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Research Article
Genotoxicity of Gamma Radiation Against Lymphocytes of Radiation Workers: The Cytokinesis-Block Micronucleus Assay

[1]Yulia Irnidayanti, [1]Marlina Azzahra, [2]Yanti Lusiyanti, [2]Devita Tetriana and [2]Darlina Yusuf

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Hasyim Asyarie Building, Rawamangun Muka, Jakarta 13220, Indonesia
[2]Research Center for Safety, Metrology and Nuclear Quality Technology, Building No. 720, KST B.J. Habibie Puspiptek St., Muncul, Setu, South Tangerang, Banten 15314, Indonesia

Abstract

Background and Objective: Gamma irradiation induces genotoxicity, characterized by the formation of extra-nuclear bodies and left behind during the anaphase stage of cell division, often referred to as a micronucleus (MN). The present work aims to monitor exposure to ionizing radiation as a genotoxic agent in the lymphocytes of workers at radiation energy centers. **Materials and Methods:** The lymphocyte cytokinesis block micronucleus assay used and analyzed the correlation between the Nuclear Division Index (NDI), age, blood type and the number of micronuclei (MN). Blood samples were collected from 20 volunteers in heparin tubes, exposed to 2 Gy gamma rays and cultured *in vitro*. **Results:** A significant difference in the number of micronuclei between blood group A and blood groups A, B and AB. The Nuclear Division Index (NDI) value for lymphocytes of radiation energy center workers after gamma radiation was significant (1.74±0.1) but still within the normal range. Neither MN frequency nor NDI values correlated with age, but MN frequency showed a correlation with blood type. **Conclusion:** The gamma irradiation did not induce a cytostatic effect but proved genotoxic to the lymphocytes of radiation energy center workers. Notably, blood type A demonstrated higher sensitivity to gamma radiation.

Key words: A blood type, genotoxic, micronuclei, gamma radiation, sensitivity, cytokinesis-block micronucleus assay

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Corresponding Author: Yulia Irnidayanti, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Hasyim Asyarie Building, Rawamangun Muka, Jakarta 13220, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the modern era, the role of ionizing radiation holds significant importance. Ionizing radiation is utilized in the medical field, particularly in cancer therapy. However, humans can also be exposed to ionizing radiation from various sources, including environmental factors, occupational activities, the use of medical devices and other sources[1]. Exposure to natural ionizing radiation is a part of life on Earth. Medical radiation workers often have more exposure to low doses of ionizing radiation (IR) worldwide[2]. Exposure to radiation or radioactive substances resulting from occupational activities can be harmful to workers in industries, medicine and research. Those who work with ionizing radiation like medical personnel are at high risk for exposure to low levels[3-5]. Hospital staff, such as doctors and nurses, encounter low levels of ionizing radiation from diagnostic X-rays and medical instruments. The escalating number of medical procedures involving radiation has significant implications for those involved in such professions.

Gamma radiation is the primary form of ionizing radiation utilized in radiotherapy. The hematopoietic system is particularly vulnerable to ionizing radiation, with even low-level exposure at one Gy capable of inducing changes in blood composition. Exposure to 2 Gy can be fatal and levels exceeding seven Gy within 4 to 6 weeks can result in hematological failure[6]. Prolonged exposure to ionizing radiation can adversely affect cell division, especially in sensitive tissues like the bone marrow. Disruptions in the bone marrow can lead to reduced blood production, impairing the transport of oxygen.

Ionizing radiation can have direct or indirect effects on cells and tissues. The direct impact involves the ionization of macromolecules in cells, while the indirect impact results from the radiolysis of water, leading to the formation of free radicals. Molecular-level effects, such as DNA and chromosome damage, present as acentric chromosomes and segregation errors of entire chromosomes. Acentric chromosomes formed during anaphase lag and do not fuse with the main daughter nuclei, thus forming micronuclei[7]. Micronuclei (MN) serve as indicators of genotoxicity, utilized in clinical and occupational biomonitoring, as well as biological dosimetry[8]. The presence of micronuclei signifies DNA damage in cells[9].

The Cytokinesis Block Micronucleus (CBMN) assay is a valuable tool for assessing DNA damage resulting from exposure to genotoxic agents in radiation worker populations. This method has gained global recognition and demonstrated high appropriateness, as proven by Zaguia *et al.*[10]. Notably, the CBMN assay is user-friendly and highly effective, positioning it as a promising tool for evaluating genotoxicity induced by both chemical and physical agents. It also proves beneficial in prioritizing medical interventions during nuclear emergencies[11]. The CBMN test, a well-established technique, requires minimal effort and involves whole blood cultures easily obtained through venipuncture, thus reducing both the time and costs associated with the procedure[12].

