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Human biomonitoring in residents of a NORM area using the comet assay, Pernambuco-Brazil

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Abstract: Geological research has found areas of uraniferous phosphorite in northeastern Brazil. This region is located along the coast of the states of Paraíba and Pernambuco, Brazil. Its distribution in Pernambuco is located in the Metropolitan Region of Recife/Região Metropolitana do Recife (RMR), covering the municipalities of Abreu e Lima and Olinda. Studies have been carried out in these municipalities, verifying the presence of Naturally Occurring Radioactive Material (NORM) in different matrices: soil, groundwater, air and fruit. Given these facts, the need arose to carry out human biomonitoring (HBM) in residents of these municipalities. Therefore, the aim of this study was to assess possible DNA damage in the residents of Olinda and Abreu e Lima by means of the comet assay using lymphocytes. The results showed high levels 3 and 4 damage to the DNA of the volunteers in the sample group who are under the influence of NORM, demonstrating a statistically significant difference in the damage index when compared to the control group. It is suggested that the genotoxic results found may be related to the NORM present in the municipality of Abreu e Lima, thus influencing the health of its residents. The research is relevant because it is a pioneer in the generation of scientific knowledge in the area of the study of uraniferous phosphorite using HBM.

Keywords: Biomonitoring human, Lymphocytes, DNA, Comet assay.

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Biomonitoramento humano em residentes de uma área NORM utilizando o ensaio cometa, Pernambuco-Brasil

Resumo: Pesquisas geológicas encontraram áreas de fosforito uranífero no Nordeste do Brasil. Esta região está localizada ao longo da costa dos estados da Paraíba e Pernambuco, Brasil. Sua distribuição em Pernambuco localiza-se na Região Metropolitana do Recife (RMR), abrangendo os municípios de Abreu e Lima e Olinda. Estudos foram realizados nesses municípios, verificando a presença de Material Radioativo de Ocorrência Natural (NORM) em diferentes matrizes: no solo, nas águas subterrâneas, no ar e em frutas. Diante destes fatos, surgiu a necessidade de realizar a biomonitorização humana (BHM) em moradores destes municípios. Portanto, este trabalho teve como objetivo avaliar possíveis danos no DNA dos moradores de Olinda e Abreu e Lima por meio do ensaio cometa utilizando linfócitos. Os resultados mostraram danos elevados de níveis 3 e 4 no DNA dos voluntários do grupo amostral que estão sob influência de NORM, demonstrando diferença estatística significativa no índice de dano quando comparado ao grupo controle. É sugerido que os resultados genotóxicos encontrados possam estar relacionados ao NORM presente no município de Abreu e Lima, influenciando assim na saúde dos seus residentes. A pesquisa torna-se relevante por ser pioneira na geração do conhecimento científico na área de estudo do fosforito uranífero utilizando a BHM.

Palavras-chave: Biomonitorização Humana, Linfócitos, DNA, Ensaio cometa.

1. INTRODUCTION

Geological research has found areas of uraniferous phosphorite in the northeast of Brazil. These rocks are associated with a layer of soil containing sandy limestones with a tabular shape, varying in thickness from centimeters to meters [1; 2]. The presence of uranium in phosphate rock depends on the existence of phosphate in its structure, which in turn promotes the deposition of uranium in the pores of phosphate minerals through chemical exchange between calcium (Ca^{+2}) and uranium (U^{+4}). In addition to the ores, the uranium-phosphate region is supplied by the Beberibe aquifer. This water, when in contact with the uranium-phosphate rocks, can be contaminated due to the leaching and transportation of uranium and other radionuclides from the rocks to the groundwater [3]. The uranium-phosphate region is located along the coast of the states of Pernambuco and Paraíba, Brazil. Its distribution in Pernambuco is in the Recife Metropolitan Region (RMR), covering municipalities such as Olinda and Abreu e Lima [4]. Studies have been carried out in these municipalities showing the presence of radionuclides in Naturally Occurring Radioactive Material (NORM) in ecosystem compartments such as soil [2], water [5, 6], cultivated fruit [7], as well as air, with an associated risk of lung cancer in the local population due to the existence of NORM [8]. This research prompted the present study, which used human biomonitoring (HMB) in residents of the municipalities of Olinda and Abreu e Lima under the influence of radionuclides in the uranium-phosphate region.

