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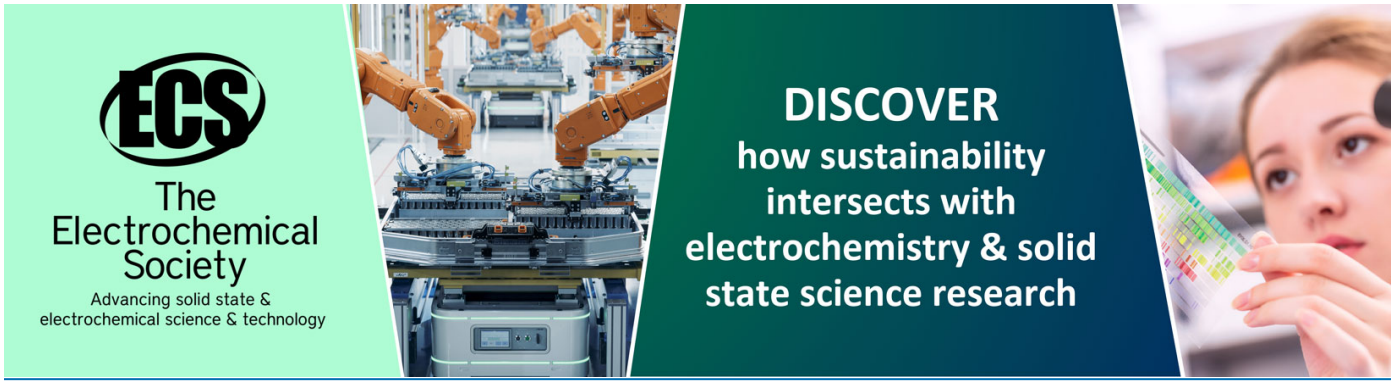

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The assessment of mitotic and nuclear division indexes as biomarkers for estimating the risk on the health of residents exposed to the high natural radiation of Mamuju, West Sulawesi

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Abstract. Cell proliferation is a potential biomarker and closely associated with the assessment of general cytotoxicity of chemical and physical agents under study. However, the utilization of these biomarkers in response to environmental stimuli such as natural radiation has not been adequately explored. This research aimed to assess the mitotic index (MI) and nuclear division index (NDI) in lymphocytes as biomarkers for predicting the risks on the health of residents living in high natural radiation area (HNRA) in Salletto and Ahu villages of Mamuju as a studied group. As a control group, people living in another region of Topoyo village were also studied. The observation of these both parameters was done according to the standard protocol as described by the International Atomic Energy Agency (IAEA). The result showed that the percentage of MI of lymphocytes obtained from the studied area was lower compared to those of the control area (6.48 vs. 9.41) whereas the percentage of NDI of lymphocytes obtained from the studied area was higher compared to those of control area (1.59 vs. 1.32). This finding is similar to previous studies in an adjacent area. The NDI obtained from manual counting was much lower than that obtained from the automatic machine for counting (1.59 vs. 22.46), of which it is due mainly to a factor in criteria for counting the cells. MI for the female is lower than that of male and there is a trend of decreasing mitotic index with increasing age in the same group. It is concluded that natural radiation exposure did not affect the proliferation of cells of local people which suggests a low risk of radiation-exposed related to inflammation.

1. Introduction

Natural radioactivity and cosmic rays that are the main sources of radiation exposures are important in the development and evolution of life. Currently the annual effective dose from natural for the world's population is about 2.4 mSv [1]. People who live in high-altitude areas such as in Yangjiang, China; Kerala, India; Guarapari, Brazil; and Ramsar, Iran [2], [3], [4], [5] are exposed to higher levels of radiation mainly due to inhalation of ^{222}Rn and its radioactive progeny. Another cause of high local radiation levels is deposited with high uranium and thorium concentrations [6], [7]. Some areas of Mamuju, a city located in Sulawesi of north-eastern Indonesia, have also been among the highest background radiation level in this country [8], [9], [10].

Little attention has been paid to the health of the local public member being at risk due to living in the natural radiation area. It is well known that radiation exposure may cause a delay in the cell cycle, thus it will decrease the cell proliferation rate, and apoptosis due to induced DNA double-strand breaks

[11]. The experiment on embryonic stem cells showed that cell populations were dropped within 24–48 hours post X-ray exposure [12]. Circulating lymphocytes are commonly used as surrogate cells to measure the cellular function/events of somatic cells in the assessment of the risk that is depended on cell cycle phases. Blood lymphocytes are typically resting in G_0 of the cell cycle and can be stimulated by phytohemagglutinin as a cell mitogen to proliferate *in vitro* [13].