Lymphocyte cells are frequently employed in CBMN assays due to their rapid response and consistent division when induced by toxic agents[13,14]. Under normal conditions, the number of micronuclei in peripheral blood lymphocytes typically ranges from 0 to 30 per 1000 binucleate cells[11]. The number of nuclei in lymphocyte cells is utilized to acquire quantitative information regarding the development of the lymphocyte cell cycle[15]. The quantitative parameter representing the numerical value of cell proliferation is known as the Nuclear Division Index (NDI) and serves as a general cytotoxicity biomarker[16]. Typically, the NDI value in peripheral lymphocyte cells ranges from 1.3 to 2.2. Lymphocytes that fail to divide exhibit an NDI value of 2.0, whereas lymphocytes with more than one nucleus indicate completed division, corresponding to an NDI value exceeding 2.0[11]. The current study aimed to monitor radiation exposure as a genotoxic agent in various occupations using the CBMN assay. Additionally, the study investigated the correlation between the NDI index, age, blood type and the number of MN.

MATERIALS AND METHODS

Study area: This research was conducted at the Cytogenetic Laboratory in 2021, at the Center for Radiation Safety Technology and Metrology of the National Nuclear Energy Agency (BATAN).

Sample: The study included 20 healthy volunteers employed at the National Atomic Energy Agency, representing blood groups A, B, AB and O[17]. The research protocol received approval from the Ethics Committee of the Institute for Health Research and Development, Ministry of Health of the Republic of Indonesia, with Ethical Approval Number: LB.02.01/2/KE.490/2020.

Collection and irradiation of blood samples: Blood samples were collected from 20 radiation workers using a heparinized vacutainer to prevent clotting. The collected blood, with a volume of 3 mL, was promptly stored at 0-4°C. Before collection, the vacutainer tube was appropriately labeled. The blood samples were irradiated with 2 Gy Gamma, for 37 sec and did not come into contact with any radioactive substances. The radiation processes followed the method

outlined by Elahimanesh *et al.*[18]. A 3 mL blood sample was placed in a Petri dish and exposed to 2 Gy Gamma radiation for 37 sec using a Gamma Chamber 4000 Irradiator. The resulting irradiated blood samples were then utilized for the CBMN test.

CBMN test procedures: The CBMN assay was conducted following the standard method[19]. A 0.5 mL blood sample was combined with 4.5 mL of RPMI 1640 media. The cell culture medium comprised RPMI 1640 solution, 0.8 mL of L-glutamine and HEPES, 0.8 mL of fetal bovine serum and 0.8 mL of penicillin-streptomycin. Additionally, 0.1 mL of phytohemagglutinin was added. Lymphocytes were cultured for approximately 72 hrs at 37°C with a flow of 5% CO gas. Cytochalasin B was introduced to the culture medium after around 44 hrs and then transferred to a culture tube, followed by centrifugation for 10 min at a speed of 800 rpm. Subsequently, the lymphocyte cells were exposed to 6 mL of cold hypotonic solution (0.075 M KCl) for 30 min and centrifuged for 8 min at 800 rpm. The resuspension process involved adding 5 mL of Ringer Carnoy's solution. Rinsing was performed 2-3 times using 5 mL of Carnoy's solution until the suspension appeared clean. This entire process was repeated once more to ensure a thoroughly clean suspension, which was then stored in the freezer overnight before use. The lymphocyte precipitate was applied onto a glass object, positioned on a slide rack at room temperature and stained with 4% Giemsa dye for 10 min. Drying is carried out by air at room temperature for approximately 12 hrs. In the final stage, the preparation is covered with a cover glass by applying Entellan to the slide.

Observations were conducted under a light microscope (Nikon Eclipse 100-Japan) with magnifications ranging from 400x to 1000x. The MN was calculated in 1000 binucleate cells[20]. The number of cells with one, two, three and four nuclei (mononucleate, binucleate, trinucleate and tetranucleate) per 500 cells was recorded to measure the cytostatic effect on radiation worker lymphocytes. The NDI value is determined based on the equation proposed by Eastmond and Tucker[21]. The NDI value is calculated using the formula:

$$NDI = \frac{(1\times M1) + (2\times M2) + (3\times M3) + (4\times M4)}{N}$$

where, M1 to M4 represents the total of cells with 1-4 nuclei and N is the total number of cells scored.

Statistical analysis: The Kruskal-Wallis's test was employed to analyze the MN frequency and NDI values of lymphocytes before and after radiation, with a significance level set at $p<0.05$ due to the non-normal distribution of the data. The data analysis was conducted using the SPSS version 25.0 for Windows software application.

