Adaptive Response of Peripheral Blood Lymphocytes in Medical Radiation Workers using the Comet Assay: A Preliminary Study

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ABSTRACT
Background: Gamma irradiation can cause DNA damage in single and double-strand breaks (SSBs & DSBs), especially in peripheral blood lymphocytes. Radiotherapy medical radiation workers can be exposed to gamma radiation related to their daily work. The comet assay is a sensitive method for analyzing DNA damage, especially SSBs. This study explores DNA damage in medical radiation workers’ peripheral blood lymphocytes as an adaptive response using the comet assay.

Methods: Blood samples were obtained from four radiotherapy medical radiation workers as a case study (MRW) and two non-medical radiation workers as controls, and then irradiated with various doses of 0, 1, 1.5, and 2 Gy. Lymphocytes were isolated by histopaque and processed by comet assay on the slide under alkaline conditions. The imaging results were analyzed using the Casplab_1.2.3b2 software. The comet assay parameters observed were Tail Length (TL), % Tail DNA (T.DNA), Tail Moment (TM), and Olive Tail Moment (OTM). The one-way ANOVA method was used to analyze statistically between treatment groups.

Results: Based on the study results, an increase in TL, T.DNA, TM, and OTM values in all samples was directly proportional to the increase in radiation dose. However, there was no significant difference (P > 0.05) between the MRW group and the control group on each parameter of the comet assay.

Conclusions: From this study, it can be concluded that the level of DNA damage of lymphocyte cells as part of the adaptive response in the MRW and control groups was relatively similar after exposure at doses of 0, 1, 1.5, and 2 Gy.

INTRODUCTION
Ionizing radiation has become an essential part of medicine, both for diagnostic and therapeutic purposes [1,2]. Medical radiation workers, such as radiotherapy operators, risk exposure to ionizing radiation despite using radiation protection devices. In addition, biological damage to normal cells is a significant health problem for radiation workers [3,4]. Radiation exposure to patients and medical workers is a direct result of using radiation to improve individual health. The trend of radiation exposure in patients and workers is influenced by the development of radiation protection and the doses used in medical practice. It is imperative to estimate the absorbed dose of individuals exposed to ionizing radiation in the workplace to perform radioprotection procedures and limit hazards to human health [5].

DNA damage as a result of exposure to ionizing radiation can occur directly or indirectly. Direct action occurs because the secondary electron energy resulting from the ionization process interacts directly with DNA. Meanwhile, indirect action happens when there is the
first radiation interaction with water molecules in the cell, which will then hit important organic molecules [6]. DNA damage can occur through several mechanisms, including loss of one of the bases or sugars making up DNA, denaturation of DNA protein segments, the formation of 8-hydroxy-2-deoxy guanosine (8-OHdG), single-strand (SSBs), and double-strand (DSBs) DNA [7–9]. These defects will result in a high frequency of chromosome aberrations, increasing mutagenesis and carcinogenesis [10].

Peripheral blood lymphocytes have a high sensitivity to ionizing radiation, so they are often used as a parameter for DNA damage. One of the fast and sensitive visual methods to quantitatively detect DNA damage in cells is the comet assay [2,11]. The alkaline comet assay can detect DNA damage up to a minimum dose of 0.6 cGy [12]. The basic principle of the comet assay is migrating denatured DNA fragments out of the cell nucleus during the electrophoresis stage [13,14].

The comet assay parameters commonly used are the tail length, % tail DNA, tail moment, olive tail moment, and head DNA [15,16]. However, Martinez et al. [3] showed a significant increase in DNA fragmentation in radiation medical workers than in unexposed administrative staff. Radioadaptive response is a phenomenon in which the cells pre-exposed to the low dose of ionizing radiation or genotoxic chemicals exhibit enhanced cellular resistance to the effect of a subsequent higher challenging dose of ionizing radiation [17].

Previous research by Musthafa et al. [7], which observed DNA damage in lymphocytes as an adaptive response in medical workers using the gamma H2AX method, showed no significant differences between the medical radiation workers group and the control group. Meanwhile, the results of micronuclei studies, apoptosis, and comet assay conducted by Mohammadi et al. [18] stated that there might be a radioadaptive response in people living in high background areas in the Ramsar region, Iran.

