Genotoxicity and DNA Repair Indicative in Blood Cells after Occupational Exposure to Ionizing Radiation

ORIGINAL

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Abstract

Occupational exposure to ionizing radiation (IR) can damage DNA. The study evaluated the genotoxic profile and repair indicatives of DNA from peripheral blood lymphocytes of health workers exposed occupationally to IR by adopting comet assay. Biomonitoring was done with ninety individuals; among them 45 were health professionals and the rests were non-professionals. Blood samples were collected after 48 h (2 d; non-exposed) and 168 h (7 d; exposed). The 7 d IR exposed group significantly increased in the rates and frequency of damage, while 2 d unexposed group exhibited more than 20% of DNA repair as compared to the respective control groups. The DNA damage was observed in more significant to the younger workers (18-27 y). However, the hematological abnormalities were not observed, despite of their positive correlation in genotoxic profile. Significant and positive correlations were observed in relation to the used medicaments, low consumption of vegetables as well as the type and place of work. In conclusion, biomarkers involved in comet assay can be applied in biomonitoring of genetic instability, including IR induced phenomena.

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Introduction

Human cells are daily exposed to risk of damage to the genetic material, sources of whose include exogenous (e.g. - plant toxins, UV, X and gamma rays, mutagenic chemicals, chemotherapeutic agents and viruses) and endogenous (reactive oxygen/nitrogen species, estrogen

Keywords

Occupational Exposure;
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metabolites, deamination, alkylations and so on). The increased levels of DNA damage and inefficient repair mechanisms are molecular events of many pathogenesis and diseases such as neurodegenerative diseases and cancer [1]. Genomic instability leads to the accumulation of mutations, is the initial step in the process of carcinogenesis. Recent data point to an association between genetic changes and cancer [2].

IR causes direct damages to the DNA, with the formation of radicals that generate lesions, such as single and double breaks in DNA strands, as well as change in bases. The overall outcomes are the bad effects on cell cycle, the repair capacity of genetic materials or apoptosis [3], and proceeding on oxidative damage to DNA, lipids, proteins and cell essential metabolites, with changes in the expression of proteins, metabolites, as well as in epigenetic events [4]. In this context, the biological effects of IR in humans exposed at low doses have being of relevant interests in relation to the effects of genetic instability, circulatory problems and in cancer [5].

The comet assay can be applied in clinical monitoring studies, an understanding of the pathogenesis of cancer and degenerative diseases, tumors prediction, radiation and chemotherapies and infertility studies, in addition to evaluation of occupational and environmental risks [1], including IR therapies, and in occupational and/or accidental exposure [2]. Its alkaline version has versatility in the detection of various lesions, for example: single and/or double stranded breaks, apurinic sites, oxidative damage and efficacy of repair [6], allowing the detection of damage at low doses, such as 25 cGy, with response to damage and DNA repair mechanism [7].

By the application of the comet assay in studies of occupational biomonitoring front to damage induced by IR in health professionals, our present study aimed to evaluate the frequency and levels of DNA damage in peripheral blood lymphocytes, along with the DNA repair rates after 7 d of exposure, and 2 d of no exposure. The data were also correlated

with the hematological parameters and to possible interference factors to the genotoxic analysis, including age, gender, lifestyle, time and place of work.

Materials and methods

Ethical aspects

In this study, the independent variables were manipulated, using blood cells and oral mucosal cells of 45 health professionals (radiologists, technologists and technicians) exposed occupationally to IR for genotoxic evaluation and study of repair. The study is following the international standards and guidelines established for research projects involving human subjects, and was approved by the Ethics Committee on Human Research, based at the Lutheran University of Brazil - ULBRA/RS, CAAE: 38570914.8.0000.5349. The participants of this study, after signing the Term of Free and Informed Consent (TFIC), aware of the importance and the individual benefits of the cellular response evaluation in non-neoplastic cells, answered a public health questionnaire, obeying the protocol published by the International Commission for Environmental Protection to Mutagens and Carcinogens (ICEPMC).

Sampling

As reported, 45 workers occupationally exposed to X-rays in two diagnostic clinics located in Teresina (Piauí), Brazil in 2015 participated in this study. For the control group, 45 people unexposed to IR were selected. All participants in the study agreed to participate and signed the TFIC. Peripheral blood samples were collected in two stages: (1) after weekly occupational exposure to IR and (2) after 48 hours of no exposure, including the weekend. Comet test was performed in the Toxicology and Genetics Laboratory of the Federal University of Piaui (UFPI), Brazil.