HMB can be defined as a method for assessing human exposure to chemical substances and their effects, by determining the concentrations of these substances, their metabolites and reaction products within the body. To do this, HMB involves the collection of biological samples such as blood, urine, saliva, breast milk, hair and fingernails [9]. Blood is the most commonly used matrix because it has advantages when it comes to assessing environmental exposures, as it travels throughout the body carrying nutrients, oxygen and chemical compounds resulting from exposure, thus providing internal exposure levels. It can be used for HMB in the fraction of whole blood, plasma or serum [10; 11]. Among the blood cells used in studies, mononuclear cells stand out, which are made up of monocytes and lymphocytes, the lymphocyte being the most comprehensive [12]. These cells are essential for biological dosimetry studies, as they are considered to be radiosensitive. They can be used in different HMB techniques, such as the comet assay [13].

The comet assay, also called single-cell gel electrophoresis, consists of microgel electrophoresis of individual cells or nuclei and is used for the detection and assessment of DNA damage caused by chemical or physical inducing agents. It can detect different types of DNA breaks, such as crosslinks between DNA bases, DNA repair error sites and the use of single-cell alkaline gel electrophoresis to detect single-strand breaks. After electrophoresis, the nuclei of the cells are stained with fluorescent reagents [13, 14]. During electrophoresis in the comet assay, the intact DNA will remain in the formed nucleus while the fragmented DNA will be transported towards the anode of the electrophoresis vat forming a comet tail. The staining of the slides can vary, with ethidium bromide, gel red and SYBR Green being used most frequently for fluorescence microscopy [15]. The results can be expressed in arbitrary units (AU), which corresponds to the weighted sum of the comets read, or as a percentage of damage (%) [15]. Due to its easy applicability and the fact that it detects different types of DNA damage, the alkaline comet assay has been used in the field of human biomonitoring [13].

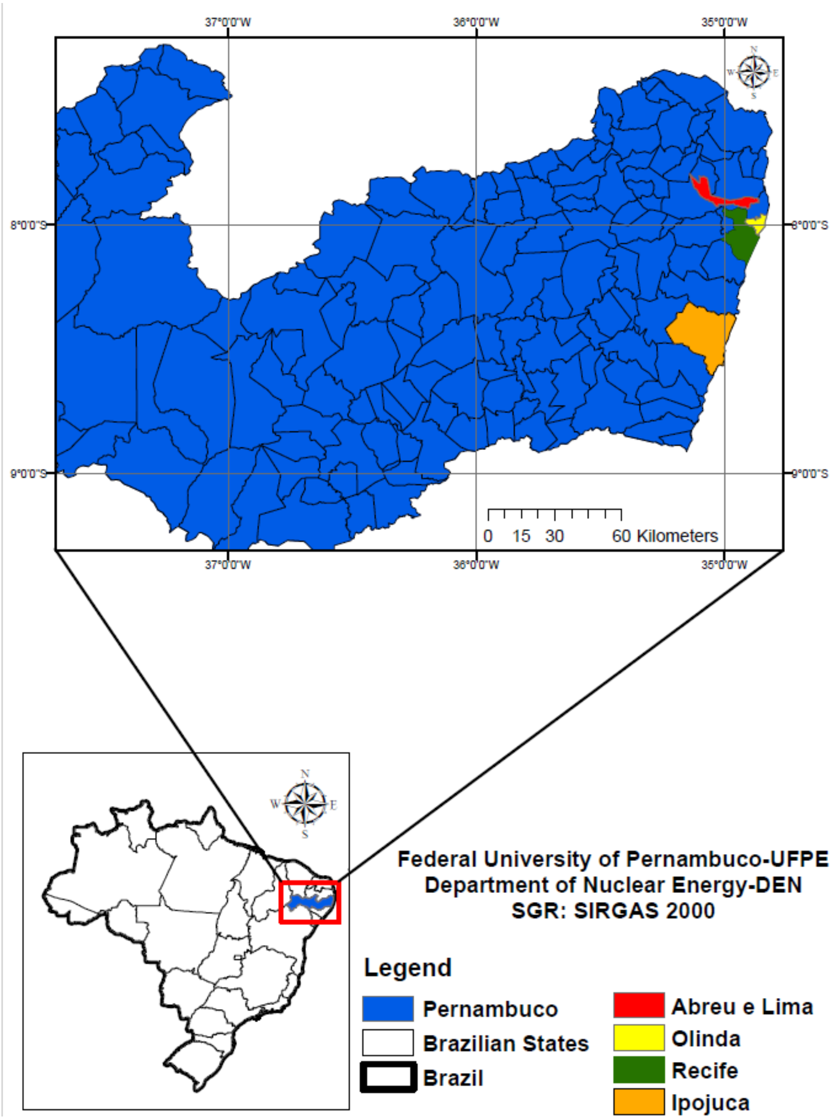
Considering the above, it is important to evaluate the genotoxicity of the population living in the regions where the uraniferous phosphorite occurs in order to assess human biomonitoring and support the development of public health policies. This study aims to assess DNA damage in lymphocytes from residents of the municipalities of Olinda and Abreu e Lima using the alkaline comet assay. The research is highly relevant as it is a pioneer in the generation of scientific knowledge using HMB in the area of study of uraniferous phosphorite.

