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Special Contribution

Cytogenetic and Molecular Damages in Blood Lymphocyte of Inhabitants Living in High Level Natural Radiation Area (HLNRA) of Botteng Village, Mamuju, West Sulawesi, Indonesia

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An evaluation on the cytogenetic and DNA damages caused by natural radiation was done in eighty eight person living in Botteng village as HLNRA and their matched control in Keang village as normal level natural radiation areas (NLNRA). Their bloods were collected with their full informed consent and culture set up for cytogenetic evaluation as well as nucleic acid damage observation with comet assay and γ-H2AX that were done according to the standard procedures. Lymphocytes were scored manually under microscopic observation for the presence of chromosome aberrations. Radiosensitivity of cells evaluation was also done by challenging these bloods to 1.5 Gy of gamma rays before being cultured. The evaluation showed that frequency of chromosome aberration in HLNRA group was lower (0.00081) compared to control group (0.00125). However, frequency of micronucleus (MN) in HLNRA group was higher (0.0204) than that in control area (0.0172). Nucleoplasmic bridge and nuclear budding are found in extremely low frequency. DNA damage observation with comet assay showed a difference between study and control group, however γ-H2AX analysis showed no any effects observed in lymphocytes for both groups. Our data did not reveal any significant difference ($P>0.05$) in HLNRA as compared to NLNRA in all parameters (cytogenetics biomarkers) studied which is supported by the nucleic acid damage evaluation. There was a less radiosensitivity observed in lymphocytes of HLNRA group in comparison with controls.

Key words: high natural radiation, dicentrics, MN, DNA damages, γ-H2AX

1. Introduction

Naturally occurring radiation can be found all around us and account for most of the radiation received by human being each year. This low dose rate of radiation is consistency of external exposures from cosmic and terrestrial sources, and internal exposures from indoor

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naturally-occurring radon that acccount for almost 42% of natural source[1]. Environmental radioactivity research revealed that the sources of radiation could vary from place to place but the dose rate was known between 80 and 150 nGy hr^{-1} world over[2, 3]. There are several areas such as in Ramsar (Iran), Yangjiang (China), Guarapari (Brazil) and Kerala (India), where the natural background radiation level is higher (sometimes 10-100 times the normal levels) than others either due to high levels of radioactivity in soils, rocks and hot springs or due to high levels of indoor radon and its decay products[4-7].

Indonesia also has a region with high dose natural ionizing radiation. Based on Gamma Dose Rate Map of Indonesia that was released by Iskandar *et al*. in 2007, Mamuju, a district in the suburb of West Sulawesi Province has a background radiation around 13 times higher than normal. This place has a highest average dose rate compared to other regions in Sulawesi island and even Indonesia area, which can achieved up to 2.800 nSv/h, as measured by carborne-radiometric using portable Exploranium GR-130[8, 9]. This radiation is the result of natural uranium content (Radium-226 and Radon gas, both of which are highly water soluble) in rock and soil. Major concern is due to its location which is near inhabitant settlement area.

About 2/3 of the radiation dose received by human beings is due to natural radiation, which causes external and internal radiological hazards due to emission of gamma rays and inhalation of radon ant its daughters. It is essential to assess the radiation doses in order to control possible health effects and has been a matter of serious concern from societal and biological stand-points. It is well known that the study of chromosome aberration in peripheral blood lymphocytes is a sensitive assay for detecting exposure to natural radiation and an indicator of cancer risk[10, 11]. Dicentric, a complex event that needs DSB in at least two different chromosomes in close proximity to each other, is considered as indicators of radiation-induced damage.

Beside that, micronucleus (MN) that reflects chromosomal damage is a useful index for monitoring environmental effects on genetic material in human cells[12]. MNs are pieces of extranuclear chromatin that may caused by genotoxic agents such as elevated level of naturally occurring radiation[13]. The frequency of MN is increased by exposure to these agents and in cells showing genomic instability[14, 15]. The increased frequencies of these aberrations has been reported in several areas with high background radiation in the world[16-18]. MN, and other biomarkers such as nucleoplasmic bridge (NPB) and nuclear budding (NBUD) or tailed nuclei assays can be used to measure chromosomal instability and DNA damage caused by genotoxic in human populations. Therefore, from 2015 to 2019, our cytogenetic and radiobiologic investigation is being carried out to determine the frequency of chromosomal aberrations such as dicentrics, translocations, rings, breaks, gaps, MN, as well as NPB and NBUD in lymphocytes of community living in Mamuju area. They are all could be used as prospective signatures of radiation exposure.

