

Correlation between γ -H2AX, micronucleus and annual occupational dose in medical radiation workers

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ABSTRACT

Background: Medical workers are exposed to long-term low levels of ionizing radiation, which makes them vulnerable to DNA damage. There are potential occupational health hazards from radiation exposure in a large occupational segment of the population. **Materials and Methods:** During this study, 69 blood samples were taken from 45 medical workers (including diagnostic radiologists, radiographers, and cathlab nurses) and 24 nonmedical workers as controls from three hospitals across Indonesia, and were analyzed for the presence of DNA damage. Detection of γ -H2AX expression as a biomarker of DNA DSB damage and the micronucleus assay were carried out by immunofluorescence microscopy and Giemsa staining, respectively. **Results:** The mean γ -H2AX foci index in workers was 0.02(0.00-0.24) and in control was 0.02(0.00-0.12), micronucleus frequency of workers (5-30 per 1000 cells) and control (12-29 per 1000 cells). The annual occupational dose of workers was recorded as (0.01-1.12 mSv). There was no statistical difference in the mean number of γ -H2AX foci and the micronuclei frequency between workers and controls, but there was a small tendency toward correlation between γ -H2AX foci and micronuclei frequency and the annual occupational dose of workers. **Conclusions:** γ -H2AX foci have the potential to be used as a biomarker to detect radiation-induced DNA DSB damage in workers caused by occupational dose exposure.

Keywords: γ -H2AX, micronucleus, occupational dose exposure, medical radiation workers.

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INTRODUCTION

The extensive use of ionizing radiation as a diagnostic and therapeutic tool represents the largest man-made contribution to the radiation dose received by the general population ⁽¹⁾. Medical workers are among the most frequently exposed to low doses of ionizing radiation ⁽²⁾. Their levels of exposure in hospitals have decreased below the regulatory limit in recent

decades, even though the increasing use of relatively high-dose procedures, such as interventional cardiology, raises some concern ⁽³⁻⁵⁾. High doses of ionizing radiation are known to induce acute and chronic effects in humans, while the potential risk of detrimental effects related to low doses of radiation is still a matter of discussion ^(6, 7).

Medical radiation workers, including doctors, nurses, and other medical staff, are exposed to

low doses of ionizing radiation from several sources, including diagnostic x-rays and other medical instruments ⁽⁸⁾. They constitute the largest occupational group exposed to man-made sources of radiation ⁽⁹⁾. Many medical uses of radiation, such as those in nuclear medicine, may cause staff exposure at higher doses. Nuclear medicine employees are continuously exposed to ionizing radiation in the workplace despite the use of radiation protection equipment ⁽¹⁰⁾.

Exposure to radiation initiates certain changes to proteins, carbohydrates, lipids, nucleic acids, and gene expression in exposed cell(s). This can be observed using various tools that are collectively defined as radiation biomarkers. In particular, ionizing radiation in a cellular system can bring about several changes such as base damage, alkylation, intercalation adduct formation, nucleotide modifications, and single-strand and double-strand breaks in the deoxyribonucleic acid (DNA) ⁽¹¹⁾. These changes may result either from direct deposition of energy on the nucleic acids (direct action), or they can be mediated by the actions of free radicals released at some point during the interaction with water (indirect action) and membranes (lipid peroxidation) within the cells ⁽¹²⁾.

The scientific evidence, both from human population surveys and animal experiments, indicates that the risks related to low doses of radiation are mainly due to stochastic effects. The prime target of ionizing radiation is DNA. Ionizing radiation produces its genotoxic effect by inducing double-strand breaks (DSBs) and non-DSB highly clustered DNA lesions consisting of single-strand breaks (SSBs), a-basic sites, and oxidized bases. However, other effects associated with exposure to ionizing radiation in the low-dose region, referred to as non-targeted effects, include damage such as chromosomal aberrations, genomic instability, and cell death ⁽¹¹⁻¹⁴⁾.

