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Micronuclei and nuclear abnormalities in fish as biomarkers of cytotoxicity and genotoxicity of gamma radiation from chromium 51 in an aquatic environment

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ABSTRACT

Context: Nuclear accidents or disasters can contaminate bodies of water. Environmental monitoring methods are needed to assess low levels of radiation. Objective: Evaluate genotoxicity in fish, Oreochromis niloticus, Nile tilapia, exposed to radioactive chromium (51Cr), as a source of gamma radiation, using the test for micronuclei and other nuclear abnormalities. Methodology: Adult tilapia were subjected to a gamma radiation dose of 20 Gy/day, through the incorporation of radioactive chromium oxide into the diet, 51Cr2O3, and administered during the seven days of the experimental period, totaling approximately 140 Gy. During this period, peripheral blood samples were collected [days 0 (control), 1, 2, 3, 4, 5, 6, and 7] and analyzed through micronucleus assay and other erythrocyte nuclear abnormalities. Results: Subchronic exposure to 51Cr caused a time-dependent increase ($p<0.05$) in nuclear abnormalities, micronuclei, deformed nucleus, nuclear bud, nuclear bridge, binucleated cells and apoptotic cells. Furthermore, the results revealed a strong correlation between the frequency of micronuclei and other nuclear abnormalities. Theoretical-methodological contributions: Nile tilapia was demonstrated to be a living organism capable of being used for biomonitoring of contamination by low gamma radiation in a freshwater environment. Social and environmental contributions: The results suggest that morphological analysis and the frequency of micronuclei in peripheral red blood cells of Nile tilapia can be considered reliable parameters in the evaluation of cytogenetic biomarkers in foods and aquatic environments contaminated with the radioactive agents 51Cr, a gamma radiation emitter.

PALAVRAS-CHAVE: Radionuclides. Biomonitoring. Nile Tilapia

1 INTRODUCTION

The release of radioactive materials into aquatic and terrestrial environments directly or indirectly contaminates the environment, through the generation of artificial radionuclides derived from nuclear accidents or tests. Ionizing radiation, gamma, from radionuclides is considered a genotoxic agent that can induce genetic mutations, chromosomal aberrations, and cell death (BAUDIN et al., 2021; MAVRAGANI et al., 2019; OKUNO, 2013; SUGIMOTO et al., 2014).

During the peak years of plutonium production at the nuclear reactors at Hanford, located on the banks of the Columbia River in Washington state and operated by the United States Federal Government, chromium-51, ^{51}Cr, was released daily from the reactors in the river (NATIONAL RESEARCH COUNCIL, 2002). This radionuclide mainly affects the gastrointestinal tract, bones, and reproductive and blood-forming organs of aquatic organisms, such as fish (ASWOOD; AL-HAMZAWI; KHADAYEIR, 2019).

Chromium (III) oxide is an inorganic compound with the formula Cr_2O_3 and is not absorbed by the gastrointestinal tract, gills, or other possible absorption route in Nile tilapia (SAKITA et al., 2015). However, $^{51}Cr_2O_3$ is an emitter of ionizing gamma radiation and has mutagenic potential (TAUHATA et al., 2013), which can lead to the ejection of electrons from atoms, ions, and electrically charged ions when located within or close to DNA molecules. This can promote chemical reactions that alter the bases in DNA, causing serious damage to genetic material, such as asymmetric rearrangements with fragment formation, non-disjunction, and breakage (KIRSCH-VOLDERS; FENECH, 2001).

To assess DNA damage in fish, the micronucleus test, MN, has been used as it demonstrates potential sensitivity (AL-SABTI; METCALFE, 1995). Furthermore, other nuclear abnormalities in cells are also indicators of mutagenicity, such as deformed nucleus, nuclear bud, nuclear bridge, and binucleated and apoptotic cells and these abnormalities correspond to different biological processes (BAUDIN et al., 2021; OSMAN; ABUEL-FADL; KLOAS, 2012).

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Thus, the current study aimed to evaluate the frequency of nuclear abnormalities, such as cytogenetic biomarkers, induced by subchronic exposure to gamma radiation and produced by the decay of chromium (^{51}Cr), in the form of $^{51}Cr_2O_3$, in Nile tilapia (*Oreochromis niloticus* L.), as an experimental model for biomonitoring.