In the present study, a comet assay analysis was performed to detect DNA damage of peripheral blood lymphocytes as a preliminary study of adaptive response in medical radiation workers.

**METHODS**

**Samples**

The present study is a preliminary study using peripheral blood lymphocyte cells as the object of research. The number of samples is very limited regarding the publication of Darlina et al. [5] with modifications. Blood samples were obtained from four medical radiation workers (MRW) as a study group and two administrative workers as a control group at Dharmais Cancer Hospital Jakarta. The study group was occupationally exposed to ionizing radiation for 5–15 years. The control group was selected from administrative areas that had never been occupationally exposed to ionizing radiation. Each research subject filled out a questionnaire and signed informed consent. The questionnaire includes personal data (age and health status) and occupational exposure to ionizing radiation at the study time (Table 1). The questionnaire also contains criteria for non-occupational exposure to potential mutagenic hazards, such as smoking, alcohol and drug consumption, viral diseases, recent vaccinations, and radio diagnostic tests.

**Irradiation**

Blood samples were obtained from six participants (MRW and controls), irradiated with gamma (Co-60) at doses of 1, 1.5, and 2 Gy, and a dose rate of 1 Gy/minute using the IRPASENA device at the Center for Isotope and Radiation Applications (PAIR-BATAN). The radiation dose determination was based on our previous study with modifications to the analyzed parameters [19].

**Isolation of Lymphocytes**

Isolation of Lymphocytes procedures for comet assay refers to the study of Panda et al. [20]. Ten ml of peripheral blood was drawn using venipuncture from each participant and inserted into a heparin tube (Becton Dickinson, N.J., USA). The blood sample was transferred into a centrifuge tube and added 3 mL Phosphate Buffer Saline (PBS), shaken gently, and then moved to a tube containing 3 mL Histopaque by slowly dropping it through the tube wall. Furthermore, the tubes were centrifuged at 1,500 rpm for 30 minutes. The formed lymphocyte ring was taken and then transferred to a tube containing 5 mL PBS for washing. Tubes were centrifuged at 1,000 rpm for 15 minutes (washing was repeated three times). The supernatant was discarded. The lymphocytes in 75 mL RPMI were added.

**Comet Assay**

The comet assay method was carried out in alkaline conditions, which refers to research by Singh [14] with modifications. A total of 10 µL of the isolated suspension was taken and mixed with 90 µL of 0.5% agarose (LMP). 75 µL (LMP and cells) was dropped on a normal agarose gel (NMP) pre-coated microscope slide, covered with a glass cover. The microscope slides were stored at 4°C for 10 minutes. Glass cover was removed, dropped with 75 µL of LMP Agarose 0.5%, and covered with a glass cover. The slides were stored at 4°C for 15 minutes. The glass cover was removed, and the slides were put into the lysing solution and stored at 4°C for 1 hour. The slides were dried, put into an unwinding buffer, and held at 4°C for 40 minutes. After cell lysis, the slides were placed in a horizontal position in the electrophoresis tank, and an unwinding buffer was added. Electrophoresis was adjusted to 25 Volt and 300 A and kept at 4°C for 20 minutes. It was washed with a neutral solution three times for 5 minutes. The slides were fixed with absolute ethanol solution (98–100%)
by immersion for 5 seconds. Finally, the slides were stored in a desiccator and allowed to stand for 24 hours before observation [14].

**Staining and Observation**

The slides were dropped with 75 µL of EtBr, covered with a cover glass, and let stand for 15 minutes in a dark room while being observed using a fluorescence microscope at a magnification of 100x. Fifty comets were observed for each sample.

**Data Analysis**

The software used for the analysis of the comet image is Casplab_1.2.3b2. The parameters analyzed were Tail Length (TL), % Tail DNA (T.DNA), Tail Moment (TM), and Olive Tail Moment (OTM) (Figure 1). The comet assay parameters used were TL, % T.DNA, TM, and OTM. TL is the comet’s tail length, which is calculated from the right side of the comet’s head to the end of the comet’s tail. % T.DNA is the percentage amount of DNA present in the comet’s tail. TM represents the center of the comet’s head to the center of the comet’s tail. At the same time, OTM shows the multiplication result of % T.DNA with TM [21].

