ORIGINAL ARTICLE

Micronucleus Assay-based Evaluation of Radiosensitivity of Lymphocytes among Inhabitants Living in High Background Radiation Area of Mamuju, West Sulawesi, Indonesia

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ABSTRACT

Naturally occurring radiation can be found all around us and account for most of the radiation received by human beings each year. Indonesia has a region with high-dose natural radiation located in the suburb of West Sulawesi province with a dose rate up to 2800 nSv/h; however, its impact was not fully understood. The aim of this study was to evaluate the radiosensitivity of 12 peripheral blood lymphocytes of inhabitant from high background radiation area (HBRA) and 10 from normal background radiation area (NBRA) based on cytokinesis-block micronucleus (CBMN) assay after challenged with 1.5 Gy of gamma ray. The analysis of CBMN was done according to standard procedure as per IAEA guidelines, and frequency of binucleate (mitotic) cells with micronuclei (MN) was scored in around 2000 binucleate lymphocytes cells per culture in microscopic analysis. Mean MN frequency for HBRA was lower than that of NBRA (0.121 vs. 0.189) after irradiation, indicating an adaptive response in HBRA group that resulted in less radiosensitivity; however, there was no statistically significant different ($P > 0.05$) between these two groups. The MN number was higher in women compared to men for both HBRA (0.15 vs. 0.09) and NBRA (0.216 vs. 0.147) groups. Besides, there was no statistically significant difference ($P > 0.05$) in Nuclear Division Index (NDI), as measured in 500 metaphase cells with published formula, between HBRA and NBRA samples (1.24 vs. 1.21). The lower MN frequency prompts us to conclude that there is an adaptive response in the lymphocytes of inhabitants as an indicator of lower radiosensitivity to the high natural radiation exposure. Further studies using large number of samples are required to obtain more comprehensive conclusion along with the assessment of other types of radiosensitivity-related biomarkers.

Key words: Adaptive response, high background radiation area, Mamuju, Micronuclei, normal background radiation area, Nuclear Division Index

Introduction

People are constantly exposed to low levels of ionizing radiation naturally present in the environment. This is the major source of human exposure to ionizing radiation which harms the body as it can ionize cell components throughout its path in the cell. The elevated levels of natural radiation such as from radon and the products of its disintegration were found to cause significant cytogenetic alterations.[1] Studies on the health of populations living in high background radiation area (HBRA) are a potential source of information on the effects of chronic low-dose-rate exposures.

Effective biomonitoring for the detection of radiation-induced genotoxicity of radioactive contamination in natural environment involves testing of blood lymphocyte of local residence for cytogenetic changes.[2] Blood, which can be easily accessed, represents a convenient compartment to assess radiation bioindicators. A variety of mutagenicity test procedures has been developed which enables the detection of DNA

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damage caused by ionizing radiation. These assays are useful in determining the adverse effects caused by radionuclides in the environment. A method which has high sensitivity to be used for biomonitoring in elevated natural radiation area is cytokinesis-block micronucleus (CBMN) that frequently used for evaluating genotoxic effects and chromosome instability in human peripheral blood lymphocytes.[3] It is one of the most reliable biomarker and established assays for biological dosimetry.[4]

MNs are masses of DNA that resemble small nuclei located in the cytoplasm of cells, rather than being within the nuclear membrane.[5] The MNs are simple structure independently and easy to identify. MN is formed during cell division that has been subject to exposure to many mutagens, including radiation, resulting in chromosome breaks or spindle dysfunction and a binucleated appearance in cells that have undergone cellular division. MN can be detected using light microscopy, with the number of cells containing MN within a cell population being related to the extent of cytogenetic damage.[6,7] The MN assay has been used in a wide range of studies, both in the laboratory and the field to assess the cytogenetic impact of exposure of different organisms to pollutants[8] and data can be generated in a short period.[9] Beside that information on the cell death by apoptotic and necrosis, and other cytogenetic damage biomarkers such as nucleoplasmic bridges and nuclear buds can be simultaneously obtained from the same slides.