MATERIAL AND METHODS

The present work was carried out at the Center for Energy in Agriculture – CENA, University of São Paulo, Piracicaba, São Paulo, Brazil and the Functional Sciences Laboratory of the University of Western São Paulo, UNOESTE, Presidente Prudente, São Paulo, Brazil (CEUA, protocol no. 551).

We used 80 sex reversed Nile tilapia adults with an average initial weight of 200.6 g ± 11.4 g, randomly distributed in 80 aquariums with a volume of 250 L. The aquariums had an independent closed water recirculation system, with aeration, controlled heating using electric heaters connected to a thermostat (26.0 ± 1.0°C), and physical and biological water filtration using a biofilter. Food remains and excreta accumulation were siphoned and the physical and chemical parameters of the water were monitored daily using a YSI 556® multiparameter probe (YSI Environmental, Yellow Spring, OH, USA). The water parameters during the experiment remained constant: dissolved oxygen 9.8 ± 2.2 mg/L; temperature 21 ± 1°C; pH 8.1 ± 0.5; and conductivity 780 ± 10 µhos.

The fish were fed twice a day (8 and 18 h), until satiety, with a basal diet composed of 30.0% digestible protein and 3,000 kcal/kg of digestible energy and included in 1.0 g gelatin capsules (NATIONAL RESEARCH COUNCIL, 2011; OFFICIAL METHODS OF ANALYSIS, 2016). Two capsules containing radioactive chromium, $^{51}Cr_2O_3$, incorporated into the diet were provided daily, one at 8 am and the other at 6 pm, and capsules containing only the basal diet until satiety. The estimated absorbed radiation dose for each fish (average weight 200g), from oral contamination of ^{51}Cr, was 20 Gy/day (WEGST, 1987).

At the beginning of the experimental period (day 0) and every 24 hours, ten specimens were randomly captured, using a sequence table generated by the R program (HARRELL, 2015) and anesthetized by immersion in benzocaine solution (100 mg/L). Blood samples were collected from the tail vein of each specimen on day 0 (control), 1, 2, 3, 4, 5, 6, and 7 using heparinized syringes, and immediately spread onto clean glass slides, air-dried overnight, and then fixed in absolute methanol for 15 minutes. Each slide was stained with 5% Giemsa solution for 20 minutes (GRISOLIA; CORDEIRO, 2000). Triplicate slides were made for each fish and 3,000 cells were evaluated by two experienced researchers, blindly using a light microscope (Leica DM 750, Leica ICC50 HD camera - Leica Biosystems Nussoch GmbH, Germany) coupled to Leica Application Suite (LAS) EZ software.

The main criteria for identifying the micronucleus (MN) were based on considering the absence of connections with the main nucleus, with a similar color and size between 1/10 and 1/30 of the size of the main nucleus (AL-SABTI; METCALFE, 1995), since most fish chromosomes are much smaller than mammalian chromosomes (SCHMID, 1975). The established criteria for identifying micronuclei were strictly followed to ensure an authentic score (MATSUMOTO et al., 2006). Cells with an oval appearance with intact cytoplasm, oval

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nuclei with an intact nuclear membrane, micronuclei smaller than or equal to 1/3 the size of the main nuclei, micronuclei clearly separated from the main nuclei, and non-refringent micronuclei were considered for counting.

The cytological abnormalities observed in the present study were classified as nuclear abnormalities (NAs). NAs include cells with a main nucleus with a clearly separated smaller nucleus called a micronucleus (MN); nuclei with "notches" in the nucleus were noted as deformed (DN); nuclei containing euchromatin and having a relatively small evagination (bud) of the nuclear membrane as nuclear bud (NBu); a bridge-like formation between two erythrocytes was identified as nuclear bridge (NBr); cells containing two nuclei and a common cytoplasm as binucleated cells (BNC); and cells with fragmented nuclei were considered apoptotic (AC) (ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012).

Statistical analysis was performed using GraphPad Prism software, version 5.02. To determine the normal distribution of data, the Kolmogorov-Smirnov test was performed. The Student's t-test was used to compare the frequency of nuclear abnormalities found at all sampling times, which were statistically compared with the control (day 0) and with each other. Pearson's correlation test was performed to determine possible relationships between MN values and possible nuclear abnormalities. In all cases, the selected significance level was less than 5% ($p<0.05$).