Statistical data analysis used IBM SPSS Statistics 24.0 software with the One-Way ANOVA method. The investigation was continued using one-way ANOVA followed by an ad-hoc test if the data were normally distributed. However, if the data were not normally distributed, nonparametric analysis was carried out.

**RESULTS**

Blood samples were irradiated with gamma irradiation at doses 1, 1.5, and 2 Gy, and a dose rate of 1 Gy/minute to see the adaptive response of lymphocyte DNA. An image of the comet assay in the MRW and control groups for each dose can be seen in Figure 2. There are changes in morphology and cell shape after irradiated gamma challenging doses of 1, 1.5, and 2 Gy compared with no radiation (0 Gy). The fragment of damaged DNA will be released and then migrate to the positive charge pole during the electrophoresis and form a “Comet”. Changes in cell morphology are proportional to the increase in radiation dose. TL of the comet will be longer at higher radiation doses, followed by changes in the T.DNA. There was no significant difference in morphological changes between the MRW and control groups.

The percentages of T.DNA, TL, and TM are the most common parameters in comet imaging. T.DNA is a parameter that is highly recommended in analyzing comet images. It can be expressed on a nominal scale from 0 to 100%. Thus, a comparison of research results using the comet assay can be carried out.

Table 1 presents a detailed description of the research subject. Subjects consisted of four MRW and two controls with an age range of 23 to 54 years. In addition, annual radiation dose data are also presented. Meanwhile, Table 2 describes, in general, the increase in values for all comet test parameters, which are directly proportional to the increase in radiation dose. The greater the radiation dose, the higher the rate of DNA damage. In almost all research parameters, the MRW group had a higher average than the control group, except for TM (13.32±8.80 and 14.91±3.37; 15.89±13.59 and 22.55±5.63) and OTM (13.59±3.90 and 18.53±4.94; 14.75±1.67 and 23.63±3.19) with radiation doses of 1.5 and 2 Gy.
In Figure 3, although the values for most of the comet assay parameters in the MRW group were higher than the control group, there was no statistically significant difference (P > 0.05). The difference in the mean value in each sample may occur due to differences in the biological response of everyone in receiving exposure to ionizing radiation that causes DNA damage.

Table 1. The description of the subject

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (Year)</th>
<th>Area of Work</th>
<th>Tare Dose 1 Year (mSv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>48</td>
<td>Medical radiation worker</td>
<td>0.26</td>
</tr>
<tr>
<td>B</td>
<td>48</td>
<td>Medical radiation worker</td>
<td>0.06</td>
</tr>
<tr>
<td>C</td>
<td>38</td>
<td>Medical radiation worker</td>
<td>0.15</td>
</tr>
<tr>
<td>D</td>
<td>32</td>
<td>Medical radiation worker</td>
<td>0.75</td>
</tr>
<tr>
<td>E</td>
<td>54</td>
<td>Administrative</td>
<td>0.22</td>
</tr>
<tr>
<td>F</td>
<td>23</td>
<td>Administrative</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 2. The level of DNA damage in the MRW and control group after gamma irradiation at doses of 0, 1, 1.5, and 2 Gy with comet assay parameters.

