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The Evaluation of Frequencies of Cytogenetic Biomarkers in Lymphocyte of Residents from High Natural Radiation Area in West Sulawesi, Indonesia

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***Abstract*— Residents living in high natural radiation areas may pose health consequences such as cancer. The study aims to evaluate the frequencies of cytogenetic biomarkers covering micronuclei (MN), nucleoplasmic bridge (NPB), and nuclear bud (NBUD) with the cytokinesis-block micronucleus (CBMN) assay. This cross-sectional study was done on 51 blood lymphocytes from the resident of Mamuju, West Sulawesi, Indonesia that was done according to standard procedure. After being stained with Giemsa solution, these biomarkers were observed on about 1,000 binucleated cells. The results showed a low frequency of MN (0.0162 in 36,091 cells) and extremely low frequencies of other biomarkers (0.00019 and 0.00061 for NPB and NBUD, respectively) in the study area, whereas these were 0.0225 MNs in 15,000 cells, and 0.00013 and 0.00120 for NPB and NBUD, respectively, in the control area. MNs and NBUDs were lower in the study area compared to control. No statistically significant differences ($p>0.05$) in NPB were found between the two areas, but not for NBUD. The frequencies of MN and NPB in female is higher than that of male in both areas. In the control group, males experienced a decrease in the number of NPB to 0.7 times compared to females, and every extra one year of age, 1.047 times more NBUDs were found. None of the confounding factors was influenced in the study group. It was concluded that there is no impact of high natural radiation to the local residents based on cytogenetics evaluation, with a note that further studies on a higher number of samples and other relevant biomarkers are required.**

***Keywords*— Natural Radiation; Cytogenetic Biomarkers; MN; NPB; Mamuju.**

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I. INTRODUCTION

Every human is exposed continuously to radiation such as sun, light, ultraviolet, microwave, ionizing radiation, and others. They are also exposed to various amounts of naturally ionizing radiation depending on where they live. This radiation covers four sources: radionuclides in the earth, cosmic radiation from external space, radioactivity inside the human body such as K-40, and radon as the biggest contributor [1]. Exposure to cosmic rays and naturally occurring radon has become unavoidable. Radon in houses type is also a problem of growing concern [2]. There is always a risk of damage to cells or tissue from exposure to any amount of or long-term exposure to ionizing radiation [3]. Therefore, improvements in the evaluation strategies in that area are valued as mandatory.

Cytogenetic analyses have been used to assess the effects of ionizing radiation in a wide variety of organisms. It is based on quantifying asymmetrical chromosome alterations (dicentrics, rings, and fragments) in phytohemagglutinin-stimulated T-lymphocytes in the first mitosis after radiation exposure [4]. Another highly standardized strategy that can also be utilized for measuring various biomarkers of hereditary harm in lymphocytes is cytochalasin-blocked MN (CBMN) assay [5], [6]. The appearance of *micronuclei* (MN) in human *T lymphocytes* indicates accumulated genetic alterations ensuing from unconstrained chromosome breakage or loss [7], [8]. MN will originate at the anaphase stage from insulation chromosome or chromatid fragments as an impact of disrepair of deoxyribonucleic acid (DNA) or unrepaired DNA breaks [6]. MNs are frequently utilized as indicators of genotoxicity caused by various xenobiotics, either chemical [9] or physical [10] agents. Even though it is not radiation-specific, laborious, and subject to scorer bias,

the assay is used widely to evaluate environmental or occupational exposure to genotoxins.

The CBMN is a multi–endpoint strategy, where it also could be utilized to know a level of dicentric and ring chromosome arrangement due to, hypothetically, these anomalous chromosomes may create a *nucleoplasmic* bridge (NPB). NPB, which reflecting chromosome rearrangements, is a biomarker of DNA disrepair and/or telomere end fusions [11]. Another biomarker is known as a *nuclear bud* (NBUD) reflecting gene amplification is the elimination of increased DNA and/or DNA repair complexes [11], [12]. The measurement of NPBs and NBUDs can improve the chromosome damage profile for predicting the risk. These three distinguished endpoints can be analyzed at the same time in this assay [6]. Their frequencies in peripheral blood lymphocytes are formed due to chromosomal aberrations arising under the action of ionizing radiation with a similar formation mechanism [13], [14]. These nuclear anomalies are associated with cancer risk. However, there is very limited data on the association of MN with NPB and NBUD in lymphocyte of residents living in high natural radiation areas [15].