2. MATERIALS AND METHODS

2.1. Blood sample collection from volunteers

This study consisted of a sample group with volunteers living in the municipalities of Olinda, Ouro Preto district (OP); Abreu e Lima, Fosfato district (AL1, AL2 and AL3) and a control group with volunteers not living in the uranium-phosphate area in the municipalities of Ipojuca, Nossa Senhora do Ó district (C1) and Recife, Iputinga (C2) and Várzea (C3) districts, with a total of seven individuals analyzed (Figure 1).

Figure 1 - Municipalities of the volunteers.



Source: Morais et al.

Blood samples were collected after answering the questionnaire, making the volunteers eligible for the study and then signing the Informed Consent Form (ICF). The exclusion criteria were: active COVID-19 patients, having been exposed to ionizing radiation by treatment or diagnosis in the last 6 months, being a smoker, being a compulsive alcoholic and an oncology patient. This study was approved by the Research Ethics Committee (REC) involving the use of human beings at the Federal University of Pernambuco (No. 45611221.0.0000.5208 and substantiated opinion 4.709.099). Seven volunteers were selected, three men and four women. Their ages ranged from 22 to 82. With regard to tobacco use, around 71.42% (n = 5) of the volunteers did not smoke and 28.57% (n = 2) had stopped smoking more than 15 years ago. With regard to alcohol use, around 71.42% (n = 5) consume alcohol sporadically and 28.57% (n = 2) do not. In terms of profession, around 42.85% (n = 3) are students, 42.85% (n = 3) are retired and 14.28% (n=1) are cooks. It is important to note that none of the volunteers work in industrial environments and/or are exposed to ionizing radiation, and that their occupational exposure is low enough to influence the experimental results. None of the sample and control volunteers had been exposed to ionizing radiation for less than 6 months. Only 42.85% (n = 3) reported having diseases such as hypertension, arthrosis and prediabetes (Table 1).

Table 1 - Data on the volunteers' characteristics (anamnesis).

Volunteers	Sex	Age	Smoking	Alcohol	Profession	Exposure to ionizing radiation	Pathologies
OP	Male	82	it stopped years ago	Rarely	Retired	No	Hypertension
AL1	Female	82	it stopped years ago	No	Retired	No	Arthrosis
AL2	Male	51	No	Rarely	Retired	No	Prediabetes
AL3	Female	44	No	Rarely	Cook	No	No
C1	Female	23	No	Rarely	Student	No	No
C2	Female	22	No	No	Student	No	No
C3	Male	29	No	Rarely	Student	No	No

Source: Morais et al.

The subjects' blood samples were collected by venipuncture using Vacutainer® heparin tubes (V= 2mL) and sent to the Radiobiology Laboratory/Laboratório da Radiobiologia (LR) at the Federal University of Pernambuco/Universidade Federal de Pernambuco (UFPE) for the genotoxic study using the alkaline comet assay.