The mutagenic and carcinogenic effects of genotoxic agents on exposed people have constituted an increasing concern. The major effect in cells induced by ionizing radiation is DNA breaks, either a single strand or both strands, beside base lesions, sugar damage, and apurinic/apyrimidinic sites. Single strand lesions are the most abundant form of DNA damage whereas DSB is believed to be much more important biologically due to that it may lead to cancer cells formation and the determination of the extent of DSB induction may help to detect precancerous and cancer cells pronesess[19]. In the last two decades, the single cell gel electrophoresis that also called Comet assay has been widely used for genotoxicity testing[20] and in molecular epidemiology studies it was used to evaluate DNA damage as a biomarker of exposure[21, 22], and its repair kinetics as well as DNA fragmentation associated with cell death or apoptosis[23]. Another sensitive marker for DSBs that had been widely used is histone H2AX phosphorylation on a serine four residues from the carboxyl terminus (producing γ-H2AX)[24, 25]. The number of γ-H2AX ionizing radiation induced foci has been observed to be proportional to the number of double-strand breaks produced[26, 27]. This is an emerging biomarker of radiation exposure and an important sign of nuclear anomalies. Some studies demonstrate excellent sensitivity of γ-H2AX assay down to a few milligray of radiation dose, its ability to identify a recent partial body exposure, and persistence of foci for several days after high dose exposure[28].

It has been suggested that certain types of chromosome aberrations have been reported to be associated with an increased genotoxic risk. Preliminary research has been done by us with a limited number of sample and did not reveal any significant difference in HLNRA as compared to NLNRA in cytogenetic biomarkers studied[29]. These observations have prompted a series of experimental studies in our laboratory on the effects of high level natural radiation on cytogenetical parameters in the inhabitants of HLNRAs of Mamuju, completed with molecular evaluation. The results will be used to provide an initial information regarding the anomaly and high dose rate area to the decision makers and the stakeholders in either local or central government.

2. Materials and Methods

Ethics

The study procedures were explained to all respondences, who provided informed consent. The protocol 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.02.01/5.2.KE.051/2015 (date of January 29, 2015).

Environmental radiation measurements and site sampling

The mapping has been successfully delineated the area of naturally occuring radiactive materials (NORM) in Mamuju or the area with thorium and uranium anomaly that identified related with multi-geological-process resulting the increase of grade into several fold from its original state[8, 9]. The measurement of gamma ray exposure was done by using portable gamma sspectrometer Exploranium GR-135 Plus. Outdoor radiation levels was measured using a portable plastic scintillometer (PM1405, Polimaster) measuring micro-Sievert per hour ($\mu Sv\ h^{-1}$), with an accuracy of ± 0, calibrated using different radiation sources (from Environmental Survey Laboratory, Radioecologic Division, PTKMR BATAN, Jakarta). Indoor radon was measured by using a passive radon monitor with nuclear track detector CR-39, an allyl diglycol carbonate, that is a plastic polymer commonly used in the manufacture of eyeglass lenses and firstly developed by Columbia Resins. The measurement was done for 3 months for each point inside the house[8].

Subjects

Eighty eight volumteers between 14 and 85 years of age who were residents of one of nine sub villages, Botteng, Te'bong, Taludu, Taludu Barat, Kassa, Kurasalimbo, Ratte, Kombiling and Tangnga of Botteng Village, with high background radiation (exposed group) and three sub villages with normal or lower background radiation as control group, Keang, Tabelalang and Lomali of Keang Village, took part in the study. All participants were informed about the nature, aims, and intention of the study and signed a consent form and questionnaire before providing blood samples. Pregnant women and any individuals suffering from an illness or taking medication were excluded from the study. The questionnaire contained questions about their diet, and any food supplements or special drinks they were taking. Subjects under 17 years old should get permission from their parents or relatives.

The people who were living for at least 10 years in this area considered as study population. All individuals filled in a detailed questionnaire covering their occupational, medical and family history, habitual behavior of food consumption, drug usage, smoking tobacco and drinking alcohol, etc.

Sample Preparation

Ten ml of peripheral blood was drawn via venipuncture from each volunteer and placed into a heparin containing test tube (Becton Dickinson, N.J., USA) under sterile conditions for cytogenetics preparation (culture) and lymphocyte separation (DNA analysis).

Culture set up and harvest for aberration analyses

The analysis was done with a standard procedures given by IAEA with slight modifications[30, 31]. Two milliliters of the whole blood samples were cultured in medium that consisted of 8.0 mL of RPMI-1640 supplemented with 10% heat inactivated feotal calf serum and 1% streptomycin/penicillin (Gibco). Into this solution, 3.0% mL of phytohemagglutinin (Gibco BRL, Grand Island, NY) was added to stimulate cell division. The culture was for 48 h and placed in incubator at 37°C with 5% CO_2. Colcemid (Gibco BRL) was added for the last 4 h of culture at a final concentration of 0.1 mg/mL to block the mitotic process of the cells at the metaphase stage. The contents of the tube were then transferred into 15 mL centrifuge tube and centrifuged for ten minutes at 1500 rpm and the precipitate was re-suspend in 8 ml of 0.075 M KCl (pre-warmed to 37°C) for twenty minutes. After that, 2 ml of cold fresh Carnoys Fixative (3:1 = methanol : acetic acid glacial) was added into the tube, and this fixation step was repeated two times (until white sediment was obtained). The yield of metaphase cells was stored in freezer at least one night until the preparation of slide was made.

Scoring the metaphases

Two to five slides were prepared for each sample, encoded, and then stained with 10% Giemsa (Merck) and mounted. The number of aberrations (well spread cells and clear chromosomes) was observed under a microscope (Nikon Eclipse Japan) connected to Olympus CCD Camera System for taking picture. By using the 100x oil immersion objective, a cell was considered as aberrant if it had one or more dicentric chromosome from each culture. Scoring was done by a single scorer in complete metaphase with more than 46 centromers only as per the scoring criteria described in IAEA[30, 32]. At least 250 first division metaphase cells were scored manually per sample. In the control samples 200-300 metaphase cells were analysed per donor.