Following exposure to ionizing radiation, DNA double-strand breaks (DSBs) are formed at sites of DNA damage. One of the initial responses to the DSB is the phosphorylation of histone H2AX protein. It forms gamma-H2AX (γ -H2AX)

foci at the DNA damage sites within minutes ⁽¹⁵⁾. Each DSB site is believed to correspond to one microscopic γ -H2AX focus that rapidly forms and, depending on the functionality of the DNA repair response, is repaired. This is indicated by the disappearance or repair of foci at these sites. These foci repair at different rates over a period of up to 48 hours ⁽¹⁶⁻¹⁹⁾. They can be observed microscopically by the immunofluorescent γ -H2AX assay which immunostains the phosphorylated H2AX histone represented as γ -H2AX. Both the intensity of the fluorescent signal at individual DSB sites and the number of γ -H2AX foci is directly proportional to the amount of DSB formed ⁽²⁰⁾.

Biological dosimetry is another method to estimate radiation exposure. It can be defined as the estimation of the received dose from past exposure to an agent through observation of biologic variables or measurements ⁽²¹⁾. For decades, biological dosimetry for radiation has been conducted by the use of the dicentric chromosome assay (DCA). DCA dose prediction is based on ionizing radiation-induced damage to DNA, which results in the formation of dicentric chromosomal aberrations ⁽²²⁻²³⁾. The chromosomal aberration, or dicentric assay, was one biodosimetry technique with low background levels and high specificity to ionizing radiation, making this assay a "gold standard" biodosimetry method and a diagnostic dose indicator recommended by the IAEA ⁽²⁴⁾. The test includes the analysis of a large number of metaphases that require highly specialized personnel to analyze. The micronucleus (MN) assay in peripheral blood lymphocytes, as an indirect assessment of chromosomal aberrations, was applied as an alternative, as it allows much faster detection of aberrations compared to dicentric assay ⁽²⁵⁻²⁹⁾. The cytokinesis-block technique (CBMN) uses cytochalasin-B as a cytokinesis inhibitor. This allows the evaluation of micronuclei frequency in dividing cells accumulated in the binucleated cells to overcome the uncertainties associated with *in vitro* cell-division kinetics that can affect MN expression ⁽³⁰⁾. In recent times, the micronucleus test has been employed more frequently to assess cytogenetic damage in

populations exposed to ionizing radiation in their occupations. The results of these studies are contradictory; they do not help to establish a relationship between the exposure dose and the increase of chromosomal damage.

In an earlier work, we studied residents living in high background radiation to determine the exposures to different professions of medical workers. The results tended to show increased numbers of γ -H2AX foci compared to a control group. These increased numbers demonstrated the process of repairing DNA DSBs damage^(31, 32). Increased frequencies of γ -H2AX foci were not considered to be a direct result of annual occupational doses of these medical workers⁽³²⁾. The purpose of this study was to analyze the correlation between the frequency of both γ -H2AX and micronuclei with respect to the annual occupational doses of medical workers. The results of γ -H2AX and micronuclei will be useful to know the potential for the use of genomic damage biomarkers in workers receiving occupational dose exposures in their daily working.

MATERIALS AND METHODS

Subjects

The research protocol for this study was approved by the National Institute for Health Research and Development No. LB. 02.01/2.KE.132 \2018. During this study, 69 blood samples were collected from 45 volunteer medical workers including diagnostic radiologists, radiographers, and cathlab nurses and 24 non-medical staff as controls across three hospitals in Indonesia. Personal dosimeters were also incorporated for evaluation of the external exposure of each volunteer. The amount of radiation exposure to each individual was measured using thermo-luminescent dosimeters (TLD) following three months of occupational exposure. The exposure was expressed in mSv as the mean of the annual effective dose for the period of exposure. Occasionally, personal dosimetry was implemented using an automatic TLD reader (Harsaw Model 6600 with LiF:Mg Ti cards;

Thermo Scientific, 81 Wyman Street, Waltham, MA 02454). The glow curve was constructed from sophisticated data processing with Harsaw CGCD (computerized glow curve deconvolution) software⁽³³⁾.

All volunteers were informed about the nature, aims, and intention of the study, and each signed an informed consent form and associated questionnaire before providing blood samples. Any individual suffering from an illness or taking any medication was excluded from the study.