Various potential prognostic biomarkers have been evaluated including mitotic index (MI) and nuclear division index (NDI) assay. These assays can reveal the role of the gene in cell cycle progression by quantifying the proportion of mitotic cells at a certain time point. MI represents the proportion of metaphases among cultured lymphocytes and also can be used to evaluate the cell proliferation rate. This cell motility is essential during development, wound healing and immune responses, and plays a prominent role during pathological conditions [14]. NDI is also a marker of cell proliferation in cultures which is mentioned as a measure of general cytotoxicity [15] and how this cell activity has been affected by radiation exposure [16]. The cells with greater chromosomal damage will either die before cell division or may be less likely to enter this phase of cell growth [17]. NDI abnormally increases or decreases depending on the proliferative capability of the cell [18].

The risk of cancer formation associated with increased mitotic and nuclear division indexes in humans is under intensive research, and it is of significant importance to evaluate related biomedical changes possibly incurred in people exposed to natural radiation area [19]. Thus biomarkers of cell proliferation are potentially valuable tools for cancer risk assessment. There is a very limited study on the assessment of MI and NDI in the lymphocyte of people living in the natural radiation area. Previous study showed that the MI and NDI in Botteng and Takandeang villages, an area with highest natural radioactivity in Indonesia, was lower compared to control (8.98 vs 9.09 for MI and 1.35 vs 1.40 for NDI), but statistical analysis revealed that the difference was not significant between both groups ($p>000.5$) [20,21]. To obtain a broader insight into the MI that may be affected by the dose rate in the local area, we were analyzed the adjacent area with a radiation dose rate of slightly higher. In this study, the MI and NDI alteration in lymphocytes cells due to radiation dose received by inhabitants of HBRAs of Mamuju was assessed.

2. Methodology

2.1 Ethics

The study procedures were explained to all respondents, who provided informed consent. The protocol of research was reviewed and approved by the Ethics Committee Review Board at the National Health Research Institute, The Indonesian Ministry of Health in Jakarta with the number of LB.0201/2/KE.063/2018.

2.2 Subjects

Forty-three samples of blood lymphocyte from residents of Salletto and Ahu Villages of Mamuju, with age between 25 and 65 years old (average±SD of 46.09±11.31), both with high background radiation as exposed group, and thirty-one blood sample from village of Topoyo (age between 18-68 years old, average±SD of 36.19±14.54) with normal background radiation as control group were used in the study. All participants (28 males and 15 females in the study area; and 17 males and 14 females in the control area) were informed about the aim and intention of the study and signed a consent form before providing their blood samples. Pregnant women and any persons suffering from an illness or taking medication were excluded from the study. The people who were living for at least 10 years in these areas considered as the study population.

2.3 Sample Preparation

Two ml of peripheral blood were drawn via venipuncture from each volunteer and placed into heparin containing a test tube (Becton Dickinson, N.J., USA) under sterile conditions for cell proliferation (culture).

2.4 Cell culture and harvesting of lymphocytes for micronuclei (MN)

The analysis was done with a standard procedure as described by IAEA with slight modifications [22]. Two milliliters of the whole blood samples were added into a medium that consisted of 8.0 mL of RPMI-1640 completed with 10% heat-inactivated fetal calf serum (FCS) and 1% streptomycin/penicillin (Gibco). Just before culture, into this solution, 3.0% of phytohemagglutinin (PHA-Gibco BRL, Grand Island, NY) was added with the aim to stimulate cell division. The culture was done by placing the solution in the incubator at 37^{o}C with 5% CO_2 for 72 h. In the 44 hours since the start cultured, 15 µL cytochalasin B (3 mg/ml) solution (Sigma) was added into a culture tube and harvested at 72 hours after the beginning of culture. For harvesting the cells, samples were transferred into a 15 mL centrifuge tube and centrifuged at 1500 rpm for 10 minutes. After the upper layer (supernatant) was removed, 8 mL of cold hypotonic solution (0.075 M KCl) was added, left at room temperature for 3 minutes, and then 3-4 drops of formaldehyde solution and cold fixative solution (methanol: glacial acetic acid = 3:1) were added. After being mixed until homogenous the solution was placed in the refrigerator (4^{o}C) for 10 minutes, and then centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and add a 6 ml cold fixative solution, centrifuged at 1000 rpm for 10 minutes. After repeated fixation (2-3 times) then the binucleated cells which may contain MN were obtained.