2.2. Comet assay on peripheral blood lymphocytes

The alkaline comet assay was performed as described by Singh et al. (1988) [16], with modifications. To obtain the lymphocytes, 2 mL of peripheral blood was aliquoted from each sample and mixed with 2 mL of phosphate-buffered saline (PBS) in a sterile 15 mL falcon tube and centrifuged (DAIKI, model 80-2B) at 3.000 rpm for 15 minutes. Ficoll® Paque Plus reagent (1:2) was then added to the mixture of blood and buffer and centrifuged again at 3.000 rpm for the same time. A layer of lymphocytes was formed in the homogenate and about 0.1 mL of this layer was collected and homogenized in 0.1 mL of low melting point agarose (Sigma-aldrich). Immediately, this new homogenate was placed on a microscope slide, previously covered with a layer of 1.5% normal melting point agarose (Sigma-aldrich), covered with a coverslip and kept at 4 °C for 10 minutes. The coverslips were then removed and the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton-X 100 and 10% DMSO, pH 10.0) for 12 hours at 4 °C. After the lysis process, the slides were placed in a horizontal electrophoresis tank containing an alkaline buffer solution, pH 13.0 (EDTA 1 mM and NaOH 300 mM). Electrophoresis was then carried out for 20 minutes at 4 °C, at 0.74 V/cm and 300 mA. At the end, the slides were neutralized with 0.4 M Tris buffer (pH 7.5) for 15 minutes and fixed with absolute alcohol for 10 minutes.

The slides were stained with 0.05 mL of a solution of SYBER safe (Invitrogen) (1:500 diluted in distilled water). 100 comets per individual were analyzed in triplicate (i.e. 300 comets in total) using a Nikon H550L fluorescence microscope at 400x magnification with an excitation filter of 450-490 nm, an emission filter of 500-550 nm and a barrier filter of 495 nm. The visual analysis of DNA damage was carried out according to the methodology of Collins et al. (2008) [17]. The comets were classified into 5 categories of DNA damage (0 to 4), depending on the extent of the damage. Category 0 indicates that no damage occurred, categories 1 to 4 indicate damage at increasing levels to the genetic material (Figure 2).

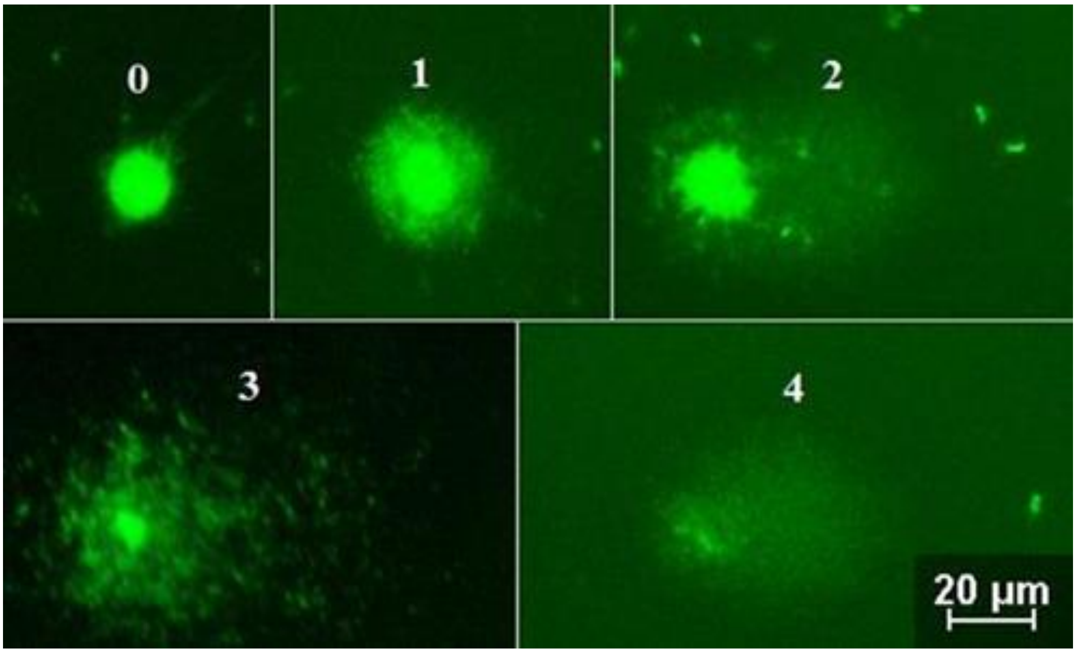
Genetic damage was assessed using two parameters: Damage Index (DI) and Damage Frequency (DF%). The DI was calculated by multiplying the level of damage by the number of comets corresponding to it, as described in Equation 1:

$$DI = 0 \times (n0) + 1 \times (n1) + 2 \times (n2) + 3 \times (n3) + 4 \times (n4) \quad (1)$$

DF% was calculated as the percentage value of all comets with DNA damage (level 1 - 4) in relation to the total number of comets (level 0 - 4), according to Equation 2:

$$DF\% = (\text{N}^{\circ}\text{ total number of comets} - \text{N}^{\circ}\text{ total number of comets 0}) \times 100 \quad (2)$$

Figure 2 - Comet damage levels 0 to 4.



Source: Morais et al. (2022, p. 4) [18]

2.3. Statistical analysis

A análise estatística para o ensaio cometa foi realizada utilizando os modelos estatísticos ANOVA e Tukey com a utilização do programa estatístico GraphPad Prism 5.0, sendo considerado $p < 0,05$.

3. RESULTS AND DISCUSSIONS

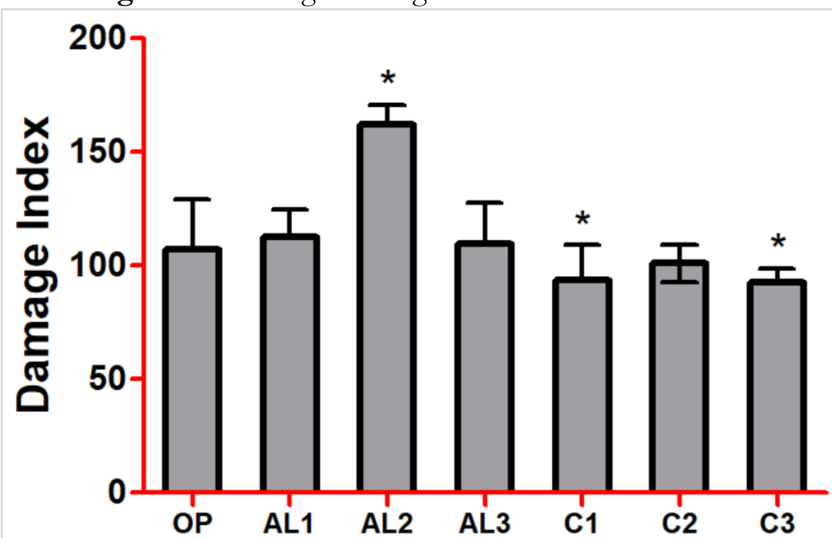
After alkaline electrophoresis, the results of the 2,100 comets counted and their respective degrees of damage were obtained. It was observed that there was a statistically significant difference between the sample group and the control group in terms of the Damage Index (DI), but there was no statistical difference in terms of the Damage Frequency (DF%). The results are shown in Table 2. The averages of the Damage Index and Damage Frequency are shown in Figures 3 and 4, respectively.

Table 2 - Evaluation of genetic damage in the lymphocytes of volunteers from the Metropolitan Region of Recife.