Culture and harvesting of lymphocytes for MN

In the 44 hours since the start cultured, 15 µL cytochalasin-B (3 mg/ml) (Sigma) was added into a culture tube and harvested at 72 hours after the beginning of culture[33, 34]. For harvesting, samples were

centrifuged at 1500 rpm for 10 minutes and the upper layer (supernatant) was removed, and then 8 ml of cold hypotonic solution (0.075 M KCl) was added, left at room temperature for 3 minutes, and then add 3-4 drops of formaldehyde solution and cold fixative solution (methanol: glacial acetic acid = 3:1). After mixed well until homogenous solution was placed in refrigerator (4°C) for 10 minutes, and then centrifuged at 1000 rpm for 10 minutes. Supernatant was removed and add 6 ml cold fixative solution, centrifuged at 1000 rpm for 10 minutes. After fixation for three times then it will be obtained binucleated cells which may contain MN. After one night stored in the freezer, then MN preparations was made by dropping 3-4 drops of cells containing lymphocytes and allowed to dry in the air. Then stained with 4% Giemsa and covered with coverglass for subsequent frequency calculated under the microscope with a magnification of 1000 times. The frequencies of MN, NPB and NBUD were simultaneously counted in 500-1000 binuclear lymphocytes of each individual according to IAEA's[30, 32] and Fenech's protocol[33].

Isolation of lymphocytes

Isolation of lymphocytes procedure for Comet and γ-H2AX assays was done according to Panda *et al.*[34] Two and half milliliters of whole blood was mixed with the same volume of Phosphate Buffered Saline (PBS) pH 7.4 and then was carefully layered in a ratio of 1:2 onto the lymphocyte-separating medium (histopaque-1077) in a centrifugation tube. Then it was centrifuged for 30 min at 1500 rpm. The lymphocytes that appeared as a gray layer between the blood plasma and the lymphoprep were then carefully transferred to a new 15 mL centrifuge tube containing 5 mL of PBS (pH 7.4) and centrifuged for 15 min at 1000 rpm. The lymphocytes were washed three times and resuspended in PBS at a density of (5–6) $\times 10^4$/mL. The cell viability was determined to be 98% by Trypan blue testing, and the lymphocyte suspension was stored in a refrigerator at 4°C. All chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

The Comet assay

The Alkaline Comet assay was carried out according to procedure described in detail by Singh *et al.*[35] Briefly, slides immersed in absolute alcohol and stored in refrigerator for at least one day was used to make sandwhich agarose layer. These frosted slides were then covered with 1% normal melting point (NMP) agarose. After solidified a second layer containing the lymphocyte isolates (4 microliters) obtained from blood sample mixed with 0.5% low melting point (LMP) agarose was placed on the slides. After solidification, slides were covered with 0.5% LMP agarose. Afterwards the slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Na-sarcosinate, pH 10) with 1% Triton X-100 and 10 % dimethyl sulfoxide added fresh to lyse cells and allow DNA unfolding.

The slides were then randomly placed side by side in the horizontal gel-electrophoresis tank, facing the anode. The unit was filled with freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and the slides were set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 min at 25 V (300 mA). After electrophoresis the slides were washed gently three times at 5-min intervals with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20mg/ml) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis.

To prevent additional DNA damage, handling with samples and all steps included in the preparation of slides for the Comet assay were conducted in the dark room. Furthermore, to avoid possible position effects during electrophoresis two parallel replicate slides per sample were prepared. Each replicate was processed in a different electrophoretic run.

γ-H2AX assay

The procedure for γ-H2AX assay was done according to the published papers[36, 37]. Medium (RPMI) containing the isolated lymphocyte from blood of residents was put in hidrophobic slides and let it for 15 minutes. Cells were then fixed in 2 % paraformaldehyde for 15 min, washed in PBS for 3×10 min, permeabilized for 5 min on ice in 0.25% Triton X-100, and blocked in PBS with 0.25% BSA for 3 × 10 min at room temperature. After removing BSA the first antibody (mixed) γ-H2AX and 53BPI as internal control (diluted in BSA 1 : 500) was dropped and incubated in dark moist chamber for 45 minutes. After removing the first antibody the slide was washed with BSA 3 x 2 minutes. After that the second antibody (488 and 594 nm diluted in 1 : 500) with DAPI (1 : 500) was added and let it for 30 minutes. After washed with PBS 2 – 3 times let it dry for 10 minutes with fan. The mounting medium Vecta Shield was dropped and mounting with cover slip and let 15 minutes in fridge. Observation was done with fluorecence microscope at magnification of 10 x 100 with immersion oil.

