P4 = 1,44 Km.

Figure 1. Chicken sampling points and location of the primary pollution source - the lead plant (COBRAC).

The samples were numbered from 1 to 4, with sample 1 referring to sampling point P1, sample 2 to P2, sample 3 to P3, and sample 4 to P4 (Figure 1).

These specimens were transported to the Health Sciences Center of the Federal University of Recôncavo da Bahia (UFRB) in Santo Antônio de Jesus (BA), where they underwent venipuncture for blood collection. The study was approved by the Animal Ethics Committee of UFRB (Protocol BL9242N).

2.2. Chromosomal damage - comet assay

Blood samples were collected by venipuncture (5 mL) in tubes with heparin anticoagulant and processed immediately. The samples were evaluated in quintuplicate, initially centrifuged at 10.000 rpm. Subsequently, the leukocytes were collected using a micropipette. The comet assay was performed according to the methodology proposed by Singh et al. (1988) with modifications in the nuclei staining, using a silver nitrate solution instead (Nadin et al., 2001). An aliquot of 5 µL was added to 75 µL of low-melting 0.75% agarose at 37 °C in a water bath. The mixture was spread on microscopy slides pre-covered with 1.5% standard agarose. The slides were then covered with a coverslip and stored in a refrigerator until solidified. After solidification, the coverslips were carefully removed. The slides were placed in appropriate glass cuvettes with cold lysis solution (2.5 M NaCl, 100mM EDTA, and 10mM TRIS, pH 10-10. 5) and protected from light. At the time of use, 1% of Triton X-100 + 10% DMSO was added. Cuvettes were placed in the refrigerator overnight.

Subsequently, the slides were placed in a horizontal electrophoresis tray covered with a cold electrophoresis buffer (300 mM NaOH and 1 mM EDTA). Electrophoresis was performed under alkaline conditions (pH > 13), at 25 V, 300 mA, in the dark for 15 minutes. After electrophoresis, the samples were neutralized with a buffer solution (0.4 M TRIS, pH 7.5) for 5 minutes. This process was repeated thrice, and the buffer was discarded at each exchange. Then, the slides were washed twice in distilled water and put to dry overnight at room temperature. The next day, the slides were placed in a fixing solution (15% trichloroacetic acid, 5% zinc sulfate, and 5% glycerol) for 10 minutes and washed three times in distilled water. The slides were put to dry at room temperature for two hours, hydrated for five minutes in distilled water, and stained for approximately 30 minutes in silver staining solution (A = aqueous solution containing 5% sodium carbonate, B = 0.1% ammonia nitrate + 0.1% silver nitrate + 0.25% tungstosilicic acid + 0.15% formaldehyde, use solution = 66 mL of A + 34 mL of B), at room temperature until the solution begins to darken. Subsequently, the slides were washed three times in distilled water and placed in a standing solution (aqueous solution with 1% acetic acid) for five minutes, finishing with washing three times in distilled water. Finally, the slides were dried at room temperature and analyzed under an optical microscope with 40x magnification to evaluate changes in the cells' nuclei and chromosomes (comets).

2.3. Quantification of serum lead

The blood samples were homogenized for 10 minutes and stored in the refrigerator. An aliquot of 100 µL of the blood samples were treated with 900 µL of 0.2% v/v nitric acid (HNO_3) and 0.2% v/v Triton X-100 solution and homogenized for 1 minute in a vortex shaker (Carvalho et al., 2005). All blood samples were processed in triplicates, stored in 5 mL Eppendorf tubes, and refrigerated. The analyses were performed using Graphite Furnace Atomic Absorption Spectrometer (GFAAS) with transverse heating equipped with Zeeman longitudinal background corrector (model AAnalyst 600, brand Perkin Elmer) with autosampler and electrodeless discharge lamp. The wavelength of 283.3 nm was selected for the lead determinations using pyrolytic tubes with the integrated platform.

2.4. Statistical analysis

The data of lead serum levels was plotted against the distance and fitted into a polynomial regression model using the software Minitab™.

3. Results and Discussion

When assessing toxic metals in blood, it is important to highlight that blood-Pb in superior animals likely represents a relevant index of exposure and health risks associated with Pb (Barbosa Junior et al., 2005). Additionally, the primary storage site for Pb in the body is inside the bones and the current existing models point out that the half-life of Pb in the three compartments are quite different, being estimated at 36 days for blood, 40 days for soft tissues and 27 years for human bones (Barbosa Junior et al., 2005; Moreira and Moreira, 2004; Souza et al., 2010). In this way, blood-Pb represents short term exposure while bone-lead content expresses chronic accumulation of this metal. From a physiological point of view, it can be associated with blood and plasma-Pb, due to the faster exchangeability of this compartment. Therefore, in this exploratory study, the detection of increased levels of lead in free-range chickens, collected at different points in the affected area, may indicate a snapshot of an active contamination in the respective locations.