Comet assay

The alkaline (pH >13) comet assay was performed with slight modification from the method described by Pu et al. [8]. Briefly, blood samples were obtained by venipuncture from the individuals. Then, the samples were immediately processed. In lab, 5 μ L of blood (whole blood with heparin) were pipetted, which were added to 95 μ L of low melting agarose, placed on slides embedded in agarose gel, and covered with cover slips. The slides were then placed in the refrigerator to prevent loss of material following by the removal of the cover slips. Four slides were made for each patient; 2 of those were exposed to 5 μ M of hydrogen peroxide (H_2O_2) for 5 min (before exposure to lysis). After 2 h on lysis solution, the slides were immersed in an alkali buffer (pH >13) for 25 min. This process allowed the unfolding of the DNA chains through the breaking of the secondary and tertiary structures present in the cell nucleus. After unfolding, the slides were subjected to an electrical current in electrophoresis unit, which induced the migration of DNA fragments in the direction of the electric current for 25 min at 25 volts and 300 mA. All these steps were carried out under indirect and dark place to avoid further damage to the DNA. After electrophoresis, the slides were immediately neutralized with 0.4 M Tris buffer (pH 7.5), then fixed, washed with distilled water and dried overnight. After hydrating with distilled water for 5 min, the slides were stained with silver solution as described by Chavan et al. [9]. The results were expressed in damage index (DI) and frequency of damage (FD). The DI was obtained by evaluating the tail type, classified from 0 to 4 (50 cells per slide in duplicate) in an optical microscope with the magnification of 100X. Intact nuclei appear round (Class 0 - no damage), while in the damaged cells, the DNA migrates from the nucleus towards the anode during the electrophoresis, showing a "tail" of sedimented fragments, like a comet: Class 1 (minimum damage) to 4 (maximum damage). FD was calculated by subtracting cells with zero damage

from 100, that is, based on the number of cells with damage vs those without damage.

Hematological parameters of IR-exposed health workers

Blood samples were collected for the complete blood count (CBC). The tests were performed according to the protocols established by Labtest Diagnóstica AS.

Statistical analysis

Normality was assessed by the Kolmogorov-Smirnov test. The t-Student test was used to test the population characteristics. To compare the damage observed in comet assay, post-test Bonferroni Multiple Comparison Test, while the Mann Whitney test was performed for micronucleus assay. The GraphPad-Prism software (version: 5.0) was used to carry out these analyzes. All statistical analyzes, including Spearman's rho correlation, were considered significant when $P < 0.05$.

Results and discussion

Characterization of the health workers

Socio-economical features and the lifestyle of the health professionals unexposed (control group) and exposed to IR are presented in **Table 1**. By the application of analysis of variance statistical test (ANOVA) and t-Students test, no significance ($P > 0.05$) was observed for age, smoking, use of medicaments, vegetable consumption, in comparison to the individuals exposed to IR. The data point about the importance of risk factors for genetic instability, such as the continued use of medications and, especially, the poor consumption of vegetables.

The nutrients existing in vegetables are considered reducers and/or protective of damage to the DNA. Studies show that supplementation with antioxidant vitamins may reduce genetic instability due to the production of low frequency of micronuclei

Table 1. General features of the IR exposed and unexposed groups.

Subject features	Not exposed group (n=45)	Exposed group n = 45)
Age ¹		
	31.73 ± 7.44 (18-45)	31.47 ± 7.34 (19 47)
Gender ²		
Male	68.9 (n=31)	66.7 (n=30)
Female	31.1 (n=14)	33.3 (n=15)
Ethnic ²		
Caucasian	28.9	4.4
Black	31.1	31.1
White	40.0	64.4
Mulatto	22.0	-
Smoking ²		
Yes	22.2 (n=10)	-
No	77.8 (n=35)	100 (n=45)
Alcohol consumption ²		
Yes	66.7 (n=30)	44.4 (n=20)
No	33.3(n=15)	55.5 (n= 25)
Prescribed medication use ²		
No	33.3 (n=15)	55.5(n=25)
No	44.4(n=20)	66.7(n=30)
Not reported	33.4(n=15)	0.00
Vegetable intake ²		
Yes	20,0 (n=9)	13.3 (n=6)
No	80.0 (n=36)	86.7(n=39)
PPE use ³		
Yes	-	100(45)

1: Mean ± standard deviation (SD); 2: Percentage; 3: Personal Protective Equipment: coat, mask, apron and boots. P> 0.05 (ANOVA) *t*-Student test; Professionals reported that consumption of alcoholic beverages is related only to an average of 3 bottles of beer on the weekend.