RESULTS

The results showed that the NDI values of workers' lymphocytes pre- and post-irradiation showed difference was not significant (Table 1). Although the NDI value in lymphocytes pre-irradiation was higher significantly (1.74±0.1) than the NDI value post-irradiation (1.65± 0.08), it was still in the normal category. In general, the NDI value ranges between 1.3-2.2, which is included in the normal category. Thus, it can be concluded that 2 Gy Gamma radiation does not affect the cytostasis of BATAN workers' blood lymphocytes. This means that 2 Gy Gamma radiation did not inhibit cell multiplication and stop worker blood lymphocyte cell growth.

The profiles of mononucleate, binucleate, trinucleate and tetranucleate lymphocyte cells were shown in Fig. 1. Mononucleate lymphocyte cells are characterized by one cell nucleus and the lymphocytes have not yet divided. Binucleate lymphocyte cells are marked by the appearance of two cell nuclei and successful division once. Trinucleate and tetra nucleate lymphocyte cells are marked by the appearance of three and four-cell nuclei, which indicates two divisions.

The frequency and the representative image of MN in blood lymphocytes of workers pre- and post-irradiation were described in Table 2 and Fig. 2. Exposure to 2 Gy Gamma radiation led to a significant increase in the frequency of MN in workers blood lymphocytes. The mean score of MN in blood lymphocytes of pre- and post-radiation workers was 9.8±4.4 and 169±32.96. The incidence of MN increases, surpassing the normal frequency range (1-30). This suggests that exposure to 2 Gy of gamma radiation is genotoxic and leads to an elevated number of micronuclei in lymphocyte blood cells.

Table 1: Mean score of mononucleate, binucleate, trinucleate and tetranuclear on blood lymphocytes of radiation workers

			∑ Nucleus				
Radiation	N	Total	Mononucleate M1 ($\bar{\chi}$±SD)	Binucleate M2 ($\bar{\chi}$±SD)	Trinucleate M3 ($\bar{\chi}$±SD)	Tetranucleate M4 ($\bar{\chi}$±SD)	NDI ($\bar{\chi}$±SD)
Pre	20	500	241.05±25.67	215.3±24.64	22.1±5.46	21.55±9.52	1.65±0.08
Post	20	500	210.25±24.57	234.95±19.38	29.2±10.38	26.1±13.34	1.74±0.1*

*Significant $p<0.05$, NDI: (M1+2M2+3M3+4M4)/500 and NDI 1.3-2.2 (normal)

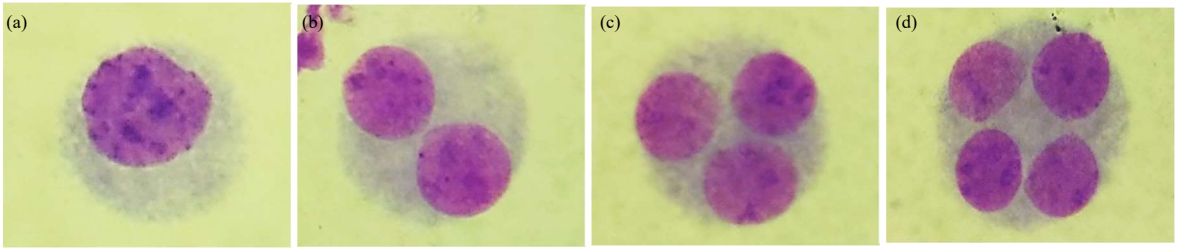

Fig. 1(a-d): Representative images of lymphocyte cells in the blood of workers after exposure to 2 Gy 1000x), (a) Mononucleated cell, (b) Binucleated cell, (c) Trinucleated cell and (d) Tetranucleated cell

Table 2: Mean frequency of micronuclei (MN) in blood lymphocytes among radiation workers

			ΣMN on binucleate cells						
Radiation	N	Total	MN 0 ($\bar{\chi}$±SD)	MN 1 ($\bar{\chi}$±SD)	MN 2 ($\bar{\chi}$±SD)	MN 3 ($\bar{\chi}$±SD)	MN 4 ($\bar{\chi}$±SD)	Total MN	MN ($\bar{\chi}$±SD)
Pre	20	1000	990.2±4.39	8.5±2.88	0.5±0.76	0.10±0.3	0.00±0.0	196	9.8±4.4
Post	20	1000	831±32.96	129.85±22.42	17.05±6.76	1.35±1.04	0.25±0.44	3380	169±32.96*
p-value									0.00