Radiosensitivity evaluation

Two set of 2 milliliters of the whole blood samples were exposed to 1.5 Gy of gamma source at a distance of 1 meter. Irradiation process was performed at the Multi Purpose Panoramic Irradiators (IRPASENA) at Center

Table 1. Radiation dose rate (nSv/h) of study site, data of individuals participated in the study (their sex, ages (year)), and the frequency of chromosome aberrations

Habitat/village	Indoor dose rate of gamma rays (range) in nSv/h	Sex (M: male, F: female) (Total)	Age (mean ± SD, years)	Mean frequency of dicentrics, rings and fragments	Mean of MN frequency (range)
Botteng	568.88*	34M, 36F	40.67 ± 16.62	0.000816	0.0204
	(330 – 1500)	(70)			(0.009 - 0.034)
Keang	162.30	6M, 12F	48.05 ± 17.43	0.001250	0.0172
(control)	(123 – 220)	(18)			(0.010 - 0.029)

* measured with Exploranium Model GR-130 surveimeter, made in Canada.

for Application of Technology of Isotope and Radiation (PAIR), National Nuclear Energy Agency of Indonesia (BATAN) in Jakarta. The challenge dose used in our experiment was according to other publication[38]. Each set was then cultured for chromosome and MN observations according to procedure above.

Statistical analysis.

The statistical different of categorical variables (gender) in inhabitants and control samples using χ^2-test, while for the continue variable (ages) was using *t*-test analysis. Unpaired *t*-test also used to compare the mean of MN value in Botteng Village inhabitants and control samples, if the data have a normal distribution. The Kolmogorov-Smirnov test was applied to know the distribution of data.

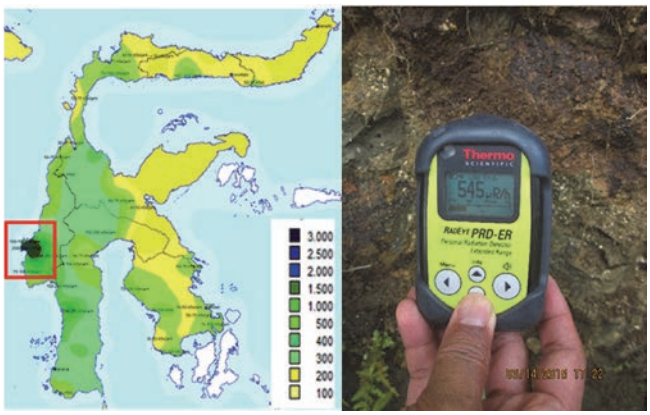


Fig. 1. Study site, Mamuju District, West Sulawesi Province (red rectangle) of Sulawesi Island with its radioactivity mapping in nSv/h (left)[9] and result of environmental radioactivity measurement with a detector in area showing 545 µR/h of dose rate (right).

3. Results and Discussion

Mamuju District is located in 1° 38' 110" – 2° 54' 552" South Latitude and 11°54' 47" – 13° 5' 35" East Longitude (Fig. 1 left) and is the widest district in West Sulawesi Province with the area of 5,056.19 km^2, consisting of 11 sub-district with 99 villages. The total community members of Mamuju District in 2016 were 272,258 people and agriculture is the main livelihood (62.17 percent) of local community member. In these area the majority houses are built from wood (57.55%) and remaining is made from brick wall (40.76%), whereas the floor made from cement is up to 39.73% and wooden is 31.62%. The most prevalent sickness reported in the area is upper throat infection with 56,243 cases[39].

Environmental radiation measurements

The measurement on the radioactivity in 63 houses in Botteng Village measured at the sampling sites using survey meter showed that the average indoor radioactivity for gamma rays was 568.88 nSv/h with the range of 330 – 1500 nSv/h, whereas for outdoor it was higher (760.95 nSv/h with range of 420-2000). An example of measurement is shown in Figure 1 right. Radon concentration that measured in 11 sub villages was between 184-380 Bq/m^3, whereas its concentration in water was between 8410 and 672,000 Bq/m^3 (as comparison in commercial bottled water it was 1830 Bq/m^3). In control area, the average indoor radioactivity for gamma rays was lower (162.30 nSv/h with the range of 123 – 220 nSv/h) compared to Botteng village.

Cytogenetics evaluation

Unstable types of chromosome aberrations (dicentrics and rings) and fragments were studied with manual observation under microscope in totally 14,695 cells of 70 residents in HLNRA and 4,000 cells of 18 residents in control area (NLNRA). On average 210 cells per subject were analyzed. Frequency of these chromosome aberration was 0.00081, whereas in NLNRA it was higher (0,00125) (Table 1). Based on dicentrics and rings, it was known that cytogenetics aberration in HLNRA was lower than that of NLNRA so that we predict there was a lower radiosensitivity of blood lymphocyte of residents living in HLNRA that results in more resistant cells exposed chronically to natural radiation. The complete results on this cytogenetics study were published elsewhere[40].