(MN) [10]. Micronutrients hold DNA protection capabilities and, when in nutritional deficiencies, occur the inducing the MN formation [11]. Breakage and loss of chromosomes involve different cellular dysfunctions, such as acentric chromosome fragments resulting from double breaks, MN formation, which

can be affected by the co-factors, for example-magnesium and calcium [12]. However, the individual response to stress may vary along with various conditions, for instance the particular function and the combination of genes, absorption and metabolism, cell death (apoptosis/necrosis, cell cycle control, DNA repair and immune response, and micronutrient deficiencies) [13]. There are reports on the associations between genetic polymorphisms and MN formation, and thus genetic variants may modulate the effects of environmental exposure to genotoxic agents, as well as age, lifestyle characteristics (alcohol, tobacco, folates), cardiovascular diseases and cancer [14].

DNA damage profile in peripheral blood lymphocytes of IR exposed individuals

Evaluation of DNA damage can be used as an accurate biomarker for quantifying toxicogenetic risks of IR, which are also related to the cancer etiologically [15]. Health workers exposed occupationally to IR showed genotoxicity in blood cells significantly ($P < 0.0001$) where an increase in the rates and frequencies of DNA damage was observed (**Figure 1**). To evaluate the susceptibility to oxidative damage,

Figure 1: Genotoxicity in health professionals. [Mean ± SD (100 cells per slide); *** $P < 0.001$ (ANOVA, Bonferroni's multiple comparison test) compared to the control group].

blood cells from health professionals were exposed to H_2O_2 and the results showed similarities between the damage seen in cells exposed and unexposed to H_2O_2 .

In an occupational study, the nuclear medicine physicians showed DNA damage in leukocytes with the application of comet assay. Correlation with smoking was not observed in the study [16]. However, Khisroon et al. suggested that the radiologists' professionals are susceptible to the IR-induced genotoxicity [17].

The comet assay is a simple, rapid, versatile and sensitive method, which indicates the DNA damage and repair capacity [7]. Thus, this assay has been applied in studies of a wide variety of chemicals, biochemicals and even IR induced genotoxic events in test systems including humans [2]. The alkaline version of comet assay is capable to detect various lesions, such as single and/or double stranded, apurinic sites, the oxidative damage in genetic materials [18] even at low IR doses (e.g. - 25 cGy) [19].

IR is evident to cause DNA damage, such as single and/or double breaks, base modifications, cross-links [20]. The double breaks are more deleterious effects induced by IR and are associated with the acute toxicity and cancer/cancer-induced situations [21], notably, genomic disorganization and phosphorylation of histone H2AX at serine 130 residues are evident in this kind of breaks [22]. IR also induces DNA damage by mechanisms associated with reactive oxygen species (ROS), which can lead to lipid peroxidation [23]. The hydroxyl radical ($\bullet OH$) can be produced by UV and IR, which may cause production of other ROS. The ultimate results are the lesions in DNA [24].

The cytogenetic impact of X-rays, even at low doses (5-3 Gy), or disclosed to induce DNA damage, especially in the assessment of chromosomal abnormalities [25]. The radio-sensitivity can be attributed to the increase of chromosomal aberrations and changes in DNA repair [26].

DNA repair profile in peripheral blood lymphocytes after IR exposure

Humans are continually exposed to IR from natural and anthropogenic sources, with the induction of several biological effects at high and low doses (50-500 mGy), but with individual variations of responses by proteomic differences between individuals [27], due to the gene polymorphism associated with DNA damage, as well as those involved in the repair [28]. Health professionals from the clinics, after a week of occupational exposure to IR, showed increased rates and frequency of damage in peripheral blood lymphocytes, when compared to non-exposed workers, as shown in **Figure 1**.