Isolation of lymphocytes

Two milliliters (ml) of whole blood was collected by intravenous injection from each individual. The Histopaque separation method to isolate peripheral lymphocytes was used, as reported in previous papers by the authors^(31, 32). The isolated lymphocytes in the buffy coat of the separated blood were transferred to a new 15 ml centrifuge tube with 5 ml of PBS to wash the cells. They were then centrifuged for a further 5 min at 1000 rpm. The cells were washed three times and resuspended in PBS at a density of $(5-6) \times 10^4/\text{ml}$ and put on basic medium (RPMI). This method was adopted from previous publications without any modification^(20, 34), centrifuged for 15 min at 1000 rpm.

γ -H2AX assay

Lymphocytes were dropped and left for 15 min on hydrophobic slides, then fixed with 2% formaldehyde for 5 min, permeabilized for 5 min with 0.25% Triton X-100, and finally blocked with 2% BSA (diluted in PBS) for 15 min at room temperature. After removing the BSA, the primary antibody (consisting of anti γ -H2AX (mouse anti-Phospho-Ser139 γ -H2AX antibody, Thermo Fisher) and 53BP1 antibodies (Thermo Fisher; used for internal control staining)) were mixed together in a 1:500 dilution in 2% BSA/PBS. The mixture was dropped on the slide-bound lymphocytes and kept for 1 h in a dark, moist chamber at room temperature. The first primary antibodies were then removed and the slides were incubated with BSA 2% three times for 15 min each. Next, the secondary antibodies (consisting of goat anti-mouse IgG

Daylight 488 and anti-rabbit-Daylight 594 nm, both from Thermo Scientific) were diluted in BSA 2% and with DAPI (diluted 1:500). Slides were then incubated for 30 min at room temperature. Once this was completed, the slides were washed 2 or 3 times with PBS to remove excess antibody, and the slides were dried for 15 min using a fan. The mounting medium Vectashield with DAPI was used and mounted with a cover slip and left in the refrigerator for 24 hours prior to analysis. The observations were performed by an experienced investigator (IK) using a fluorescence microscope (Nikon) equipped with red, green, and blue fluorescence filters and using 100× magnification under immersion oil ⁽³²⁾.

Micronucleus assay

The micronucleus assay was performed in cytokinesis-blocked lymphocytes using cytochalasin-B (Cyt-B), following as the method described by Fenech ⁽²⁹⁾ with only a small modification. Blood cultures were incubated for 72 h. After 44 h of incubation, cytochalasin-B (Cyt-B Sigma) was added to the cultures at a concentration of 6 g/ml to block cytokinesis. The cells were isolated and collected by centrifugation, and a mild hypotonic solution containing 0.075 M KCl was then added for 3 min. After centrifugation and removal of the supernatant, the cells were fixed with a freshly prepared mixture of methanol/acetic acid (3:1). Centrifugation and resuspension were carried out three times and the cells were then dropped onto clean microscope slides for detection of micronuclei by conventional staining with 5% Giemsa. The evaluation was performed with a minimum of 1000 binucleated cells for each person scored for the presence of micronucleus

according to the criteria described by Fenech ⁽³⁰⁾.

Statistical analysis

After data were collected and analyzed, the Kolmogorov-Smirnov Test was applied to ensure the normality of distribution. The Mann Whitney Test was used to analyze data between γ -H2AX foci and micronuclei index between the workers and the nonmedical staff (controls). Rank Correlation and Fit with Regression Line were used to analyze the correlation between γ -H2AX foci and micronuclei index, and to compute the annual dose estimate of each medical worker. All data were analyzed with MedCalc Software 12.7.00.

RESULTS

The frequency of micronuclei in workers and controls ranged between 5-30 and 12-29 respectively. The range of mean γ -H2AX foci per cell in workers and controls was 0.00-0.24 and 0.00-0.12 respectively. There was no statistical difference between mean γ -H2AX foci/cell and the micronuclei frequency between workers and controls ($p > 0.05$). Related data such as age, sex, and the habits of the volunteers can be seen in table 1. Even though there was no statistical difference in the γ -H2AX and micronuclei index between control and exposed (figure 1a and 1b), no statistical correlation between the mean γ -H2AX and micronuclei index was found. It is interesting to note the small tendency toward a negative correlation between γ -H2AX and the emergence of micronuclei (figure 2a), and the tendency toward a positive correlation between γ -H2AX and annual occupational doses (figure 2b).