Natural sources of ionizing radiation are correlated with increased MN frequencies in a blood sample of coal miners inhaled to high radon concentrations [16]. However, limited evaluation has been paid to the well-being of the residents being at risk due to living in places like Mamuju, West Sulawesi Province, Indonesia. A previous study revealed very low frequencies of these biomarkers in such a place [15]. MN assay to determine individual radiosensitivity has also been evaluated [17]. The assessment of residents' health and physical conditions is an important source of information on the possibility of negative effects of chronic low dose-rate exposures [18]. This long-term exposure may result in health effects like cataracts, cancer, and cardiovascular disease [19].

In this research, MN, NPBs, and NBUDs were evaluated by recording the co-existence of these endpoints within personal lymphocyte cells obtained from the resident staying in the high background radiation of Mamuju.

II. Material and Methods

A. Ethics

This cross-sectional research protocol was introduced to every respondent who was given educated assent. The Local Ethics Committee Review Board mentioned the protocol used in this research for Health Research with Ethical Approval: LB.02.01/5.2/KE.167/2017, April 5, 2017.

B. Subjects

Fifty-one blood lymphocyte samples were obtained from the Transmigration Village of Botteng and Mamuju City, West Sulawesi, Indonesia. Both areas have high natural radiation (denoted as a studied group) (the average of environmental gamma dose rate is 847 nSv/h), and villages of Topoyo with low background radiation (±80 nSv/h and as a control group) were utilized in the research. All participants explained the study's purposes and signed an assent form and survey before blood sampling. The survey covered questions about habitual foods they were consuming, occupational, medical and the sickness family history, smoking tobacco, and drinking alcohol habit.

C. Sample Preparation

Two milliliters of peripheral blood were taken under sterile condition via venipuncture from each participant and placed into a heparin-containing test tube (Becton Dickinson, N.J., USA) for the culture process.

D. Cell culture and harvesting of lymphocytes

The CBMN analysis was conducted with a standard protocol released by IAEA [20]. First, the whole blood samples were put in a Falcon (50 ml) tube containing medium that supplemented with 8.0 mL of RPMI-1640, 10% fetal calf serum, 1% antibiotics (Gibco), 3.0% (0.25 mL) of phytohemagglutinin (Gibco BRL, Grand Island, NY). The solution was then placed in an incubator at a temperature of 37^{o}C with 5% CO_2 for 72 h. At 44 hours of culture, 15 µl cytochalasin B (3 mg/ml) (Sigma) was added, and cells were harvested at 72 hours of culture time [21]. For cell collecting, samples were poured into a 15 mL centrifuge tube and centrifuged at 1500 rpm for 10 minutes at room temperature. After the upper layer was discarded, the cells pellet was mixed, and 8 ml of cold 0.075 M KCl solution was added and kept at ambient temperature for 3 minutes. At that point, 3-4 drops of formaldehyde solution and 6 ml cold fixative solution (methanol: glacial acetic acid = 3:1) were added into tube. After mixed well the solution was placed in 4^{o}C for 10 minutes, and then centrifuged at 1000 rpm for 10 minutes. After cell fixation process for 2-3 times then it would be obtained binucleated cells solution.

E. Slide Preparation, Giemsa Staining, and Scoring

After at least one night, stored in -20^{o}C, MN, NPB, and NBUD preparations were performed by putting 3-4 drops of harvested cells on the cleaned glass slide and dried in the air. The slides were then stained with 4% Giemsa for 12 minutes and covered with cover-glass for subsequent frequency evaluation under the microscope with a magnification of 1000 times. The numbers of MN, NPB, and NBUD in 1000 binucleated cells (BNC) were counted in the lymphocytes of each individual according to Fenech's protocol [21]. The criteria of MN counted was only when it had a similar intensity of staining, did not touch the BNC, and nearby, where two nuclei of BNC have to be the same size and surrounded by a clear border cytoplasm [21]. The criteria of NPB and NBUD counted are according to published papers [22].

F. Statistical analysis

Mann-Whitney U test was utilized to analyze the statistical differences between study and control areas between males and females in the same area and the age group of respondents. $P<0.05$ represented a significant difference. Furthermore, the associations of MN, NPBs, and NBUDs frequencies with age and gender were further examined using the Poisson regression models.

G. Health and physical examinations

Data earned were done by observing and interviewing all respondents by providing a standard questionnaire. Cataract observation was conducted with a shadow test by lightening

the eye at an angle of 45° in the dark (dimly lit) room. The observations of the ear, nose-throat, teeth, any abnormalities in mucosa and hygiene in the mouth, and any lesion on the back skin were also done. For *anamnesa*, any sickness or uncomfortable condition and any difficulties in sightseeing, sprue, history of asthma, and serious sickness/malignancies were asked. Blood pressure and frequency of pulse were measured with special devices. Any enlargement on the thyroid gland and lymph node by palpation on these glands on the neck was also examined. The data were presented as the number of the case found.