Frequency of chromosome aberration (dicentric, fragment and ring) in our study is different with other studies conducted in areas of high natural background

Table 2. The distribution and total of MN numbers in Botteng and control samples based on age group (less and more than 40 years)

Group	Age (Years)	No.sample	Mean Age ± SD	Mean MN numbers per binucleated cells ± SD	Total MN
HBRA	< 40	37	29.77 ± 4.23	43.59 ± 15.83	1605
	> 40	33	52.94 ± 5.91	44.44 ± 11.31	1493
Total		70			3098
Control	< 40	6	31.88 ± 2.36	35.50 ± 5.84	200
	> 40	12	54.15 ± 7.34	43.35 ± 14.86	483
Total		18			683

Table 3. The mean MN numbers in females and males from Mamuju (HBRA) and control samples

Group	Gender	No. sample	Mean MN numbers per binucleated cells ± SD
HBRA	Females	34	44.81 ± 12.80
	Males	36	43.68 ± 14.91
Control	Females	12	38.83 ± 12.46
	Males	6	36.17 ± 5.81

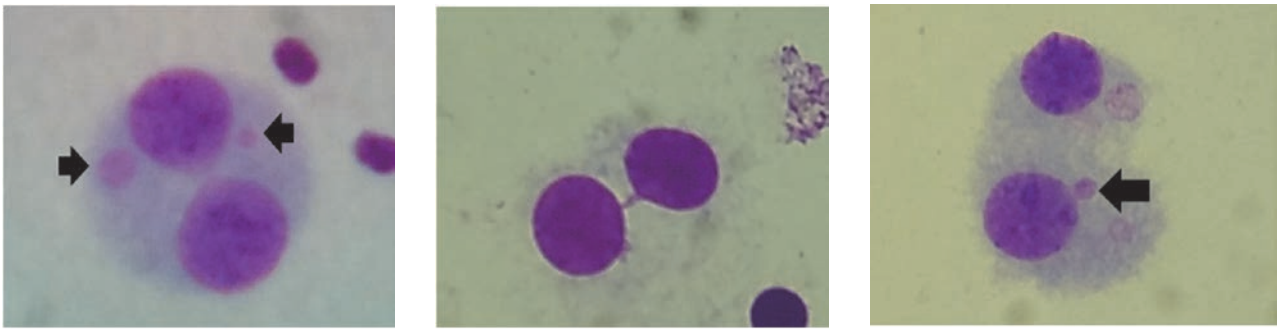
radiation[4, 5], of which an increased frequency of chromosome aberrations has been noted. However in this study there is no evidence of increased cancers or other health problems arising from these high natural levels. There is also no increase in the frequency of cancer documented in populations residing in areas of high natural background radiation. It is known that this study showed that the high level of natural radiation received by inhabitants of this area, Mamuju, paradoxically don't have significant health effect. One possible reason that base damage might not accumulate is that radiation-induced DNA damage may be rapidly repaired.

For MN the results showed that no effects of natural radiation where the frequency of MN was in normal range. Frequency of MN in Botteng Village was ranged between 0.009 and 0.034 with the mean of 0.0204, whereas in control area it was ranged between 0.010 and 0.029 with the mean of 0.0172 (Table 1). This is different with results of the study by Muhammadi *et al.*[41] who found a smaller number of MN in HLNRA compared to NLNRA that could be the results of higher resistance to radiation stress and a more rigorous at checkpoint at cell division in HLNRA group. A more detail results on MN numbers in HLNRA and control samples based on age group are presented in Table 2. Our study showed that there were not significant difference in the mean MN numbers per binucleated cells between Botteng and control groups ($P>0.05$). Based on age group, for both groups (HLNRA and NLNRA) MN numbers are found higher in older than 40 years group of residents, (Table 2). From this data it is known that the MN number is found to increase systematically with the age where it corresponds to the decreased efficiency of DNA repair which is typical in older subjects[42].

Based on the sex, the mean MN numbers per BNC cells in females was higher compared to males (44.81 *vs* 43.68 in Botteng and 38.33 *vs* 36.17 in control) (Table 3). Even though not significantly different ($P>0.05$) our data was supported the assumption that MN frequency in females commonly higher compared to males. It was known that MNs frequency in females tends to be relatively higher to males by a factor approximately 1.4. It is suspected that the higher of MNs frequency in female correlated with greater tendency of the inactive X-chromosome to be lost as an MNs relative to other chromosomes, and to the fact that females have two copies of the chromosome compared to only one in males[43, 44].

Beside MN, observation on NPB and NBUD showed extremely low frequencies (2-3 in 20,000 BNCs for each sample) of these biomarkers in both group (data not shown). NPB originate from dicentric chromosomes, which may occur due to misrepair of DNA breaks, telomere end fusions, and could also be observed when defective separation of sister chromatids at anaphase occurs due to failure of decatenation. Whereas NBUD represent the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells[45]. This very low frequencies of both biomarkers lead us to speculate that high or elevated environmental radiation in Botteng village might avail efficient DNA repair mechanism as a first line of defense against radiation exposure, or that the dose rate was not suffice to induce such an aberration. Examples of microscopically observation of MN, NPB and NBUD are presented in Figure 2.

For translocation chromosome evaluation, seven samples in HLNRA and 4 samples in CA were done and observed under metasystems. We found no increase



(A) (B) (C)

Fig. 2. Examples of microscopically observation of MN (arrows) beside BNC (A), NPB (B) and NBUD (arrow) (C) in peripheral blood lymphocytes of Botteng village (HLNRA) residents.

in the frequency of dicentrics and rings in HLNRA compared to those in the control area. There is also no increase of translocations in HLNRA compared to those in the controls. Our preliminary results also showed no significant difference even in the case of the inhabitants who lived in houses with extraordinarily elevated levels of natural radiation (up to 14.59 mSv/year). The frequency of chromosomal aberrations was reported per 300 cells. The frequencies of dicentrics were 1.87 ± 0.14 (mean ± SD) in HLNRA and 1.88 ± 0.34 in NLNRA, which were not significantly different from each other. We did not found that HLNRA results in increase in the frequency of chromosome aberrations in the circulating lymphocytes of persons living in this area, moreover its carcinogenic effect is still unproven. Wider distribution of chromosome aberrations in NLNRA is due to the quite less size of control sample used here (7 vs 4). It is better if the sample size is increasd, so that the variability or distribution expected is decreased.