*Significant p<0.05 and MN 0-30: Normal

Table 3: Correlation coefficient between the NDI values of lymphocytes exposed to 2 Gy Gamma radiation and the age of the workers

		Correlation coefficient (r) of NDI			
Age	N (individual)	Pre-radiation	p<value	Post radiation	p-value
<40 years	7	0.04	0.93	0.16	0.72
>40 years	13	0.39	0.18	0.11	0.71

p<0.05

Table 4: Correlation coefficient between the micronucleus frequency of lymphocytes exposed to 2 Gy gamma radiation and the age of the workers

		Correlation coefficient (r) of MN			
Age	N (individual)	Pre-radiation	p-value	Post radiation	p-value
<40 years	7	0.18	0.68	0.28	0.54
>40 years	13	0.28	0.34	0.16	0.59

p<0.05

Table 5: Average score of micronuclei frequency in lymphocytes pre- and post-radiation 2 Gy Gamma radiation based on blood type

		MN ($\bar{\chi}$±SD)	
Blood type	n	Pre-radiation	Post-radiation
A	5	9.8±4.6	201.8±30.99[a]
B	5	11.8±6.76	158.2±33.12[b]
AB	5	10.2±2.86	167.2±28.95[b]
O	5	7.4±2.07	148.8±16.16[b]
p-value		0.57	0.04

[a,b]Significant p<0.05

The correlation between the NDI index in lymphocytes and the age of workers is represented in Table 3. The NDI value of post-irradiation worker lymphocytes in both age groups (40≤ and 40≥) did not show a significant correlation (p>0.05). Likewise, the correlation between the frequency of MN worker lymphocytes after 2 Gy Gamma radiation in both age groups was not significant either (p>0.05). Table 4 shows that there is no correlation between age and genotoxic damage to lymphocytes after exposure to 2 Gy gamma irradiation (Table 4).

Pre-irradiation micronuclei frequencies were as follows: 9.8±4.6 for blood group A, 11.8±6.76 for blood group B, 10.2±2.86 for blood group AB and 7.4±2.07 for blood group O. However, a significant increase in the average frequency of micronuclei in lymphocyte workers was observed after irradiation. The post-irradiation mean micronuclei frequency score in blood group A was 201.8±30.99, in blood group B was 158.2±33.12, in blood group AB was 167.2±28.95 and in blood group O was 148.8±16.16 (Table 5). Notably, the mean MN score in blood group A is

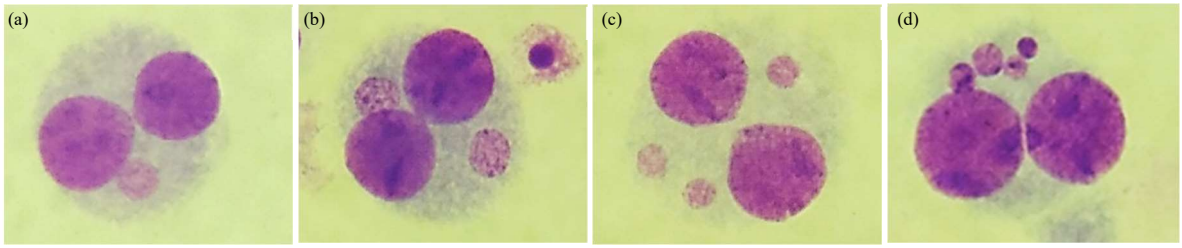

Fig. 2(a-d): Representative image of micronuclei in binucleated cell of post-irradiation workers' blood lymphocyte (1000x), (a) One micronuclei (1MN), (b) Two micronuclei (2MN), (c) Three micronucleus (3MN) and (d) Four micronucleus (4MN)

significantly higher than in the other blood groups, indicating that individuals with blood type A are more sensitive to radiation.

DISCUSSION

This study found that exposure to gamma radiation increased the frequency of MN formation and significantly increased the NDI index in radiation workers. Exposure to 2 Gy of gamma radiation causes DNA damage in the lymphocytes of workers, leading to the formation of micronuclei, indicating its genotoxicity. The frequency of micronuclei (MN) in lymphocytes of blood cells is not correlated with age. However, the frequency of MN in blood group A is higher than that in other groups. It appears that individuals with blood type A may be more sensitive to radiation compared to other blood types.