3 RESULTS AND DISCUSSION

Nile tilapia is one of the most widely used freshwater fish in toxicological studies, as it presents a series of characteristics that can make it a suitable model for use as an indicator species in biomonitoring programs (FIGUEIREDO-FERNANDES et al., 2007; OSMAN; ABUEL-FADL; KLOAS, 2012).

In the current study, variation in the frequency of micronuclei was observed between individuals on day 0 (control), 0.23 ± 0.15%, in addition to other cellular anomalies, deformed nucleus 0.31 ± 0.12%, nuclear bud 0.31 ± 0.12%, nuclear bridge - microcyte 0.29 ± 0.15%, binucleated cells 0.19 ± 0.33%, and apoptotic cells 0.24 ± 0.44% [Table 1 and Figures 2A - Micronuclei (MN), 2B - Deformed nucleus (DN), 2C - Nuclear bud (NBu), 2D - Nuclear bridge - Microcyte (NBr), 2E - Binucleated cell (BNC); and 2F - Apoptotic cell (AC)]. The frequency of micronuclei can vary considerably from one individual to another (0.03 to 0.47%), and these differences depend on genetic and environmental factors, as well as diet, age, and sex (PASTOR et al., 2002) and to reduce genetic and environmental effects, as well as effects of management, age, and sex, we used specimens from the same spawn, sex reversed (males), and with similar basal diet and management.

Testing for micronuclei testing and other abnormalities in fish erythrocytes should be performed at various times after exposure, thus making it feasible to track possible increases in alterations in frequencies (ANBUMANI; MOHANKUMAR, 2011). In the current study, collections were performed daily during the seven days of the experimental period and thus, the increase seen in the frequency of micronuclei must be due to poor repair of DNA double-strand breaks, leading to symmetrical and asymmetrical exchanges of chromatids and chromosomes or fragments that fail to be included in the daughter nuclei at the end of

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telophase during mitosis because they did not attach to the spindle during the anaphase segregation process (FENECH et al., 2011). A statistically significant increase in MN formation was also observed in studies with another fish species, *Ictalurus punctatus*, in a cooling pond at a Chernobyl-contaminated site (SUGG et al., 1996), in *Esox lucius* at nuclear facilities and chemical complexes in Siberia (ILYINSKIKH; ILYINSKIKH; ILYINSKIKH, 1998) and in *Catla catla* kept in aquariums (ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012).

Table 1. Frequency (mean ± standard deviation) of nuclear characteristics in erythrocytes of Nile tilapia (*Oreochromis niloticus* L.) exposed to 7 days of gamma radiation resulting from the decay of ^{51}Cr, $^{51}Cr_2O_3$*.ção gama resultante do decaimento de ^{51}Cr, $^{51}Cr_2O_3$*.

Exposure (days)	Frequency of nuclear abnormalities**					
	MN***	Deformed nuclei	Nuclear bud	Nuclear bridge - Microcyte	Binucleated	Apoptotic
0 (control)	0.23 ± 0.15^{A}	0.31 ± 0.12^{A}	0.31 ± 0.12^{A}	0.29 ± 0.15^{A}	0.19 ± 0.13^{A}	0.24 ± 0.14^{A}
1	0.45 ± 0.21^{AB}	0.38 ± 0.17^{AB}	0.37 ± 0.14^{Ab}	0.37 ± 0.16^{A}	0.28 ± 0.20^{Ab}	0.37 ± 0.19^{A}
2	0.65 ± 0.30^{BC}	0.47 ± 0.20^{ABC}	0.46 ± 0.10^{B}	0.47 ± 0.19^{AB}	0.41 ± 0.14^{BC}	0.42 ± 0.29^{AB}
3	0.83 ± 0.43^{CD}	0.57 ± 0.20^{BC}	0.51 ± 0.11^{BC}	0.54 ± 0.22^{ABC}	0.54 ± 0.19^{CD}	0.54 ± 0.12^{B}
4	0.97 ± 0.11^{D}	0.62 ± 0.26^{BC}	0.57 ± 0.15^{BC}	0.62 ± 0.26^{BC}	0.57 ± 0.19^{CD}	0.62 ± 0.25^{BC}
5	1.08 ± 0.15^{D}	0.67 ± 0.25^{C}	0.61 ± 0.11^{BC}	0.67 ± 0.14^{BC}	0.67 ± 0.12^{D}	0.70 ± 0.14^{C}
6	1.18 ± 0.16^{DE}	0.72 ± 0.19^{C}	0.66 ± 0.13^{BC}	0.74 ± 0.20^{BC}	$0.75\pm0.25D^{d}$	0.76 ± 0.21^{C}
7	1.27 ± 0.21^{E}	0.75 ± 0.25^{C}	0.70 ± 0.14^{C}	0.80 ± 0.23^{C}	0.80 ± 0.25^{D}	0.82 ± 0.17^{C}