Previously, we have shown that the DNA of people living in areas with high radon concentrations may be affected by substantial genotoxicity. This was reflected in our study by an increased frequency of cells with MN, particularly in binucleated peripheral blood lymphocytes (0.6% of cells with MN in the exposed group vs. 0.3% in the control, $P<0.001$)[46]. The same findings were discovered by another group[47].

Radiation responsiveness (radiosensitivity) evaluation

Here we also predicted a beneficial effect from low-level radiation (up to about 10 mSv/yr) of residences living in Mamuju. This radiation hormesis may be due to radiosensitivity or radiation responsiveness of the cells. In the case of carcinogens such as ionizing radiation, the beneficial effect is seen in resistance to the effects of higher doses. However, our study revealed that an *in vitro* challenge dose of 1.5 Gy of gamma rays that was administered to the blood sample showed do

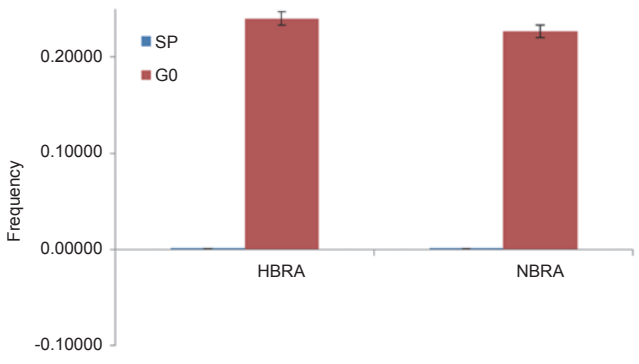


Fig. 3. Frequency of chromosome aberration in lymphocytes either spontaneously analyzed (SP) or irradiated with gamma rays of 1.5 Gy at G0 phase (G0) for HLNRA and NLNRA samples.

not significantly reduced frequency for chromosome aberrations of people living in high background compared to those in normal background areas in Mamuju. This is different with study in Ramsar, a city in northern Iran, by Mortazavi *et al.*[38] that showed a significant radioadaptive response in the residents of high-level natural radiation areas. Five out of seven inhabitants exhibited a reduction in induced chromosomal aberrations following exposure to a 1.5 Gy challenge dose of gamma radiation. It is logic due to the fact that cumulative dose in HLNRA of Ramsar is 2534 mSv, which 173 times higher than in NLNRA (14.6 mSv). Whereas in our study the dose rate of outdoor in Botteng village was 569 nSv/h, which is only 3,5 times higher compared to control (162.3 nSv/h). This difference may be due to some technical factors such as in the handling the samples and lifestyles.

From graphic in Figure 3 it can be seen that frequency of chromosome aberration in pheripheral blood lymphocytes either spontaneously analyzed or irradiated with gamma rays of 1.5 Gy at G_0 phase for HLNRA

Table 4. Tail length, percentage DNA in comet tail and tail moment measurements from samples in HLNRA and NLNRA

Comet Parameters	Normal Level Natural Radiation Area	High Level Natural Radiation Area
Age (years)	38.2 ± 16.79	41.80 ± 14.75
Tail Length (TL)	21.48 ± 9.33	17.75 ± 4.72
Percentage DNA in comet tail (%T)	16.94 ± 10.34	12.84 ± 5.47
Tail Moment (TM)	5.34 ± 4.29	2.64 ± 1.32

samples were not significantly different with these of NLNRA. Beside that the frequency of chromosome aberration at G_0 phase of HLNRA even higher (0.24) compared to NLNRA (0.23). From these we predicted that there is no alteration in radiation responsiveness in blood lymphocyte cells of HLNRA residents.

The similar results was obtained in genetic studies on human population residing in HLNRA of Kerala coast conducted by Das[48] which did not reveal any significant difference in HLNRA as compared to NLNRA in both spontaneous frequency of MN among newborns and telomere length of adult population studied. The frequency of dicentrics in adults was also not significantly different between two areas.

Study by Kumar *et al.*[49] was conducted on the peripheral blood mononuclear cells from inhabitants of Kerala in southwest India that were challenged to higher dose gamma radiation. Initial levels of DNA strand breaks observed after either a 2 Gy or a 4 Gy challenging dose were significantly lower in subjects of the high dose group (HDG) from HLNRA compared to subjects of NLNRA (2 Gy, $P = 0.01$; 4 Gy, $P = 0.02$) and low dose group (LDG) (2 Gy $P = 0.01$; 4 Gy, $P = 0.05$). Subjects of HDG from HLNRA showed enhanced rejoining of DNA strand breaks (HDG/NLNRA, $P = 0.06$) during the early stage of repair (within 7 min). However at later times a similar rate of rejoining of strand breaks was observed across the groups (HDG, LDG and NLNRA). Preliminary results from our study suggest *in vivo* chronic low-level natural radiation provides an initial exposure that allows an adaptation to a subsequent higher radiation exposure, perhaps through improving DNA repair via an unknown mechanism. Therefore, further investigations would be necessary in this population to understand the biological and health effects of chronic low-level natural radiation exposures.