It was also observed that, after 48 h, in the weekend, these damage frequencies have been reduced by about 20%, suggesting repair, as noted by significances between the increased frequency of injury observed after one week of exposure and the ones observed after 48 h (without exposure). These data were corroborated with the percentage of DNA repair, calculated by considering the damage observed during one week of exposure, which reached, on average of a 20% repair (**Figure 2**). In its alkaline version, the comet assay estimates the DNA injury

Figure 2: Frequency of Damage (FD) and percentage of DNA repair in peripheral blood lymphocytes of health workers exposed to ionizing radiation (IR).

Values are mean $\pm$ SD (100 cells per slide). FD = frequency of damage. *** $P < 0.001$ (ANOVA, Bonferroni's multiple comparison test) compared to the group 48 h after exposure to IR. The % of DNA repair was calculated using the formula: % R = [(FD after a week of exposure - FD 48 h after a week of exposure) / FD after a week of exposure] X 100.

Figure 3: Possible DNA repair in blood cells of health professionals exposed occupationally to the IR, considering the types of damage (0-4).

Values are mean $\pm$ SD (100 cells per slide). * $P < 0.05$ (ANOVA, Bonferroni's multiple comparison test) compared to the group 7 days of exposure (a) and 48 h after exposure (b). Damage types 0 (A), 1 (B), 2 (C), 3 (D) and 4 (E). Photomicrograph profile of control group (F), 7 days of exposure (G) and 48 h after exposure (H).

and also determines the repair effectiveness with predictive value for clinical studies on IR effects, with responses in individualized cells, once it is a rapid method for detecting DNA damage and repair possibilities in cell lines that decrease the length of tail after 60 minutes application of radiation [29].

The biochemical mechanisms of DNA repair have been well characterized by Aziz Sancar and Paul Modrich[30], but how these mechanisms are regulated, still to be found out [31]. As characterized in the previous item, IR induces different DNA lesions, once the double breaks are the most common and lead to genetic instability, cell death and cancer. The double breaks induced by the IR repair by homologous and non-homologous recombination [32], in the G2 phase of the cell cycle [33].

Repair indicative were also observed by the analysis of the types of damage (0-4) of the DNA du-

ring the observation period. The data presented in **Figure 3** indicate significance ($P < 0.05$) in damages graded by 1, 2, 3 and 4 regarding to the control group. More frequent damages were observed in type 1, 2 and 4.

It should be emphasized that failures in these repair mechanisms can lead to DNA damage and apoptosis [34], as well as changes in DNA (mutations, chromosomal aberrations, translocations) [35]. However, in tumor cells, the repair is constituted a resistance to radiotherapy [36]. An understanding of cell responses to the DNA damage is important for the comprehension of the molecular mechanisms for post-transcriptional modification factors involving in responses to the cellular damage, primarily related to double strand breaks (**Figure 4**). The repair path for double failures can be attributed to the repair by homologous recombination (HR) and non-homologous recombination (NHR), which occurs in the G2 period of the cell cycle. The phosphorylated RPA replication protein is a marker of response to IR [33]. The double breaks are potentially lethal, since they cannot be repaired, leading to genomic instability. One study indicates that the ATM proteins are the sensor for IR and responsible for HR repair [37].

The responses to DNA damage are important for the maintenance of the genome, such as DNA repair, apoptosis and senescence. The apoptosis are often used as cancer risks and are controlled by the p53 protein [38]. Notably, p53, p63 and p73 proteins, and transcription factors involved in the induction of the cell cycle arrest are important, emphasizing that no repair or incorrect repair leads to apoptosis [7]. The repair of nuclear injury induced by IR depends on the mitochondria, as they consume energy (ATP), and cells exposed to radiation increase oxygen consumption, with a reduction in the kinases phosphorylation (CDK1), which operate in the cell cycle and nuclear DNA repair. These aspects are important for the understanding of genotoxic stress conditions [39].

[illegible]

7

Table 3. Hematological parameters of exposed and unexposed groups to ionizing radiation.