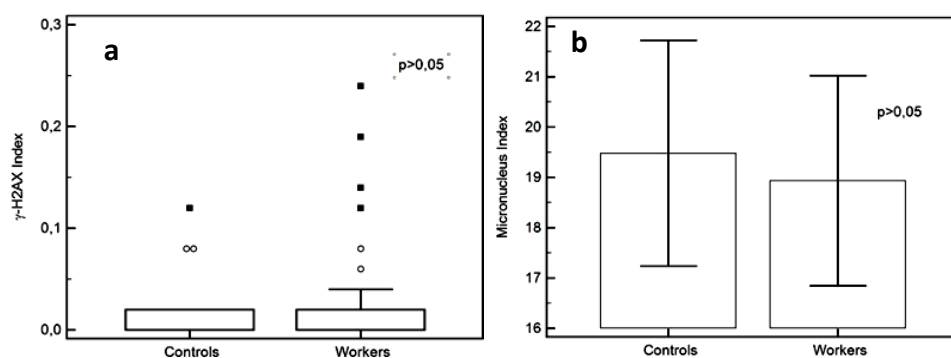


Figure 1. Mean γ -H2AX index in controls and workers (a) and micronucleus index in controls and workers (b).

Table 1. Distribution γ -H2AX, Micronucleus, and Occupational Dose of Workers and Controls.

No	Code/Name	Ages (Years)	Sexes	γ -H2AX	Micronucleus	Doses/Year (mili Sievert)	Smoking/No
1	BS1	32	W	0,04	20	1,15	No
2	BS2	50	M	0,24	21	0,71	No
3	BS3	53	M	0,14	15	0,33	No
4	BS4	26	M	0	15	0,47	No
5	BS6	47	W	0,04	24	1,04	No
6	BS7	43	M	0,12	17	0,51	No
7	BS8	45	M	0,19	20	0,45	No
8	BS9	46	W	0	20	0,38	No
9	BS12	35	W	0	15	0,32	No
10	BS15	40	W	0	25	0,7	No
11	BS16	44	M	0	21	0,2	No
12	BS17	57	M	0,02	29	0,15	No
13	BS18	54	M	0,02	17	0,29	No
14	BS19	50	M	0,02	13	0,35	No
15	BS22	30	M	0	5	0,78	No
16	BS26	27	W	0,08	10	0,47	No
17	BS27	42	W	0	26	0,34	No
18	BS32	56	M	0	30	0,26	No
19	PD-1	35	M	0	15	0,31	No
20	PD-2	43	M	0,02	8	0,45	No
21	PD-3	43	W	0	10	0,32	No
22	PD-4	35	M	0	11	0,31	No
23	PD-5	54	W	0	6	0,3	No
24	PD-6	34	W	0	13	0,33	No
25	PD-7	43	W	0	21	0,45	No
26	PD-8	37	W	0	13	0,33	No
27	PD-9	33	W	0	15	0,36	No
28	PD-10	33	W	0,06	15	0,3	No
29	PD-11	32	W	0,02	25	0,36	No
30	PD-12	44	W	0	25	0,36	No
31	PD-13	51	M	0	12	0,51	No
32	PD-14	38	M	0	29	0,33	No
33	PD-15	44	M	0	28	0,51	No
34	PD-16	27	M	0,02	14	0,39	No
35	PD-17	55	M	0	22	0,53	No
36	PD-18	49	M	0	18	0,41	No
37	PD-19	43	W	0	25	0,37	No
38	PD-20	49	W	0	20	0,44	No
39	PD-21	29	M	0,02	26	0,41	No
40	PD-22	56	W	0	27	0,38	No
41	BS11	58	W	0,08	24	Control	No
42	BS13	56	M	0,02	25	Control	No
44	BS14	47	M	0	25	Control	No
45	BS20	58	W	0,08	23	Control	No
45	BS21	56	M	0	27	Control	No
47	BS24	41	W	0	29	Control	No
48	BS25	35	W	0	19	Control	No
49	BS28	40	W	0	18	Control	No
50	BS29	42	W	0	18	Control	No
51	BS30	41	W	0	14	Control	No
52	BS31	30	W	0,12	19	Control	No
53	PD-25	57	W	0,02	23	Control	No
54	PD-26	54	W	0	29	Control	No
55	PD-27	50	W	0	21	Control	No
56	PD-28	30	M	0	9	Control	No
57	UL-1	28	M	0	12	Control	No
58	UL-2	27	W	0,02	16	Control	No
59	UL-4	42	M	0	15	Control	No
60	UL-7	56	M	0	15	Control	No
61	UL-9	53	W	0	14	Control	No
62	UL-11	26	W	0,02	16	Control	No
63	UL-12	25	W	0,02	20	Control	No
64	UL-20	28	M	0	17	Control	No
65	UL-23	32	M	0	25	Control	No
66	UL-24	35	W	0	14	Control	No