The finding above was supported by categorizing data of HLNRA and NLNRA samples based on chromosome aberration at G0 phase irradiated with 1.5 Gy that demonstrated that only one of eight samples of HLNRA predicted to not sensitive or may be an adaptive response was induced in that sample. This very low number of sample is not enough to generalising that there is an adaptive response in HLNRA samples. It is suggested that there is an indication of radioadaptive response phenomena in blood lymphocyte of Mamuju

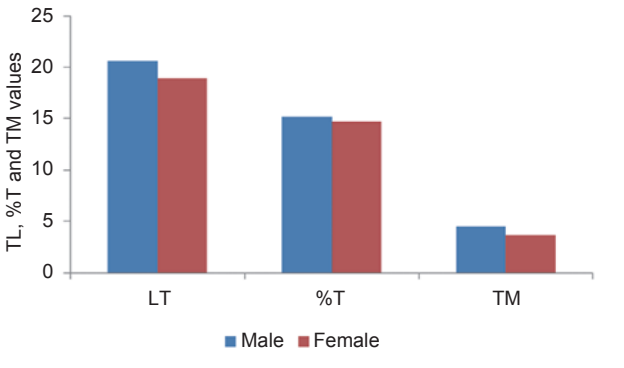


Fig. 4. LT, %T and TM values in male and female from all donor samples.

people that needs further investigation. Lymphocytes of Mamuju residents when subjected to 1.5 Gy of gamma rays showed fewer induced chromosome aberrations compared to residents in a nearby control area whose lymphocytes were subjected to the same radiation dose. This is similar with other report[40].

DNA damage evaluation with Comet assay

The results on the evaluation of DNA damages that was focused on tail length (TL), percentage DNA in comet tail (%T) and tail moment (TM) from control and HLNRA groups were showed in Table 4. T-test analysis showed there were insignificant differences in TL, %T and TM ($P = 0.449$, 0.456, 0.216, respectively) between control and HLNRA groups. It also showed in this research that TL, %T and TM in male at all samples were slighly higher compared to those of female (Fig. 4).

As seen in Figure 4 that DNA damage in males was higher compared to females. This finding was in a good agreement with another study by Kopjar *et al.*[50] who found a little higher DNA damage, as represented by comet tail length, in males compared to females that was influenced by smoking habit. Smoking can induce DNA damage by generating free radicals.

The TL, %T and TM values from Mamuju samples obtained in our research were in a good agreement with Kumar *et al.*[49] who evaluated inhabitants of high background radiation group in Kerala, India. Interestingly the TL, %T and TM values from HLNRA samples were

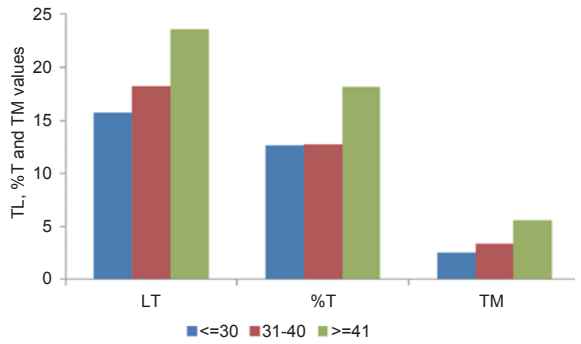


Fig. 5. LT, %T and TM values in three different age groups from all donor samples.

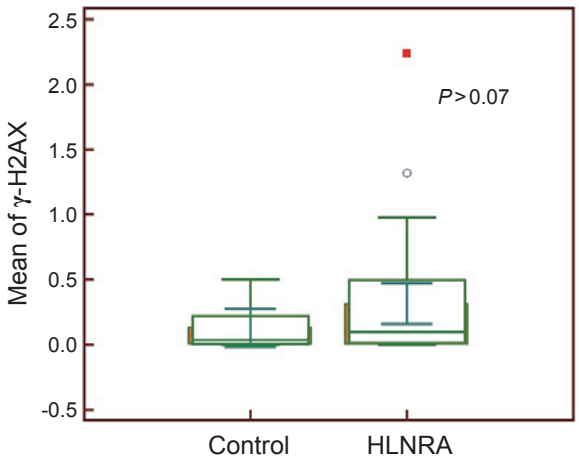


Fig. 6. Mean of γ-H2AX foci in control area and HLNRA of Botteng Village, Mamuju.