Volunteers	Degrees of Damage Comets					Damage Index Average	Damage Frequency% Average
	0	1	2	3	4		
OP	77	146	58	17	2	**107,00 ± 38,04**	**74,33 ± 18,34**
AL1	49	177	63	10	1	**112,33 ± 21,55**	**83,67 ± 14,29**
AL2	31	138	64	47	20	**162,33 ± 14,47**	**89,67 ± 0,71**
AL3	114	88	61	29	8	**109,67 ± 31,26**	**62,00 ± 9,85**
C1	44	231	25	0	0	**93,67 ± 27,02**	**85,33 ± 19,35**
C2	25	247	28	0	0	**101,00 ± 14,18**	**91,67 ± 9,90**
C3	60	207	28	5	0	**92,67 ± 9,87**	**80,00 ± 16,26**

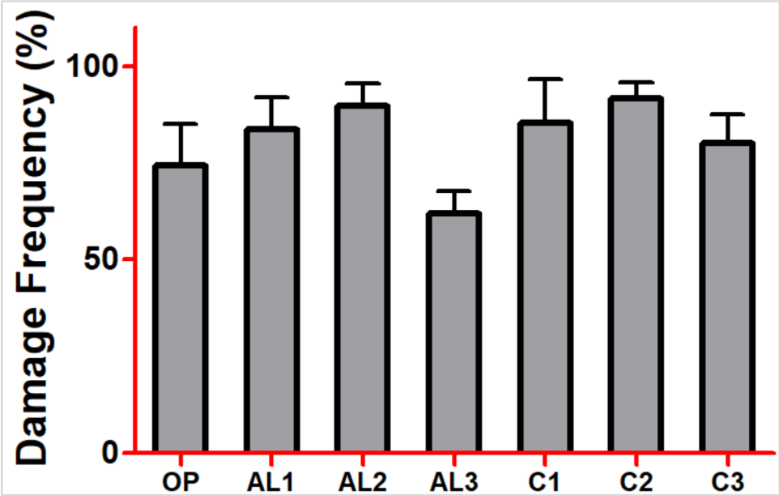
Source: Morais et al.

Figure 3 - Average Damage Index of the volunteers.



Source: Morais et al.

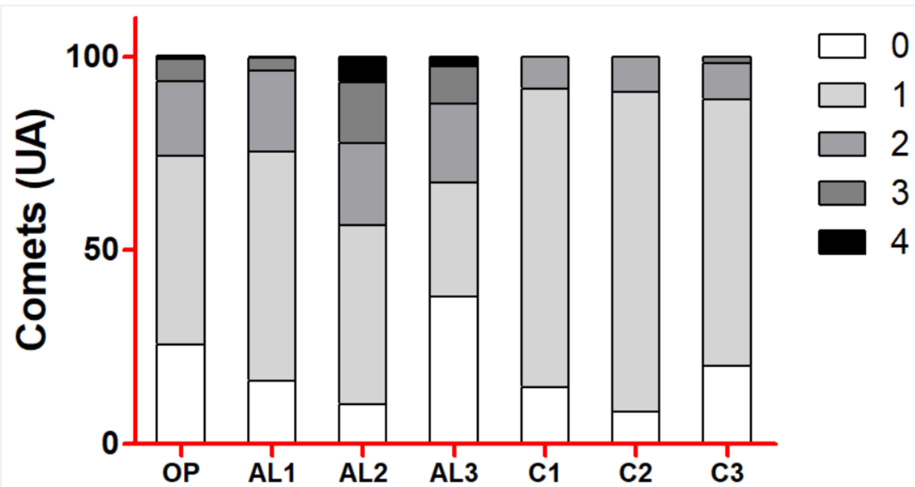
Figure 4 - Average Damage Frequency of the volunteers.



Source: Morais et al.

A statistically significant difference was found between volunteer AL2 from the sample group and volunteers C1 and C3 from the control group in terms of the Damage Index (DI). Individual AL2 from the NORM area had the highest average damage index (ID = 162.33 ± 14.47) compared to the other individuals, with a large number of grade four comets (20 AU). In addition to this volunteer, volunteer AL3 from the same area also had a high number of grade four comets (8 AU). The control group had a large number of grade 1 comets and so the ID and FD% calculations were high. However, when analyzing the blood of the Abreu e Lima (AL) sample group, there was an increase in the severity of DNA damage, with significant numbers of grade 3 and 4 comets (Figure 5).

Figure 5 - Average degrees of comets found.



Source: Morais et al.