* Source: Authors. ** The results in the column with different superscripts differ significantly (Student's t Test; $p<0.05$). *** Micronucleus.

The exposure period in this study was seven days and the results revealed a significant increase ($p<0.05$) in the frequency of MN in a time-dependent manner. A study using ^{60}Co as a source of gamma radiation in freshwater fish, *Catla catla*, showed that the MN frequency peaked on day 12 and that this is probably the time required for the production of nascent cells that undergo DNA damage to reach the peripheral circulation of the kidney cephalic or spleen under stressful conditions (ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012). However, in other studies on the micronucleus rates of various fish species (*Tilapia rendalli*, *Oreochromis niloticus*, and *Cyprinus carpio*), the results demonstrated that this increase occurs between the first and fifth day after the start of exposure (GRISOLIA; CORDEIRO, 2000).

The results also revealed a positive correlation between MN frequencies and nuclear abnormalities (Figures 1A, 1B, 1C, 1D, and 1E), suggesting the importance of other nuclear abnormalities as prospective biomarkers (OSMAN et al., 2010). The simultaneous expression of nuclear morphological abnormalities (NAs) together with MN are considered as indicators of cytotoxicity and genotoxicity, respectively (GRISOLIA et al., 2009). Nuclear abnormalities arise

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from acentric chromosomal fragments or entire chromosomes that end up being lost during mitosis and are usually formed due to exposure to clastogenic and/or aneugenic activities of substances. Furthermore, the speed at which these alterations begin to occur may depend on the species, genotype, DNA repair mechanisms, deficient chromosomal segregation, immunological status, sex, and age of the individual (ZÚÑIGA-GONZÁLEZ et al., 2001).

Few studies have been carried out using fish exposed to gamma radiation (ANBUMANI; MOHANKUMAR, 2011; ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012; ILYINSKIKH; ILYINSKIKH; ILYINSKIKH, 1998), and exposure to radiation in these studies occurred with radioactive waste diluted in an aqueous medium and extensively released into rivers or laboratory tanks. However, the current study differed, as it used the radioactive element as a source of ionizing radiation incorporated into the diet, with the aim of simulating the ingestion of contaminated food.

Along with MN, several other nuclear abnormalities, such as deformed nucleus, nuclear bud, nuclear bridge, binucleated cells, and apoptotic cells were observed in fish during subchronic exposure to gamma radiation generated by the decay of ^{51}Cr and administered orally to Nile tilapia fish (depicted in Figures 2A, 2B, 2C, 2D, 2E, and 2F). Nuclear morphological alterations may be due to cells that have duplicated but not completed cytokinesis and gene amplification and will be eliminated by budding or incomplete mitotic division (FENECH, 2007). The presence of binucleated erythrocytes (in mitotic division) in peripheral blood may be associated with a temporary increase in oxygen transport capacity, or with a form of cellular senescence (HOUSTON; MURAD, 1995). Furthermore, gene mutation in the nuclear lamina can result in nuclear abnormalities (STRUNJAK-PEROVIC et al., 2009).