2.5 Counting NDI

Cells with one, two, three, and four nuclei were scored for calculating the nuclear division index (NDI) in order to evaluate the proliferative status of each volunteer's lymphocytes according to the formulation provided by Eastmond and Tucker [23] and Ipek *et al*. [24]. NDI=(M1+(2×M2)+(3×M3)+(4×M4))/N, where M1, M2, M3, and M4 represent the number of cells with 1 to 4 nuclei, and N represent the total number of scored cells (Figure 1).

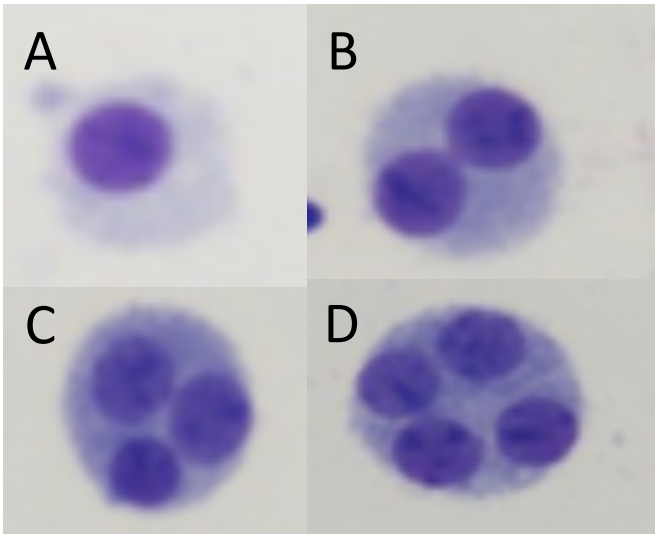

Figure 1. A microscopic view of Giemsa stained cells with one nucleus (mononucleate, A), two nuclei (binucleate, B), three nuclei (trinucleate, C), and four nuclei (tetranucleate, D) within cytoplasm as the base for counting the NDI.

2.6 Counting manual and automatic MI

The slides were analyzed based on the protocol provided in the IAEA publication (IAEA, 2011). The number of metaphase cells per totally 1000 metaphase and blast cells was counted under 400x magnification using a light microscope (Figure 2). The number of metaphase cells then was converted

to a percentage in order to calculate the MI. Automatic MI calculation for the whole slide was conducted using metaphases finder module from Metafer 3.11.2 imaging system (MetaSystems, Altlussheim, Germany) with a Zeiss Axioplan 2 Imaging epifluorescence microscope connected to a Cool Cube. The mitotic index of the cell was determined with programmed or automated metaphase finder according to the Manual of the machine.

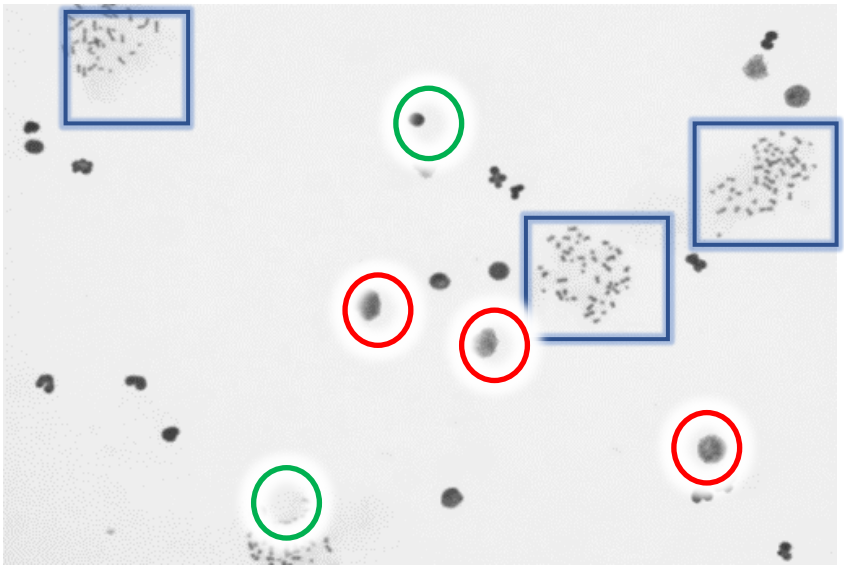

Figure 2. A microscopic view of Giemsa stained slide that containing metaphase chromosome spread (blue boxes), blasts cells (red circles) and nuclei which are not counted (green circles) as the base for the observation of mitotic index (magnification of 400X).