Figure 2 shows the results of mean concentrations of serum lead (Pb) in the samples at each sampling point. It is possible to demonstrate the strong association ($R^2 = 0.96$) between lead concentrations in the blood of animals and the distance between the collection site and the source of contamination (COBRAC). The shorter the distance from the source of contamination, the higher the serum concentration of lead in the blood of the investigated birds. Thus, these results reinforce that, even deactivated, COBRAC continues to be a source of acute exposure to lead and that this risk is even greater in the vicinity of the plant for all living beings, including humans.

The chickens evaluated in this study were raised freely, feeding on grain provided by the farmer, as well as vegetation, insects and small animals available.

According to Macedo et al. (2016), who found high levels of lead in muscle tissue of chickens from Santo Amaro, the possible source of exposure for these animals was the contaminated soil, as well as the eating habits of these birds that scratch and peck at the ground. The association between eating habits and lead levels in the blood of different groups of birds was demonstrated by Garcia-Fernandez et al. (1995) where it was revealed that omnivorous species (such as chickens) and scavengers showed higher levels of contamination by this toxic metal when compared to piscivorous and insectivorous birds. In this way, in order to avoid chicken subclinical intoxication, the results of a recent work suggest that to retain chicken blood Pb below 20 µg/dL soil Pb needs to be < 166 mg/kg (Yazdanparast et al., 2022). Other studies carried out specifically in Santo Amaro showed higher concentrations of lead in the soil, close to the source of contamination (COBRAC) and lower concentrations at greater distances from the source (Machado et al., 2013), in agreement with the results found in our study of the inverse relationship between the concentrations of lead in the blood of birds and the distance from the source of contamination.

The identification of tolerable levels of heavy metals in the blood of birds is a major challenge that has been discussed for decades (Monclús et al., 2020). More conservative studies pointed out that lead concentrations in the blood of raptor are considered high when exceed 30 µg/dL and that clinical signs of poisoning are identified when higher than 60 µg/dL (Pain et al., 1993). In this sense, even higher blood lead concentrations (35 µg/dL) were considered normal in birds by Klein and Galey (1989) in all bird species.

On the other hand, a large portion of the most recent studies have indicated that lead concentrations in the blood of birds that do not show subclinical signs should be less than 20 µg/dL (Bauck and LaBonde, 1997; Dumonceaux and Harrison, 1994; Fenstad et al., 2017; Franson and Pain, 2011; López-Perea et al., 2019; Monclús et al., 2020) and as threshold concentrations of clinical poisoning and lethality probability set as 50 µg/dL (Monclús et al., 2020; Temamogullari et al., 2022). Other studies even present more cautious results, highlighting that blood lead level above 10 µg/dL is considered a risk limit for the health of chickens (Yuan and Tang, 1999).

Studies by Osweiler (1998) suggested that normal serum lead concentrations in birds must be below 10 µg dL^{-1}, they also reported non-toxic exposure to this metal in concentrations that ranged from 10 to 40 µg dL^{-1}. Likewise, Yazdanparast et al. (2022) found that chicken serum lead levels higher than 50 µg dL^{-1} are associated with toxic effects and significant impairment of metabolic functioning.

As seen, there is divergence in the literature regarding safe levels of lead in bird blood and, in this sense, the present study provides new information on this topic. In our experimental design, the comet test was used to detect possible damage to the DNA of chickens freely exposed in an area contaminated by lead.

Figure 3 shows intact leukocyte nuclei without verifying DNA migration from a specimen sampled in point P3.

The comet assay identified chromosomal damage in only one animal specimen (sampling point P4) and the Figure 4 shows comet-like nuclei in two different fields of observation of the material from this same animal. The comets found in the blood samples of Santo Amaro chickens were compared with the results of comet assays published in the scientific literature, consistent with each other (Gajski et al., 2019; Vallverdú-Coll et al., 2019)

The results of the present study showed that concentrations of approximately 34 µg dL^{-1} lead to chromosomal damage, which would possibly be aggravated at higher concentrations. The data obtained by the comet assay and the serum lead analysis are consistent since chromosomal damage was identified for higher concentrations of this metal and specimens sampled closer to the pollutant source. These results pointed out the comet assay's potential for diagnosing early genotoxic effects caused by lead in animals.