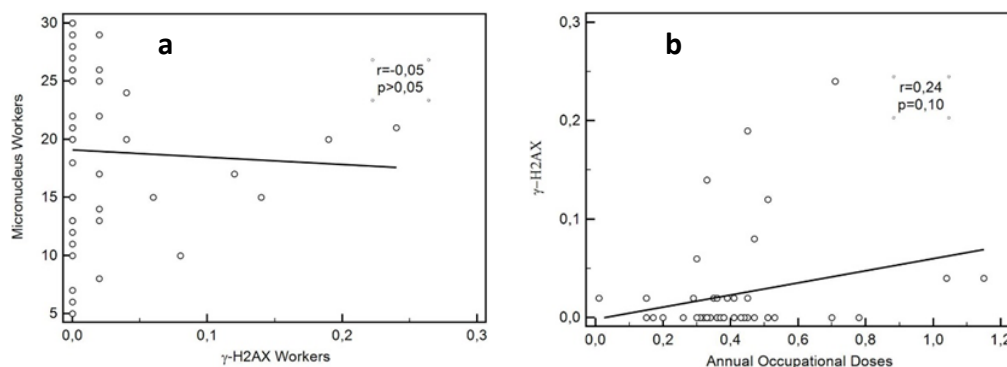


Figure 2. Correlation of γ -H2AX index and micronuclei index (a) and γ -H2AX index and annual occupational doses in workers (b).

DISCUSSION

Exposure of living organisms to genotoxic agents can induce several kinds of DNA damage, including DSBs. DSBs are considered to be lethal events as they result in the formation of exchange type chromosome aberrations and lead to clonogenic and mitotic cell death⁽³⁰⁾. However, in response to the formation of DSBs, some molecules of histone 2A (H2AX) occur in the chromatin part of the chromosome, flanking the break site, and they are rapidly phosphorylated. These in turn recruit several other molecules, facilitating DNA repair and chromatin remodeling. The site of DSB along with the protein molecules including the phosphorylated H2AX can be seen as foci which can be measured with flow cytometry and/or microscopy⁽¹⁸⁾. The assay was adopted to quantify DNA DSBs in cells exposed *in vitro* and *in vivo*^(34,35).

Micronuclei formation in cells cultured to division is another established biomarker of radiation exposure^(29,30,37,38,39), and the MultibioDose and RENEB networks have used this assay during triage simulation⁽⁴⁰⁾. The development of the cytokinesis-block technique has transformed the human-lymphocyte micronucleus assay into a reliable and precise method for assessing chromosome damage. Micronuclei are acentric chromosomal fragments, or whole chromosomes, lost during cell division because due to DNA damage. Formation is dependent on the capacity for DNA repair and the accumulation of DNA damage^(41, 42).