3. Results and Discussion

The result showed that the percentage of MI (%MI) of lymphocytes obtained from studied area was 6.48, which is lower compared to those of control area (9.40) whereas the percentage of NDI (%NDI) of lymphocytes obtained from studied area was 1.59 and it is higher compared to those of control area (1.32). So it is reported a reduced mitogenic stimulation of peripheral blood lymphocytes in exposed cases compared to non exposed controls. The increase of NDI may indicate the cumulative effect of low-level chronic exposure to radiological and chemical materials. The mean NDI found here is within the recommended value (1.2 - 2.2) [25]. All samples were also within this range and it is assumed that cell division was comparable in the cultures.

Various potential prognostic markers have been evaluated, including MI. The arrest of cells in the G_2 phase in response to radiation damage leads to suppression of the mitotic index to protect cell viability. Malignancy is characterized by accelerated cell division or high mitotic index. Study results showed that the %MI and %NDI in the studied area (Salletto and Ahu Villages) was lower compared to the control group. Statistical analysis revealed that the difference was insignificant ($P>0.005$).

This %MI finding is similar to previous studies that have been conducted in the adjacent area (Botteng Village of Mamuju) of which it was known that the mean MI observed manually was lower compared to the control group (4.96±2.25 vs. 5.93±2.14) [20]. In contrast, the mean automatic MI (20.37±10.49 vs. 18.87±7.49) and NDI (1.555±0.174 vs.1.523±0.112) in Takandeang Sub-Village inhabitants was higher compared to the control group [21]. Statistical analysis revealed that the difference of mean manual MI, automatic MI and NDI in Takandeang Sub-Village inhabitants was not significantly different compared to the control group ($p>0.05$).

Table 1. The mean MI value in lymphocytes of residents from Salletto and Ahu villages of Mamuju, West Sulawesi as study group and that of Topoyo village as a control group.

Group	Number of samples	Total counted cells (metaphase + blast)	Total number of metaphase cells	Mean MI ± SD (%)
Salletto+Ahu	43	45,999	2,999	6.48 ± 1.93*
Control	31	34,236	3,236	9.41 ± 2.04
All samples	74	80,235	6,235	

* Students t-test: P >0.05 (not different from control group)

For %NDI itself, the index obtained from manual counting was much lower than that obtained from the automatic machine for counting (1.59 vs. 24.08), of which it is due mainly to a factor in criteria for counting the cells as determined in the machine used. MI for the female is lower than that of male and there is a trend of decreasing mitotic index with increasing age in the same group. This study was small (74 for both high and normal areas) in the number of subjects studied and other hosts or environmental factors were not considered in the analysis such as tobacco and drinking habits.

Table 2. The mean NDI value in lymphocytes of residents of Salletto and Ahu villages of Mamuju, West Sulawesi as study group and that of Topoyo village as the control group.

Group	n*	Total M1	Total M2	Total M3	Total M4	Total N	Mean NDI ± SD
Salletto+Ahu	43	24,031	15,077	2,347	2,215	43,670	1.59 ± 0.15*
Control	31	33,125	8,169	355	373	31,000	1.32 ± 0.15
All samples	73	57,156	23,246	2,702	2,588	74,670	

* Students t-test: P >0.05 (not different from control group)

In this research the proliferation index, which characterizes the rate of cell division, was higher in the exposed group (1.59 vs 1.31). This increase may be caused by a compensatory or adaptive mechanism to promote a more rapid renewal of the proliferative pool under the genotoxic effects of natural radiation. The mean percent of mitotic cells in blood lymphocytes was significantly higher in cases (3.59%) than in controls (3.26%, $P < 0.01$, Table 2). There is also a tendency of both MI and NDI to decrease with the age of residents living in the study area with the relationship as $y = -0.0192x+7.3665$ and $y = -0.0025x+1.7162$, respectively (Figure 3). For MI the tendency of reduction is 0.0192/year of age whereas for NDI it is lower than IM (0.0025/year). This fact is due to the general dysfunction of the mitotic machinery or apparatus of aged cells, indicating cell fitness. Transcriptome analyses of a panel of lymphocyte cultures from young and old individuals revealed changes in the expression of genes that controlling the mitotic machinery [26]. This is closely related to proliferation defects, gene signature of the environmental stress response, multiple forms of genomic instability, and proteotoxicity, of which these are hallmarks of cellular aging.