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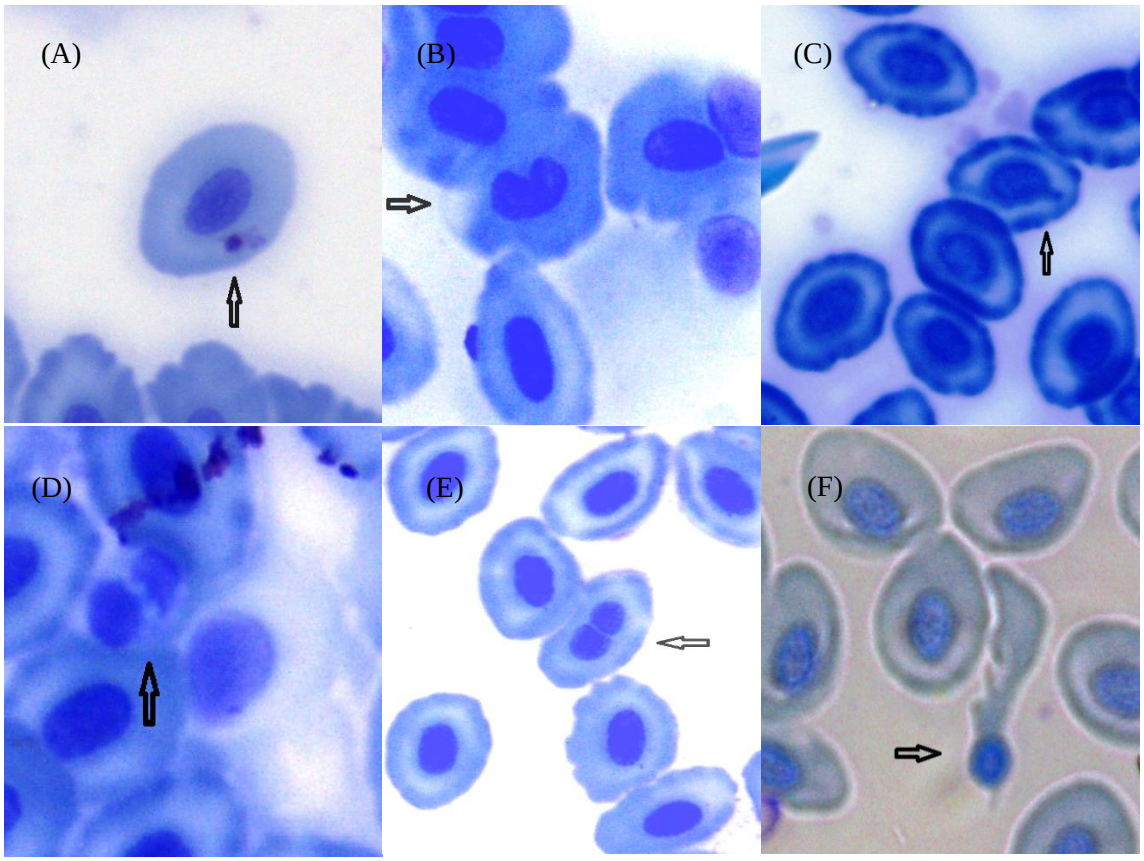
Figure 1. Representative images of various erythrocytic cellular abnormalities in Nile tilapia fish exposed to acute gamma radiation (^{51}Cr). The nuclear anomalies observed were (A) Micronuclei (MN), (B) Deformed nucleus (DN), (C) Nuclear bud (NBu), (D) Nuclear bridge - Microcyte (NBr), (E) Binucleated cell (BNC) and (F) Apoptotic cell (AC).



Source: Authors.

In the current study, bridges between binucleated cells and the aforementioned nuclear alterations were observed, although at low frequency, in peripheral erythrocytes, which was also observed in another study using *Catla catla* (Ham.) fish exposed to gamma radiation (ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012). Ionizing radiation is known to induce double-strand breaks in DNA, causing the formation of dicentric chromosomes that manifest as nucleoplasmic bridges in cytokinesis (FENECH, 2007).

Pearson correlation results showed a significant positive correlation between MN frequencies and other nuclear abnormalities: Deformed Nucleus [r(Pearson)=0.9983, 95%CI = 0.99 to 1.00, R^2=0.9965, p<0.0001 (Fig. 1A)], Nuclear Bud [r(Pearson)=0.9974, 95%CI = 0.97 to 1.00, R^2=0.9948, p<0.0001 (Fig. 1B)], Microcyte [r(Pearson)=0.9958, 95%CI = 0.98 to 1.00, R^2=0.9916, p<0.0001 (Fig. 1C], Binucleated [r(Pearson)=0.9954, 95%CI = 0.95 to 1.00, R^2=0.9909, p<0.0001 (Fig. 1D)], and Apoptotic [r(Pearson)=0.9945, 95%CI = 0.95 to 1.00, R^2=0.9891, p<0.0001 (Fig. 1E)]. Similar observations, evidenced by the correlation between the frequency of MN and other nuclear abnormalities, were also found in other studies (CAVAS; KONEN, 2008; RYBAKOVAS; BARSIENE; LANG, 2009).