The annual effective radiation dose from natural and man-made sources for the world's population is about 3 mSv, where nearly 80% of this dose comes from natural background radiation, although levels of natural radiation can vary greatly, even within countries as well.[10,11] The primary radioactive elements in the earth's crust that lead to human exposure are potassium, uranium, thorium, and their radioactive decay products (e.g., radium, radon, etc.). Mamuju has been reported for its high radiation dose rate due to these element contents in the soil of that area. On gamma dose rate map of Indonesia, this suburb area of West Sulawesi shows the highest average dose rate compared to other region in Sulawesi Island and even Indonesia, which can be at 2800 nSv/h.[12] Although extensive knowledge of radiation risks has been acquired in previous studies, the health effects of chronic low-level radiation exposure in that area are still poorly understood.

Preliminary studies by Nurhayati *et al*.[13] and Syaifudin *et al*.[14] on the cytogenetic effects revealed that MN number in lymphocyte of HBRA was higher compared to that of normal background radiation area (NBRA) with no significant difference was found between groups. The present study evaluated the radiosensitivity of lymphocytes of local residents exposed chronically to low-dose radiation exposure in HBRAs of Mamuju, which has an annual effective dose (H_E) of 6.15 ± 0.81 mSv[15], after being irradiated with a challenge dose.

Materials and Methods

Blood sampling and ethical approval

Twelve healthy adult participants (6 males and 6 females) from Botteng Village as HBRA at Mamuju District of West Sulawesi Province and 10 (4 males and 6 females) from normal background radiation area (NBRA) of Keang Village of the same district as NBRA were included in this study. Peripheral blood samples were collected by venipuncture using heparinized vacutainer tubes (BD Vacutainer systems). The study was approved by the Ethics Committee of the National Institute of Health Research and Development, Indonesian Ministry of Health, with the number of LB.02.01/5.2.KE.051/2015, January 29, 2015. Informed consent was obtained from all donors. A detailed questionnaire was provided to obtain information on age and occupation and other biodata. All procedures performed in this study were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Irradiation of the blood as challenge dose

The blood samples were irradiated at dose of 1.5 Gy with gamma rays from the ^{60}Co source at a dose rate of 0.38 Gy/min. Irradiation process was performed in the Secondary Standard Dosimetry Laboratory at Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia (BATAN) located in Jakarta. The sample was placed inside the acrylic box with dimension of 30 cm × 30 cm × 30 cm containing water with maintained temperature of 37°C during irradiation.

Micronucleus assay and Nuclear Division Index

Peripheral blood samples were cultured according to the MN assay protocol in IAEA publication.[16] Two cultures were setup in 15 mL centrifuge tube for each blood sample. Whole blood samples were cultured for 72 h in the incubator at 37°C containing 5% CO_2. The culture medium consisted of 4.5 mL of Rosewell Park Memorial Institute 1640 supplemented with 20% heat-inactivated fetal bovine serum, 1% streptomycin-penicillin, and 0.1 mL of phytohemagglutinin (PHA). At 44 h, post-PHA stimulation, cytochalasin B (an inhibitor of the spindle assembly) at a final concentration of 6 μg/ml was added to all the culture tubes. The cells were then centrifuged for 10 min at 1000 rpm and re-suspended in 7 ml of 0.075 M cold (4°C) KCl. The cells then were centrifuged again for 8 min at 1000 rpm and re-suspended in freshly made fixative consisting of methanol: acetic acid (10:1) diluted 1:1 with Ringer's solution. The cells then were washed with two to three further changes of freshly prepared fixative until the cell suspension is clear. The cell suspension then was stored in-20°C at least for one night until the slide preparation was conducted. Fixed cells were dropped onto clean, wet slides, dried, and stained with 4% Giemsa solution (pH 6.8). The scoring criteria were based on the methods described by IAEA[16], Fenech,[9] and Vral *et al*.[17] and have been implemented in our previous studies.[18-20] Two thousand binucleated lymphocytes were scored at the magnification of 1000 times under microscope observation and immersion oil.