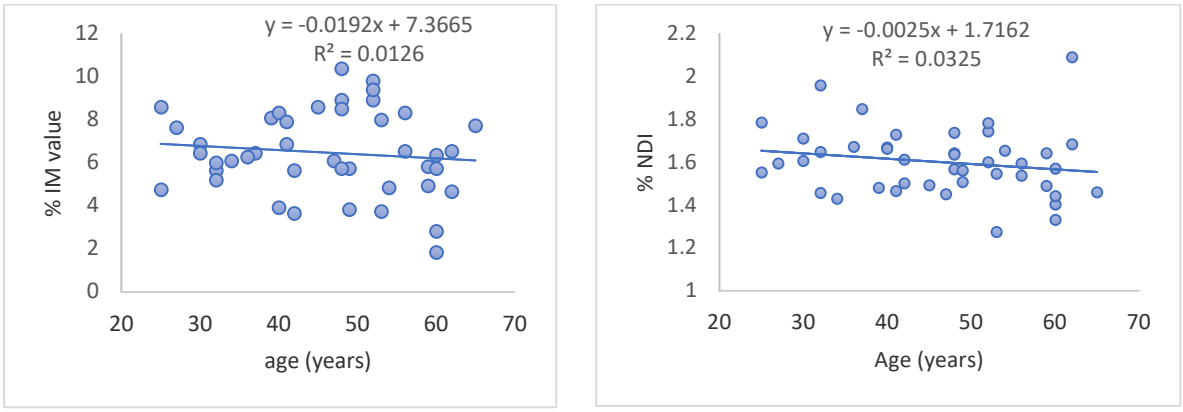

Figure 3. The correlation of MI (left) and NDI (right) with the age of residents living in the study area. The thick line is the result of a linear regression analysis of the data.

The natural radiation is considered as the major sources of ionizing radiation exposure in human life that may disrupt the tissue homeostasis through the depletion and accumulation of genetic abnormalities in cells, mainly for radiosensitive cells, followed by cell division disruption, which may result in the loss of tissue function [27]. The DNA of people living in areas with high radon concentrations such as in Mamuju may be affected by such potential genotoxicity agents. Cytogenetic methods are valuable and valid tools to assess this radiation-induced loss of tissue function and prognosis of radiation casualties [10, 21,28]. In the study presented here, the radiation response of human peripheral blood lymphocytes with respect to cell differentiation or proliferation has been investigated. This study also useful to assess or predict the risks of inhabitants in such area and if possible followed by giving recommendations based on lessons learned from such studies to enhance the use of studies data in the area under study. Therefore, assessing the effects or risks of radon and other natural radionuclides exposed people in areas such as in Mamuju is of particular interest to support the existence of a natural radiation laboratory for an epidemiological study.

Moreover, estimating the long-term effect of continuously and chronically small doses of ionizing radiation is a complicated issue that affects not only radiobiology but also social and economic issues. It has been reported that >60% of the ionizing radiation received by people annually living in this globe can be caused by natural sources of radiation, and more than 50% of this radiation comes from radon and its disintegration products. In addition to using methods of dosimetry such as chromosome aberrations to evaluate the risk of harmful radiation effects, the severity of such a risk should also be evaluated by a biological marker such as cell proliferation rate (MI and NDI). Biological test systems can be used to evaluate the risks of radiation and provide an assessment of the quality of the environment and its suitability for human living [29].

Considering the effective dose presented by Sohrabi (2013) [4] in comprehensive review articles and proceeding papers of the chain of conferences, it is noted that the annual effective doses from external and internal exposures in Brazil are less than about 12 mSv/y. Whereas the average annual effective dose in China is reported to be 6.4 mSv with an external dose of 1-3 mSv (average 2.1 mSv) and the internal dose of 4.3 mSv; about three times higher than that of control areas. It also reported that the annual effective dose in India is 4.5 mSv/y from gamma-rays with about 2.4 mSv/y effective dose from internal exposures [4, 30]. However, in certain locations on the coast of India, the radiation levels may be as high as 70 mGy/y. A higher dose was reported in Iran where the mean effective dose is 6.00 mSv/y in high background natural radiation areas of this country with the range of 3.0 to 202 mSv/y [2]. From these, it is important and urgently how to protect the affected inhabitants of such areas with improved methodologies that may provide the needed information.

4. Conclusion

In conclusion, it is known that based on NDI and IM indexes evaluation the natural radiation exposure did not affect significantly the proliferation of cells of local people under study which suggests a low risk of natural radiation-exposed related to the inflammation.

Acknowledgments

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