Groups	Gender	Parameters	Minimum	Maximum	Mean	SD	Reference values
Not exposed	M	Hemoglobin	14.0	17.6	15.5	1.0	13.0 - 27.5
		Erythrocytes	4.4	5.5	4.9	0.2	
		Platelets	22000.0	420000.0	281452.5	60114.4	
		Hematocrit	41.0	50.0	45.6	2.4	
		Leucocytes	2700.0	7600.0	5588.2	1073.9	
		Basophil	0.00	0.00	0.0	0.0	
		Eosinophil	0.00	5.0	1.6	1.0	
		Rod cells	0.00	2.0	.1	0.4	
		Segmented Neutrophils	43.0	68.0	58.0	5.8	
		Lymphocytes	30.0	48.0	37.0	4.7	
		Monocytes	1.0	5.0	3.0	1.2	
	F	Hemoglobin	11.1	16.0	13.7	1.2	
		Erythrocytes	4.0	5.1	4.6	0.2	
		Platelets	200000.0	430000.0	281756.4	55867.7	
		Hematocrit	36.0	45.0	42.2	2.1	
		Leucocytes	3200.0	52000.0	7888.4	11064.7	
		Basophiles	0.0	0.0	0.0	0.0	
		Eosinophil	1.0	5.0	2.2	1.2	
		Rod cells	0.0	2.0	0.2	0.5	
		Segmented neutrophils	46.0	68.0	56.4	5.3	
		Lymphocytes	33.0	46.0	39.5	4.4	
		Monocytes	1.0	5.0	2.4	1.3	
Exposed	M	Hemoglobin	11.1	16.0	14.1	1.1	
		Erythrocytes	4.00	5.10	4.6	0.2	
		Platelets	190000.0	430000.0	270346.8	42845.6	
		Hematocrit	36.0	48.0	43.0	2.9	
		Leucocytes	3200.0	52000.0	7212.5	9498.8	
		Basophiles	0.0	0.0	0.0	0.0	
		Eosinophil	0.0	5.0	1.9	1.1	
		Rod cells	0.0	2.0	0.2	0.5	
		Segmented neutrophils	43.0	68.0	57.7	6.2	
		Lymphocytes	30.0	48.0	37.7	5.4	
		Monocytes	1.0	5.0	3.0	1.4	
	F	Hemoglobin	11.1	15.0	13.2	1.0	
		Erythrocytes	4.0	5.1	4.5	0.3	
		Platelets	22000.0	420000.0	299785.7	84683.4	
		Hematocrit	36.0	45.0	40.3	3.0	
		Leucocytes	4050.0	7900.0	5473.6	1100.6	
		Basophiles	0.0	0.0	0.0	0.0	
		Eosinophil	0.0	4.0	1.2962	0.8	
		Rod cells	0.0	1.0	0.2	0.4	
		Segmented neutrophils	47.0	65.0	58.0	5.8	
		Lymphocytes	31.0	43.0	36.3	3.5	
		Monocytes	1.0	4.0	2.8	1.0	

Hematological evaluation of patients exposed to IR

The biological effects of IR are primarily derived from the generation of electrons that generate free radicals and subsequent attack DNA, proteins and lipids. However, this situation can be derived directly by the deposition and energy, generating systemic effects [40]. Studies on doses and effects of IR in relation to the cytopenia (leucopenia, thrombocytopenia), as hematopoietic cell responses front to the chronic exposure, have been proposed for different types of blood cells to measure the damage [41]. In our study, no changes were observed in hematological parameters in exposed and unexposed individuals within the observation period (Table 2). However, a decreased level in hemoglobin content and red blood cells were observed in the exposed group in comparison to the control group (data not shown).

Blood cells reduce oxidative stress in DNA damage from blood mononuclear cells by phosphorylation of histone H2AX, recruit repair, which indicates that a large amount of hemoglobin protects against oxidative damage to DNA [42]. The phosphorylation of histone H2AX is an earlier event to repair DNA breaks induced by IR to maintain the genetic stability [43].

Correlations between the genetic damage in relation to age, gender and lifestyle with genotoxic and hematological biomarkers

Low doses of IR inducing oxidative damage, initiate responses partially by endogenous antioxidants, with heterogeneity in biological matrices, species, and age. However, there are adaptations to antioxidant responses [44]. Oxidative damage can cause disruption of biopolymers [45], and may affect proteins, protein complexes and lipids [46].