<table>
<thead>
<tr>
<th>Radiation Dose (Gy)</th>
<th>0</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical Radiation Workers (MRW) n=4</td>
<td>Tail Length (µL)</td>
<td>44.09±8.31</td>
<td>50.38±14.63</td>
<td>50.82±29.75</td>
</tr>
<tr>
<td></td>
<td>Tail DNA (%)</td>
<td>11.76±3.74</td>
<td>13.81±2.76</td>
<td>14.51±5.94</td>
</tr>
<tr>
<td></td>
<td>Tail Moment (µL)</td>
<td>7.16±2.76</td>
<td>13.11±6.96</td>
<td>13.32±8.80</td>
</tr>
<tr>
<td></td>
<td>Olive Tail Moment (µL)</td>
<td>6.93±2.07</td>
<td>11.09±5.64</td>
<td>13.59±3.90</td>
</tr>
<tr>
<td>Controls n=2</td>
<td>Tail Length (µL)</td>
<td>33.22±13.58</td>
<td>39.45±3.61</td>
<td>43.62±7.44</td>
</tr>
<tr>
<td></td>
<td>Tail DNA (%)</td>
<td>10.37±5.98</td>
<td>11.25±1.87</td>
<td>12.58±3.40</td>
</tr>
<tr>
<td></td>
<td>Tail Moment (µL)</td>
<td>5.62±5.81</td>
<td>8.18±5.04</td>
<td>14.91±3.37</td>
</tr>
<tr>
<td></td>
<td>Olive Tail Moment (µL)</td>
<td>4.36±3.71</td>
<td>10.34±8.32</td>
<td>18.53±4.94</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of comet assay between the MRW and the control group on various parameters. (A) TL, (B) %T.DNA, (C) TM, and (D) OTM. ns: not significant (P<0.05)
DISCUSSION

The research uses a sample of peripheral blood lymphocytes to measure the level of DNA damage. Peripheral blood cell lymphocytes are known to be very sensitive to radiation. The radiosensitivity of lymphocyte cells dramatically affects DNA damage caused by radiation with the comet assay. Generally, the lymphocyte cells used in the comet assay are peripheral blood mononuclear cells (PBMC). The research conducted by Rady et al. [21] stated that TM and OTM also provide a positive DNA damage [4]. In their research, Rady et al. [21] compared the effects of electrophoresis at 4°C and 20°C. Their results showed that DNA damage due to ionizing radiation will be more massive as the radiation dose increases. It can be seen from changes in the morphology of lymphocyte cells through observation with a fluorescence microscope.

Based on Kumaravel et al. [24] research, gamma-irradiated lymphocytes with a dose range of 0–8 Gy showed positively correlated comet images, especially TL and OTM parameters. TL is a parameter commonly used for the detection of radiation-induced DNA damage by comet assay. In alkaline conditions with a pH > 13, it will break the DNA strand. This process results in the release of damaged DNA fragments and weakens the DNA loop bonds near the center of cell division. The DNA fragments and DNA loops travel towards the anode, which produces the comet tail [15,25].

In this study, the electrophoresis process was carried out in a refrigerator (4°C) to avoid increasing the gel temperature to 15°C. McKelvey-Martin et al. [26], using irradiated and non-irradiated lymphocytes, said that there were differences in comet morphology when the electrophoresis process was carried out at different temperatures, namely 5°C and 10°C. Meanwhile, DNA migration was significantly increased when the electrophoresis process was carried out at a high temperature of more than 20°C. These results are consistent with Sirota et al. [27] research, which compared the effects of electrophoresis at 4°C and 20°C. % T.DNA is a unit derived from TL, determined by the amount of DNA that migrates out of the cell nucleus. Thus, it can provide information about the relative proportions of DNA in different regions of the comet’s tail. In addition, % T.DNA is directly proportional to DNA damage [4]. In their research, Rady et al. [21] stated that TM and OTM also provide a positive correlation with the dosage of the genotoxic agents used, both chemicals and radiation.

Almost all the mean values of each parameter indicate that the MRW group is higher than the control group. It suggests that chronic low-dose exposure received by the MRW group failed in forming a radioadaptive response in lymphocyte cells. Comet measurements may reflect both individual repairability and damage level. Because the measured damage level is a result of equilibrium between damage infliction and repair, a low damage level as assessed experimentally in an individual may result from an actual low number of lesions or high efficiency of repair [5].

However, DNA damage in the MRW group and control was relatively similar. Several studies have shown that adaptive responses were formed after the induction of chronic ionizing radiation exposure in nuclear, labor, or environmental accidents [11,28].

CONCLUSIONS

Competing of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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Ethics Approval
This research had passed the ethical review from The Institutional Review Board at the Faculty of Medicine, Universitas Indonesia (Ethical approval number: 910/ UN2.F1/ETIK /2017)

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