In this study, nuclear buds were observed, a nuclear abnormality that is formed when a nucleoplasmic bridge between two nuclei breaks and the remnants shrink towards the nuclei

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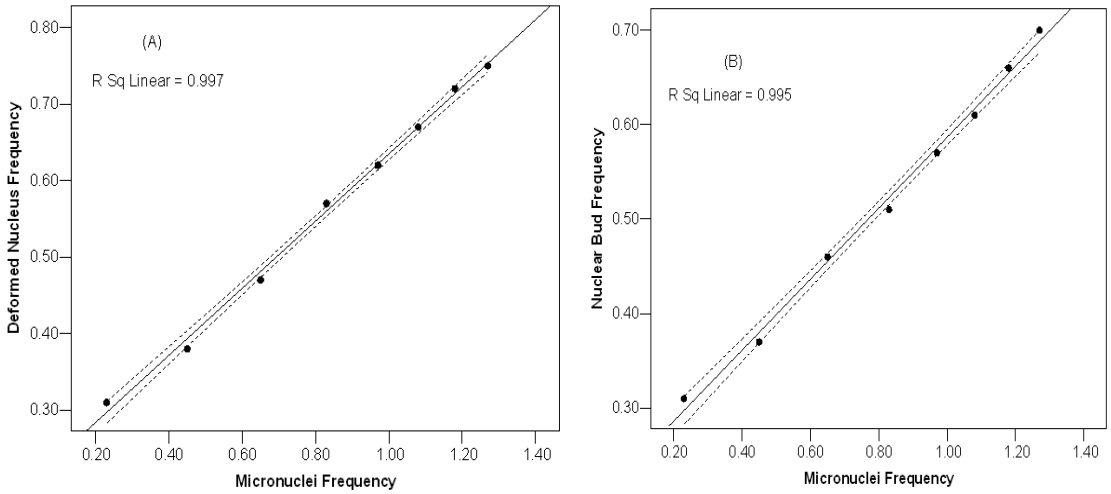
(ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012; UTANI et al., 2010). These and "tail nucleus" abnormalities have also been observed in fish red blood cells and in human lymphocytes exposed to gamma radiation from the decay of radioisotopes from the Chernobyl nuclear accident (KRAVTSOV et al., 2000).

In addition, we observed that subchronic exposure to gamma radiation from radionuclide decay during the seven-day experimental period resulted in a significant increase in nuclear abnormalities and apoptosis. These abnormalities have also been reported in studies with humans (human peripheral blood lymphocytes) and fish (erythrocytes) exposed to acute or subchronic radiation and have been shown to induce a complex cascade of events, such as chromatin condensation, cell shrinkage, formation of blebs, and fragmentation (ANBUMANI; MOHANKUMAR; SELVANAYAGAM, 2012; YADAV et al., 2021). Furthermore, the results revealed a high positive correlation in the frequency of binucleated cells, which may be due to tubulin failure, difficulty in polymerization, and formation of the mitotic spindle caused by the aneugenic action of toxicants (DE CAMPOS VENTURA; DE ANGELIS; MARIN-MORALES, 2008).

Figure 2. Pearson's correlation coefficient test ($p<0.05$) of micronucleus (MN) frequency and nuclear abnormalities in erythrocytes of Nile tilapia exposed to gamma radiation and from feed contaminated with ^{51}Cr (gamma radiation). (A) Correlation between the frequency of MN and the frequency of Deformed Nuclei. (B) Correlation between MN frequency and frequency of nuclear bud. (C) Correlation between MN and frequency of microcyte. (D) Correlation between MN and frequency of binucleated red blood cells. (E) Correlation between MN and frequency of apoptotic red blood cells.