For Nuclear Division Index (NDI), each slide in each sample, the proportion of mononucleated, binucleated, trinucleated, and tetranucleated cells per 500 cells scored was assessed. NDI was calculated based on the following formula where M1, M2,

M3, and M4 indicate the number of cells with one, two, three, and four nuclei and N is the total number of cells analyzed.[9,16]

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

Statistical analysis

Using SPSS 22.0 (IBM United States Software Announcement 213-309) statistical software, unpaired *t*-test was used to compare the MN number between HBRA and control groups before and after irradiation if the data have a normal distribution. The Kolmogorov–Smirnov test was applied to determine the distribution of data.

Results

This small-scale and simple evaluation was conducted to evaluate the frequency of genotoxic effects of natural radiation in HBRA of Mamuju by focusing on the MN induction within very limited number of subjects. MN can be produced by chromosome breaks and failure in DNA repair machinery is summarized in Table 1. As shown in the Table 1, MN number and its frequency in HBRA group after challenged with gamma ray at dose of 1.5 Gy were lower than that of NBRA (240.92 vs. 377.60 and 0.121 vs. 0.189), indicating an adaptive response of lymphocyte cells that lead to less radiosensitive cells in HBRA group.[21] However, there was no statistically difference ($P > 0.05$) between these two groups as measured in independent sample *t*-test. From these, chronic low-dose radiation in Botteng, Mamuju did not have a significant effect on MN frequency among respondents.

The mean background MN in this evaluation was 46.54 from 26,175 binucleated cells and 42.40 from 22,312 cells in HBRA and NBRA, respectively. There is slightly higher MN was found in study area. For nonirradiated samples, it is comparable with previous research,[13,14] where the mean MN number were 44.25 ± 13.78 from totally 154,443 BNC that were scored in 70 individuals from HBRA. It means that 1.5 Gy irradiation as challenge dose caused the increase of MN frequency up to around 6 times (0.121 vs. 0.020). In control group of that previous study, a total 39,866 BNC were scored in 18 individuals from NBRA, with the mean MN numbers per binucleated cells were 37.94 ± 10.59, so that 1.5 Gy irradiation increased up to 11 times higher frequency of MN (0.189 vs. 0.017). Again this means that high natural radiation in Botteng village might have a role in the lesser radiosensitivity of the blood lymphocyte of residents.

Age and gender may be important demographic variables in determining the MN frequency. With the assumption that younger individuals may be more susceptible to the effects of environmental radiation, we divided the samples into two groups, <50 years old (8 samples for HBRA and 5 for NBRA), and >50 years old (6 samples for HBRA and 5 for NBRA). The MN frequency between younger and older individuals is not significantly ($P > 0.05$) different [Figure 1].

Ramachandran *et al*.[22] observed significantly lower frequency of MN in elder (>40 years) individuals from high-dose group of HBRA as compared to the young (≤40 years) individuals after 1.0 Gy ($P < 0.001$) and 2.0 Gy ($P = 0.002$) of challenging doses. However, the basal frequency of MN was also comparable across NBRA and HBRA group ($P = 0.64$). An example of MN in a cytoplasm containing binucleated cells as shown in Figure 2.

When the results were grouped according to the gender, the MN number was found to be higher in women compared to men for both HBRA (0.15 vs. 0.09) and NBRA (0.216 vs. 0.147) groups [Figure 3]. This is comparable to previous study[13] where the mean MN numbers per BNC cells in females was higher compared to males in both groups. This is also observed by Alsbeih *et al*.[23] that revealed that females and males differed significantly in radiosensitivity ($P = 0.004$) that impacted genetic or single nucleotide polymorphisms association where only XRCC1 gene remained significant in both genders ($P < 0.05$).