For the analysis of possible differences between the mean ages in relation to genotoxic damage, the ID and FD were used. The data not only points

Figure 5: DNA damage according to age of the individuals in peripheral blood lymphocytes. A: ID (index of damage); B: FD (frequency of damage). ANOVA-Bonferroni's test. *** $p < 0.001$ compared to control group (N=45). ^{a,b} compared to age intervals of 18 to 27 and 28 to 37.

genotoxicity in workers exposed to X-rays, when compared to unexposed group, but also indicate that workers in mean age of 18-27 y were more susceptible to DNA damage induced by occupational exposure to X-rays (Figure 5A and 5B). Studies show that the environmental, occupational, dietary and lifestyle effects may be important in clinical analysis of risk of DNA damage, which can be evaluated by the comet assay [47]. It is noteworthy that, the repair capacity of single and double strand breaks in peripheral blood decrease with the age of the individuals [7], despite of having a significant positive association [48].

Studies show an association between the risk of IR (average of 1.5 Gy) on the induction of menin-

gioma in smokers and nonsmokers as well as in relation to gender. The data indicate significant risks for smokers and for women [49].

Although an exposure to IR is relevant for diagnosis, but the cytotoxic and genotoxic harms may occur with a number of chemical agents, individually or synergistically with IR [50]. Otherwise, the adaptive responses are the phenomena with IR exposure, related to the variability in the expression of genes, endocrine and hormonal factors [51, 52]. In this context, not only diagnostic assays but also the understanding of the influence of environmental factors, lifestyle and clinical investigations are crucial for factors in bio-genetic monitoring [47].

With the application of statistic of Sperman's rho correlation, positive and significant correlations between lifestyle (**Table 1**) with the genotoxic biomarkers were observed. Corroborating with the hypothesis that occupational exposure to IR has genotoxicity risks, rising indices and frequency of damage (**Figure 1**), the data showed positive correlations between these parameters of genotoxicity with the type and time of work. However, the genotoxicity observed was also correlated with the use of prescription drugs and with the low consumption of vegetables (**Table 3**). In our study, a positive and significant correlation was observed between hematological parameters and genotoxicity biomarkers.

Multiple nutrients and its interactions, optimize genomic stability and DNA repair capacity [53]. The increased levels of DNA injury and inefficient repair mechanisms are molecular events of many diseases, including cancer and neurodegenerative diseases [1].

Pharmaceutical drugs can cause genotoxic damage and/or carcinogenic effects, and therefore should be considered in the evaluation of the risk/benefit ratio [54]. Many pharmaceutical formulations are evident to induce toxic phenomena in various organs. Therefore, investigations of the molecular mechanisms are necessary, aiming to the benefits of the therapeutic potential [55]. Hopefully, regulatory agencies, notably in Europe and in US are always

Table 3. Correlations between the life style and biomarkers.

Parameters	Sperman's rho	p value
Lifestyle versus biomarkers		
Time of work vs index of damage	0.637	0.001**
Work place vs index of damage	0.309	0.039*
Work place vs frequency of damage	0.324	0.030*
Index of damage vs prescribed medication	0.339	0.000**
Frequency of damage vs prescribed medication	0.688	0.000**
Vegetable intake vs index of damage	0.324	0.000**
Vegetable intake vs frequency of damage	0.161	0.000**
Haemoglobin mg/dLvs index of damage	0.249	0.000**
Haemoglobin mg/dLvs frequency of damage	0.153	0.000**
Erythrocyte count vs index of damage	0.161	0.000**
Erythrocyte count vs frequency of damage	0.115	0.000**

conscious about this matter [56]. Among the other adverse effects, carcinogenic potentials are the prime concerns in risk assessment. Chronic effects are of major concerns in this context, as it may lead varieties of deleterious events including cancer [1].

Conclusion

Occupational exposure to IR, even at low doses in health professionals may cause genotoxicity. In this study, we performed alkaline comet assay to investigate two important biomarkers as damage index and frequency of damage of DNA in peripheral blood cells (lymphocytes) in an exposed group and an unexposed group. Our findings suggest that, the DNA damage was more prominent in younger professionals in relation to the old in the exposed

group. However, an average of 20% DNA repair was observed, when compared to the frequency of damage after a week of exposure. We founded a correlation between the genotoxic damage and damage type with duration of work, drug as well as micronutrient users. In conclusion, comet assay may be a helpful tool to monitor the genotoxicity induced by IR.

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