The cell division can provide information related to the assessment of cell cytotoxicity. Cell division status can be determined through the Nuclear Division Index (NDI)[22] and is usually used as an indicator to evaluate division status in living cells[20]. This value provides numerical information directly related to DNA replication and the number of cell cycles during the progression of lymphocytes in cell culture[23]. Additionally, NDI is a biomarker of the mitotic and cytostatic effects of mitogenic agents[15] and also a biomarker for predicting health risks. Cells with extensive chromosomal damage may either die before cell division or may be unable to enter this phase[24]. Therefore, when cells accumulate genetic damage, they lose the ability to divide, will die before cell division, or are less likely to enter the mitotic phase. In this study, the NDI value was 1.74. This value shows that there are living cells that have completed one division and some have not yet completed their division. If all cells have completed division, then all cells will have two nuclei, resulting in an NDI value of 2.0. Therefore, it was concluded that 3 Gy Gamma radiation did not affect worker lymphocyte cell proliferation[25].

The micronucleus is used as a biomarker to detect DNA damage caused by genotoxic agents[26]. Ionizing radiation, as a genotoxic agent, induces various types of genetic damage, including single strand breaks (SSB) and double strand breaks (DSB). The DSB damage that cannot be completely repaired can result in the formation of micronuclei and at the most severe level, it can lead to mutations in cells[27]. The frequency of spontaneous micronuclei in a healthy population can range from 0 to 40 per 1000 binucleated cells[28]. An increase in the frequency of MN numbers indicates genetic damage, in the form of acentric fragments. Fenech *et al.*[7] showed that an elevation in the number of MN due to radiation exposure led to mutations, one of which was characterized by the presence of acentric fragments. These fragments cannot fuse with the daughter cell nucleus at the anaphase stage, forming a micronucleus. It concludes that gamma radiation is the cause of the increase in MN frequency. This damage is a biological response, which can activate cell signaling pathways and ultimately can cause cell cycle arrest or apoptosis[29].

In addition, the radiation dose can affect the number of micronuclei formed. Research by Lusiyanti *et al.*[30] showed that cytogenetic damage due to exposure to doses as low as 0.05 Gy in human lymphocytes was visible in the presence of MN and doses of 1.0 Gy and 2.0 Gy caused an elevation in the frequency of MN. However, the higher the dose, such as 5 Gy, there is a decrease in response, where most cells are damaged and unable to carry out mitosis. Lymphocyte proliferation is a form of immune response to radiation that triggers DNA damage. Everyone has a different immune response and DNA repair abilities[31]. The adaptive immune response of everyone can also influence the number of micronuclei in lymphocyte cells.

In the present research, a significant difference was observed in the frequency of micronuclei (MN) in workers' lymphocytes after radiation exposure ($p<0.05$) and this difference was found to be related to blood type. The increase in MN frequency in blood type A was higher than in other blood types. Blood type A has higher radiosensitivity than other blood types[18]. Therefore, exposure to 2 Gy Gamma radiation in blood type A causes a decrease in lymphocyte cell proliferation or division[32]. A decrease in cell division can be

shown by the NDI value. Even though the increase in the NDI value is significant, it remains within the normal category. The normal NDI value category is 1.3-2.2. The NDI value below 1.3 indicates that most lymphocytes are mononucleate and the NDI value above 2.2 indicates that most lymphocytes have more than two nuclei.

Research by Ramadhani *et al.*[33], Cheng *et al.*[34] and Miloševic-Djordjevic *et al.*[35] demonstrated that age does not correlate with NDI values or micronuclei (MN) frequency. Additionally, the frequency of MN is influenced by various factors, including lifestyle, gender, nutritional status, smoking habits, diet and alcohol consumption[36]. Some studies have shown an increase in the frequency of MN with age in individuals exposed to radiation[37-39]. However, in the present study, age did not show a correlation between NDI values, age and MN lymphocyte frequency in workers after exposure to 2 Gy of Gamma radiation.

CONCLUSION

The study found that exposure to 2 Gy Gamma radiation led to an increase in the frequency of micronuclei (MN) formation. Before radiation exposure, the average number of MN in the lymphocytes of workers was within normal limits, but after radiation, there was a significant increase in the number of MN. There is no correlation between human age and NDI values or MN frequency. However, MN frequency is significantly higher in blood group A than in other blood groups.

SIGNIFICANCE STATEMENT

Radiation workers usually have jobs related to radiation throughout their lives, such as doctors, nurses and workers in industry, medicine and research as a result of their work activities. Although the majority of radiation workers or hospital staff are exposed to low doses of ionizing radiation, the occupational hazards of these individuals are not limited to the time they are on duty, but their effects will increase with more exposure to ionizing radiation and according to the available evidence, may affect future generations. Therefore, it is necessary to monitor the health of workers who are in contact with ionizing radiation to prevent adverse effects which are a serious matter through the CMBN test.

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