Beside the frequency of MN according to age and sex/gender groups described above, the influence of natural radiation exposure to the nuclear division was also studied. Here, the NDI was calculated with published formula [Table 1], and there was no statistically different ($P > 0.05$) found in NDI, as calculated in

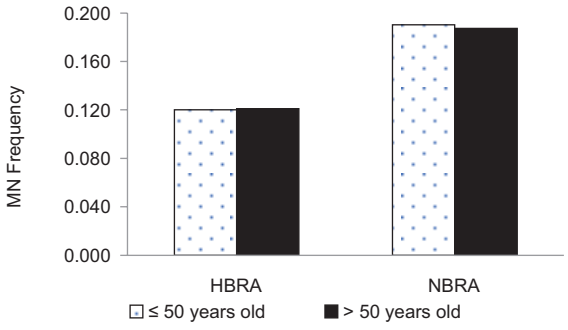

Figure 1: Frequency of Micronucleus for high background radiation area and normal background radiation area according to the age group of respondents

Table 1: The mean micronuclei numbers and nuclear division index of high background radiation area as study group and normal background radiation area as control samples (female; male) after being irradiated with gamma rays at 1.5 Gy

Group	Number of sample (sex)	Mean age±SD (range, years)	Background number of MN±SD (frequency)	Mean MN±SD after irradiation (frequency)	Mean NDI±SD
HBRA	12 (6 female, 6 male)	43.50±15.38 (27-70)	46.54±14.71 (0.0212)	240.92±83.95 (0.121)	1.240±0.11
NBRA (control)	10 (6 female, 4 male)	48.50±14.49 (17-70)	42.40±10.82 (0.0190)	377.60±99.50 (0.189)	1.206±0.09

NDI: Nuclear division index, HBRA: High background radiation area, NBR-A: Normal background radiation area, MN: Micronuclei, SD: Standard deviation

500 metaphase cells, between HBRA and NBRA samples (1.24 vs. 1.21). It is suggested that natural radiation did not significantly affect the nuclear division of lymphocytes. Examples of mononucleated, binucleated, trinucleated, and tetranucleated cells with clear cytoplasm under microscope are shown in Figure 4.

Discussion

Humans are exposed to ionizing radiation from many sources, including naturally occurring radionuclides, such as cosmic and terrestrial, for example, radon gas.[24] Radioactivity is a part of our earth where naturally occurring radioactive materials are present in every things in our life. Background levels of radiation can vary greatly from one location to the next. The average global exposure to natural radiation is 2.4 mSv/year. Doses over 100 mSv can have a harmful effect on humans such as a higher incidence of developing cancer.[25] Integrated molecular analysis conducted by Olipitz *et al*.[26] indicates undetectable change in DNA damage in mouse after continuous irradiation with dose at about 400-fold natural background radiation.

Ionizing radiation induces gene mutations and cellular death in the target cells. Efforts have been made in the development of techniques for measuring radiation damage in biological systems. Among these techniques, MN test has shown as a good biomarker of DNA damage, being widely used in environmental monitoring to detect genotoxic agents[27] and also very useful in monitoring a large number of population[28] because of its simplified technique. MN frequencies in human T lymphocytes represent accumulated genetic changes resulting from the spontaneous or induced chromosome breakage/loss induced by physical and chemical agents. Its size in human lymphocytes varies according to inducing agent,[29] and the frequency of MN is in a radiation dose-dependent. Besides its reliability and good reproducibility, CBMN assay has become one of the standard cytogenetic tests for genetic toxicology tests in human and mammalian cells.[9] The collaborative study group for the MN test based on strain and sex differences, as well as single versus multiple dosing, including mouse peripheral blood erythrocytes stained by acridine orange have been done.[6] Using this technique, differentiation can be done between the MN induced by spindle-damaging agents such as vincristine and colcemid, and those caused by agents that directly damaging the chromosomes such as mitomycin-C and X-rays. This may be because the spindle-damaging agents preferentially produces chromatid fragments while mitomycin-C and X-rays produce chromosome fragments.[29]

Karuppasamy *et al*.[30] found that the overall frequency of MN was 11.7 per 1000 BN cells. The frequencies of MN in the HBRA was 11.7 and NBRA was 11.6 and were not statistically significantly different ($P = 0.59$). However, a statistically significant ($P < 0.001$) age-dependent increase in MN frequency was observed among individuals from both HBRA and NBRA. Whereas Ramachandran *et al*.[31] studied in a total of 1,267,788 metaphases from 27,295 newborns of mothers aged 17–45 years were analyzed during 1986–2007. Frequencies of dicentrics in high and normal level radiation areas were 1.90 ± 0.14 and 2.01 ± 0.26 per 10,000 cells, respectively. Frequencies of other biomarkers such as chromosomal aberration and karyotype anomalies between the newborns from the HBRA and NBRA were very similar.