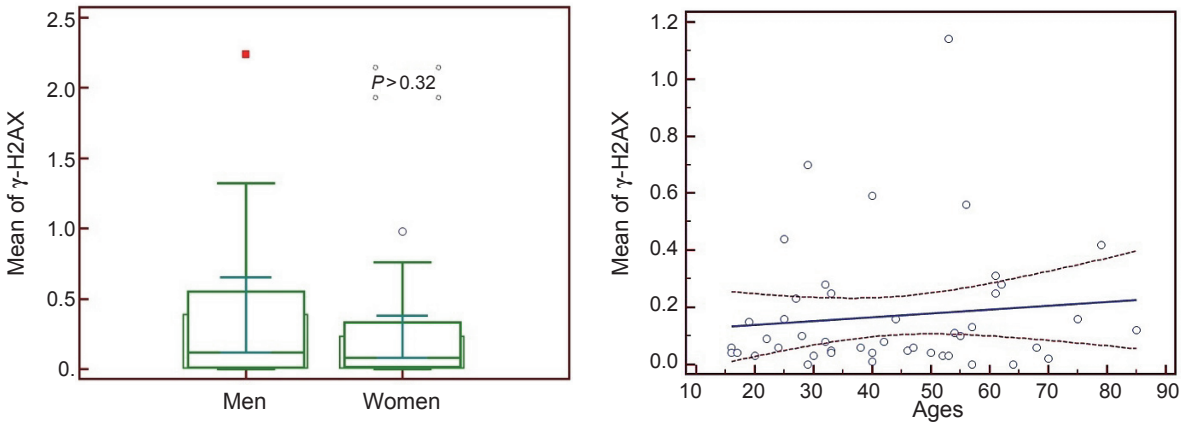


Fig. 7. Correlation between sex (left) and ages (right) of respondents with γ-H2AX expression.

lower compared to normal background radiation area (control). This phenomenon also found in the research by Kumar *et al.*[49] The increased of TL, %T and TM simultaneously with age also found in our research (Fig. 5). It is logic that the cells of older people are more susceptible to be damaged by exogenous agents. The results from this assays need to be interpreted with caution as a number of confounding factors including age of the subject, smoking and diet have been reported.

DNA damage evaluation with γ-H2AX

Results showed that the mean γ-H2AX foci both in study (37 respondents) and control (8 respondents) area was still in normal range and there was no statistical difference between both groups ($P>0.07$) (Fig. 6)[51]. In this preliminary evaluation it is assumed that high background radiation do not effectively induce double strand break of DNA in resident living in Botteng village, it needs higher dose of radiation to produce DNA effects manifested as γ-H2AX. Rothkamm *et al.*[51] stated that dose of 1 mGy produces one persistent DNA double-strand break in a small percentage of cells, and these breaks can be detected using γ-H2AX staining of the broken ends. This γ-H2AX is an extremely sensitive technique, and potentially could be used as a biomarker of such very low doses rate in HLNBR. In addition, the technical limitations of this foci based method need to be solved so that this method could be implemented in routine clinical labs[52].

Mean γ-H2AX foci is found almost similar between men and women for both groups (Fig. 7 left). It is supported by the statistical analysis that showing no significant difference between these groups ($P=0.32$). It is also same with others. Mean γ-H2AX foci tends to increase with the increasing of age (Fig. 7 right). Here we demonstrate that tissues of younger residents are slightly less susceptible to IR-induced DNA damage. Younger residents exhibited quite lower levels of γ-H2AX formation which partially correlated with cellular proliferation and expression of DNA repair proteins[52]. So it is well known that γ-H2AX response and DSB repair capacity decreased with

increasing age.

In the present stude, we evaluated the effects of long term low dose rate radiation on the cytogenetic and DNA damages using several highly methods for both biomarkers. Olipitz *et al.*[53] studied low dose-rate radiation of 0.0002 cGy/min which is almost 400-fold background radiation to the mice continuously for over 5 weeks. They did not observe any changes in the levels of the DNA nucleobase damage products and no DNA fragmentation induced by low dose-rate radiation was observed based on the micronucleus assay, and also there was no evidence of double strand break–induced HR. They concluded that lowering the dose-rate will suppresses the potentially deleterious impact of radiation in an *in vivo* animal model and calls attention to the need for a deeper understanding of the biological impact of low dose-rate radiation.

Ghiassi-nejad *et al.*[54] also studied people in Ramsar Iran living in area with an annual radiation absorbed dose from background that is up to 260 mSv/y that is substantially higher than the 20 mSv y^{-1} for radiation workers. Cytogenetic studies show no significant differences between people in the high background compared to people in normal background areas. However different with our finding, an *in vitro* challenge dose of 1.5 Gy of gamma rays that was administered to the lymphocytes showed significantly reduced frequency for chromosome aberrations compared to those in normal background areas. Specifically, inhabitants of high background radiation areas had about 56% the average number of induced chromosomal abnormalities of normal background radiation area inhabitants following this exposure. This suggests that there is less radiosensitivity of cells to chronic exposure of natural background radiation that resulted in lower proliferation and growth rate.

4. Conclusion

In conclusion, the elevated level of natural background radiation in Botteng, Mamuju as assessed by cytogenetics analysis and DNA damage evaluation has no significant effect in blood lymphocytes of the population residing in this area. Data on the effects of high-level natural radiation on some cytogenetic parameters in the habitants of HLNRA presented here are still very much limited. Therefore, higher number of samples are needed to obtain a comprehensive results.

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Conflict of Interest Disclosure

The authors declare that they have no conflict of interest.

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