These two individuals (AL2 and AL3) are not influenced by external factors such as smoking, compulsive alcohol consumption, advanced age, occupation, etc. Since the comet assay can be influenced by these factors [19]. In addition, several studies have shown the presence of NORM in the municipalities of Olinda and Abreu e Lima, such as Amaral et al. (2005) who analyzed the concentration of natural uranium and Ra^{226} in food grown in rural areas of the municipality of Abreu e Lima, where the high presence of these radionuclides in grains, fruits, roots and tubers was found [7]. In 2018, the same author and other collaborators studied the same municipality, analyzing the outdoor dose rate from the soil using a portable gamma detector, and found the presence of high doses of background radiation [2]. The study carried out by Amaral et al. (2019), which analyzed the presence of radon (Rn^{222}) in residential well water from groundwater in the same municipality, found a high presence of this radionuclide in some wells [5]. Paiva (2019) analyzed the dispersion and mobility of radionuclides in the Beberibe aquifer in the municipality of Olinda, finding high activities of radon and the presence of strontium (Sr) in wells [6]. And the most recent study by Ramos et al. (2023), which analyzed the occurrence of lung cancer due to the presence of radon in the homes of the inhabitants of these two municipalities [8].

Among the research cited above, Amaral et al. (2019) [5] found a high radon concentration of 74 $Bq.L^{-1}$ in the well water of the Abreu e Lima volunteers in this study, which is above the permitted limit of 11,1 $Bq.L^{-1}$ [20]. In addition, Ramos et al. (2023) [8] also found significant concentrations of atmospheric radon in the phosphate neighborhood of Abreu e Lima, ranging from 311 to 1.174 Bq/m^3, above the permitted limit of 100 Bq/m^3 [21]. It is suggested that the DNA damage detected by the comet assay may be associated with contact with the environment, either through exposure to air or through the handling and collection of water from artesian wells located in the uranium phosphate region. In this exposure, both radon and other trace elements can cause changes in cellular DNA.

In the literature, some studies also corroborate the present study, as they found genotoxicity caused by radon, resulting in grades three and four comets. Such as Ciorba et al. (2010), who observed, in vitro, that increasing the exposure time of lymphocytes to radon increases the appearance of grade three and four damage [22]. Walczak et al. (2020) evaluated the population of the city of Kowary under the influence of NORM with a high concentration of radon in Poland, finding genotoxic damage in residents of homes above 100Bq/m^3 with grades three and four comets [23]. And Darlina et al. (2022), who analyzed the population of the town of Mamuju with a high natural radioactive background in Indonesia, found high genotoxic damage from comets in residents due to chronic exposure to radon in the area (184 to 380 Bq/m^3) [24]. This demonstrates that even at lower concentrations than Abreu e Lima (311 to 1.174 Bq/m^3), radon is capable of causing significant genotoxic damage, as observed in the current research.

In Brazil, few research studies on human biomonitoring and NORM can be found in the literature. Guimarães et al. (2010) evaluated the populations of the cities of Monte Alegre, Prainha and Alenquer, in the state of Pará, which are located in a NORM area due to uranium mineralization, but found no statistically significant difference between their groups [25]. Sombra et al. (2011) evaluated the same region, studying the influence of NORM on the DNA repair genes (XRCC1, XRCC3 and GSTM1) of the local population, but found no influence, with the genes being the same as the other populations in Brazil [26]. And the study by Xavier et al. (2023), which analyzed oxidative stress in residents of an area with a high concentration of radon, in the state of Rio Grande do Norte, found high oxidative stress and polymorphism in the DNA repair gene hOGG1 in these residents [27]. These three studies reveal the scarcity of studies in the area of human biomonitoring and NORM in Brazil, demonstrating the importance and relevance of the present study in contributing to the enrichment of the literature in this field, which could boost future researchers.

4. CONCLUSIONS

In view of the above, it is suggested that the genotoxic results found may be related to the NORM present in the municipality of Abreu e Lima, thus influencing the health of its residents. Future research using dicentric chromosome assays is recommended to improve the accuracy of the results due to their specificity and sensitivity to ionizing radiation.

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CONFLICT OF INTEREST

All the authors declare that they have no conflicts of interest.

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