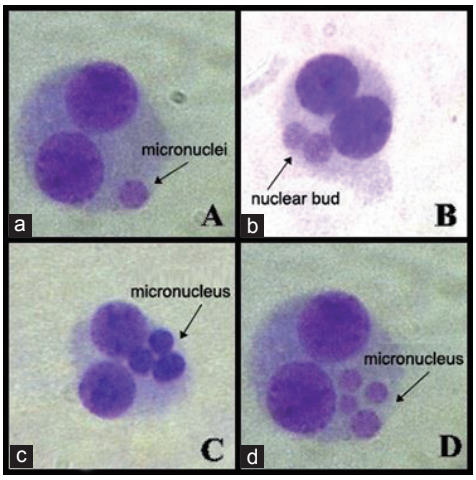

Figure 2: Microscopic view of binucleated cells with 1 MN (a), 2 MNs (b), 3 MNs (c) and 4 MNs (d) (arrows) at × 1000. MN: Micronucleus

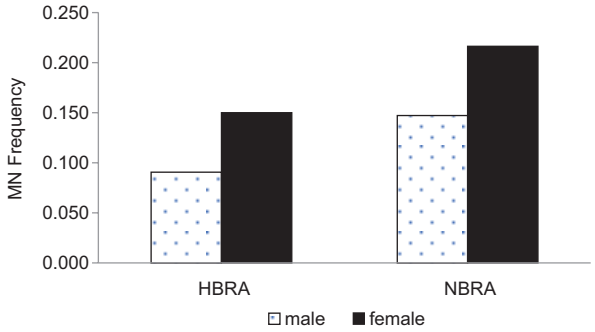

Figure 3: Frequency of micronucleus of male and female respondents from both high background radiation area and normal background radiation area

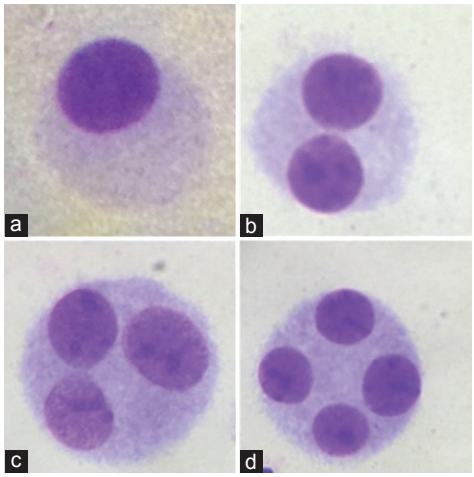

Figure 4: Microscopic view of lymphocyte with one/mononucleate (a), two/binucleate (b), three/trinucleate (c), and four/tetranucleate (d), cells.

In this study, nuclear abnormalities in peripheral blood of individuals living in Mamuju was only focused on MN, whereas

other forms of nuclear abnormalities such as nuclear bud or blebbed cells and binucleated cells with nucleoplasmic bridges were not considered.[9] The results obtained here are from a limited number of cases, and some major confounding factors were not controlled for. It should be noted that above and beyond the unavoidable difficulties associated with study of exposure to HBRA, the estimation of cancer risk will be extremely difficult to accomplish with high precision. Understanding the health impacts of low-level chronic public exposure is also critical to provide a rational basis for regulating radiation exposure in today's society.

Conclusion

Overall, the present study concludes that the MN frequency in lymphocytes of HBRA was lower than that of NBRA where this biomarker was affected by age and sex. Further investigations into different types of DNA damage biomarkers are warranted to fully elucidate the role of environmental radioactivity to the health status of inhabitants living in Mamuju.

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Conflicts of interest

There are no conflicts of interest.

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