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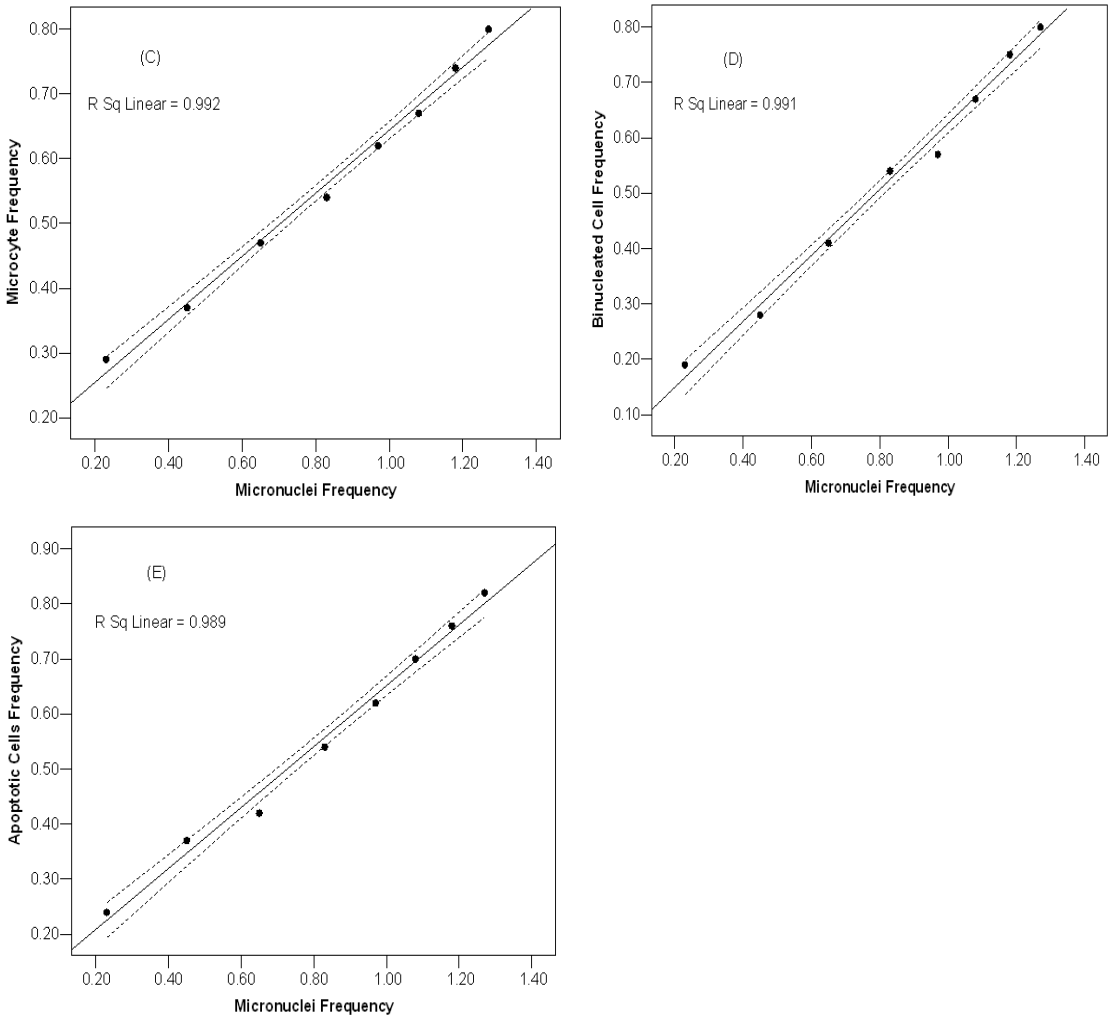

Source: Authors.

4 CONCLUSION

The results allow us to conclude that Nile tilapia, exposed and contaminated by feed incorporated with the mineral element in radioactive form, are sensitive to gamma radiation, resulting from the decay of ^{51}Cr. The polluting substance caused erythrocyte alterations and nuclear abnormalities, due to the ionizing radiation present in the feed and aquatic environment and a strong correlation between erythrocyte alterations and nuclear abnormalities. Therefore, this species demonstrated the possibility to be used as a sensitive tool to monitor areas affected by radiation.

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