The current observations suggest that occupational exposure to ionizing radiation does not cause significant DNA DSB damage, as observed by γ -H2AX and micronucleus induction in peripheral blood leukocytes of medical workers compared to controls. However, the range within the data suggests that the ranges of both γ -H2AX and micronuclei in workers are larger than the ranges in controls. Dobrzyńska *et al.*⁽⁴¹⁾ reported that there were no statistical differences in DNA damage within females compared to males in both control and exposed persons. Pajic *et al.*⁽⁴²⁾ evaluated several cytogenetic parameters: chromosomal aberrations, premature centromere division, and micronuclei, and observed higher frequencies in the exposed (worker) group compared to controls. Moreover, it is not clear whether this could be related to cytogenetic damage observed by micronucleus appearance, as DNA DSB damage detected as γ -H2AX foci also were higher in workers when compared to controls. The lack of a significant difference between γ -H2AX foci and micronuclei frequency in workers and controls may be influenced by several confounding factors. Two published papers have reported that the expression of internal γ -H2AX foci is influenced by both the gender and age of volunteers, reported by Sharma *et al.*⁽⁴⁴⁾ and Siddiqui *et al.*⁽⁴⁵⁾. As with γ -H2AX foci, micronuclei expression is also influenced by some confounding factors such as gender, age, and smoking habit. Gender, age, and smoking habit did not influence γ -H2AX frequencies in both control and exposed groups, except for parameters of micronucleus, where

mean values were higher in females, smokers, and subjects older than 45 years of age ^(41,42).

Interestingly, the current data suggest there was a tendency toward a negative correlation between expression of γ -H2AX foci and micronuclei frequency. This may be associated with DNA DSB repair processes that prevent the formation of micronuclei related to any damage to cell division or chromosomal aberration⁽²⁴⁻²⁸⁾. Expression of γ -H2AX foci showed a positive correlation to annual occupational dose estimates in workers, caused by the level of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), playing an important role in the biological effects of ionizing radiation⁽⁴³⁾. This could mean that a higher dose than expected was received by the workers, which could have the potential to affect the level of DNA DSB damage and may still be repaired, as shown in these results, the negative association between expression of γ -H2AX foci and micronucleus frequency. No direct correlation between γ -H2AX foci and the occupational dose measured by an individual dosimeter was observed. The enrolled workers were only slightly exposed to IR. However, increased DNA repair activity was found in the IR-exposed group, and only subjects highly exposed to IR accumulated DNA damage in their circulating cells (figure 1). DNA damage has been considered as the most likely event to “kick-start” the multistep carcinogenic process ⁽⁴⁴⁾. Therefore, an effective DNA repair system is essential to prevent mutations and, ultimately, cancer. Various DNA repair pathways are known to prevent the persistence of such DNA lesions. In particular, 8-oxoguanine glycosylase 1 (OGG1) is one of the DNA repair enzymes associated with the susceptibility to tumor development ⁽⁴⁶⁾. Gourabi and Mozdarani showed that radioresistance to higher doses of radiation in pre-exposed lymphocytes might be due to initial DNA damage and an induced DNA repair mechanism ⁽⁴⁷⁾. These findings support the “radiation hormesis model,” which assumes that adaptive/protective mechanisms can be

stimulated by low-dose radiation and that they can prevent both spontaneous and toxicant-related cancers, as well as other adverse health effects ⁽⁴⁸⁾. Evidence of hormesis throughout defined low-dose ranges has been described ⁽⁴⁹⁾. Although hormesis induced by chemical/physical exposure occurs in humans, such hormesis cannot be promoted to the public as a protective mechanism due to unresolved issues of safety, including the impossibility of controlling a “tolerable exposure” ⁽⁴⁸⁾. Publications reporting a correlation between γ -H2AX foci annual occupational doses and micronuclei frequencies in the same subject are not common, and most of those published papers report only the relation of DNA DSB damage to radiation exposure ^(15-20, 30, 31).

In conclusion, γ -H2AX foci have the potential to be used as an effective biomarker to detect DNA DSB damage in occupationally exposed IR workers. More intensive investigation is needed by reducing some of the confounding factors such as age and gender to fully appreciate the results of this study.

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Conflicts of interest: Declared none.

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