Thus, the results of the present research contradict more conservative values considered safe in bird blood, such as 35 µg/dL (Klein and Galey, 1989) and 30 µg/dL (Pain et al., 1993), while corroborating with the most recent blood lead level value of 20 µg/dL considering a normal threshold value for subclinical signs (Bauck and LaBonde, 1997; Dumonceaux and Harrison, 1994; Fenstad et al., 2017; Franson and Pain, 2011; López-Perea et al., 2019; Monclús et al., 2020).

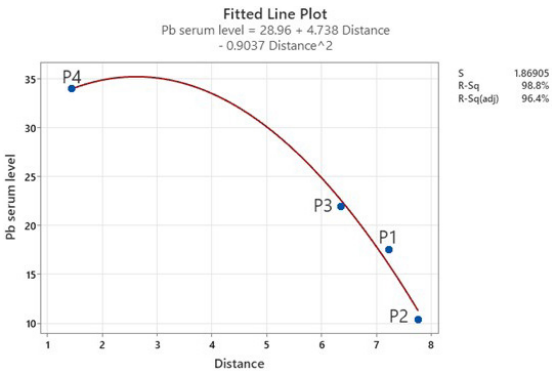

Figure 2. Relationship between chicken serum lead levels and the distance between sampling points and the polluting source (COBRAC).

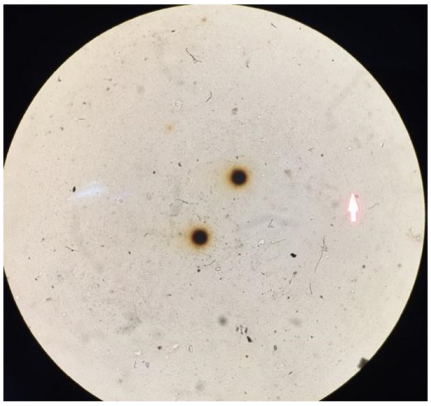

Figure 3. Intact nuclei from the specimen sampled at P3 point with 40x magnification.

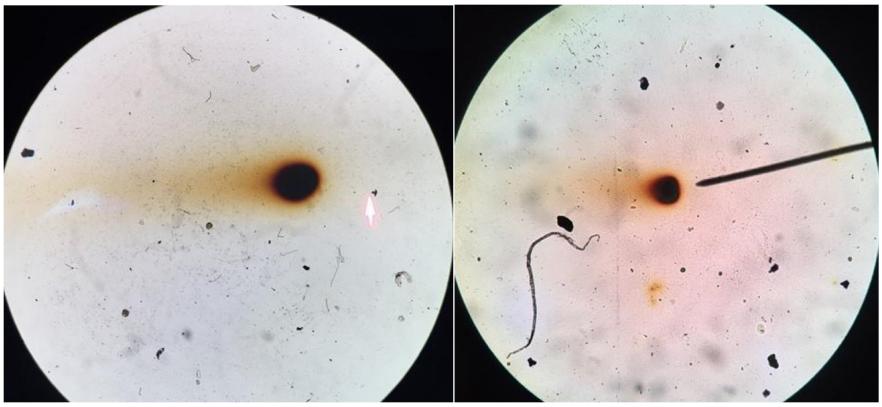

Figure 4. Comet-like nuclei observed in the specimen sampled at P4 point with 40x magnification.

It should be noted that a limitation of the study is the small number of birds included in the samples. Therefore, considering that no statistical sampling was carried out, the results are not necessarily representative of the entire contaminated area, being the first exploratory indications of genotoxic damages in birds caused by lead contamination of this area. In conclusion, the inverse relationship found between lead concentration levels in the blood of free-range chickens and the distance from the source of contamination indicates the potential of exploratory studies with chickens to detect contaminated areas. In this sense, the presented results showed a relation between high plasma lead levels with DNA damage, therefore highlighting the comet assay as a potential damage biomarker in lead-contaminated birds. Altogether, the present study brings elements that indicate a biological active contamination of the points sampled, as well as a potential risk in the consumption of the birds produced in the contaminated area.

Considering the results of this study, it is important to expand the research including new results from more chicken specimens and associate them with results of serum lead levels of the inhabitants that depend on these animals as primary sources of animal protein. In this way, this new research could evaluate the lead accumulation as a consequence of nutritional habits associated with a highly metal-contaminated environment.

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