III. Results and Discussion

The result showed that the frequencies of MN were 0.0162 in 36,091 cells counted for the study area, whereas this was 0,0225 MNs in 15,000 cells for the control area. It can be seen that the frequency of MN in the study group was lower than that of the control group. This shows that a chronic radiation exposure within a certain dose range, such as in high natural radiation, results in more resistant hematopoietic cells due to its adaptive response. There was an activation of barrier anti-oxidative stress mechanisms after continuous radiation exposure. Individuals living in high background areas may pose an evolution that has been occurred in the presence of ionizing radiation.

Besides, the frequency of NPB biomarker in the study area was 0.00019 in the same number of cells counted from 36 subjects enrolled in the study, and the frequency of NBUD was 0.00061 in that area, whereas there were 0,00013 and 0,00120 for NPB and NBUD, respectively, in 15 respondents from the control area (Tables 1 and 2). Thus, NPB in the study area was higher than control, but NBUD was much lower than control. No significant differences frequency of NPB biomarker was found between study and control area. However, it is significantly different for NBUD.

Here, the frequency of NPB and NBUD (an example of this biomarker is presented in Figure 1) was extremely low and approximated that of dicentric chromosomes (data not shown) but was considerably lower than that of *dicentric chromosomes* (data not shown) MN. This result was extremely low compared to the frequency of NPB in sentinel fish exposed to 2.5 Gy (2.71) of *gamma-ray,* and it increased to be 6.40 after exposure to 5 Gy as measured by a similar method (*erythrocyte micronucleus cytome assay*) [23]. This low number of NPB was similar to the number of dicentric found in this research.

It is different from the previous study that showed that MN frequencies in Takandeang Village (a study area as an adjacent place of the current study) residents were significantly higher than control. Higher NPB than control was also found in that previous study [24]. It is predicted that the high background radiation in this area caused both *clastogenic* and *aneugenic* effects in lymphocytes. In contrast, the mean of NBUD levels was lower than control.

The frequency of MN in female respondents was higher than that of males either in the study or control areas (0.0171 vs. 0.0155 in the study area and 0.0231 vs. 0.0219 in the control area). This was also similar to NPB frequency and previous studies. Beside that cohort study to the survivor of the atomic bomb in Japan showed that the excess relative risks for cancer induction was higher during childhood and decreased progressively at 30–40 years old. Therefore, we grouped the population under study into more than 40 years old and less or the same with 40 y.o. and analyzed these biomarkers. MN and NPB frequencies of older than 40 years group were higher than younger than 40 y.o. Statistical analysis showed that there was no significant difference ($p>0.05$) between younger than 40 y.o. group and older than 40 y.o. in the study area of Transmigration of Botteng village and Mamuju City (Table 2). It was important to note that these higher MN levels in older people may signify age-related alterations or longer times of radiation penetration.

In this research, the data distribution was not normal. However, the statistical test showed that overall there was a significant difference in NBUD (cell budding) of the study group compared to the control group. NPB frequencies in males were not significantly different compared to that of females of the study area, and in the control area, this was also found in NBUD frequency ($P>0.05$).

Health and physical observation and measurement were obtained from 42 respondents in the study area consisting of 24 males and 18 females. Of blood pressure measurement, 18 (42.8%) respondents were categorized as normal, 17 (40.5%) were *prehypertension*, 16 (28.1%) were hypertension grade 1, and 1 was hypertension grade 2. From the *anamnesa*, there were complaints of easy palpitations (1 respondent), vanished fever (1), *hernia scrotalis reponibilis* (1), back pain (1), blurred vision (1), and the rest did not complain about anything. Therefore, it was believed that no direct relationship between these indicators and the radiation factor.

From the history of the disease that was asked, it was obtained that two respondents have had Dengue fever, 2 had malaria, whereas hepatitis, typhoid, inguinal hernia, and dysentery were each found in 1 respondent. Eye examination revealed lens turbidity found in 5 respondents, pterygium in 2, and 1 respondent who had undergone cataract surgery. Acne vulgaris was observed in 2 respondents, onychomycosis in 2, tinea versicolor in 2, and tinea corporis in 1 respondent, of which these were not related to excessive radiation exposure. On dental and oral examination, it was found that poor oral hygiene was observed in 9 respondents, and tonsillitis in 5, which were not complained of by respondents.

Considering the intense radiological impacts of ionizing radiation on humans, the health risk to residents living in high natural radiation areas is important to understand. This study notices prove how simple analyses among genetic endpoints in the CBMN assay can give data on possibly structural chromosomal rearrangements induced by natural radiation in Mamuju. In this area, environmental radioactivity was from 176 to 10,000 nSv/h as measured by carbon-radiometric using portable Exploranium GR-130 [25], [26].

MN biomarker, which can also be induced by an agent other than ionizing radiation, is extensively studied and reported in humans [9], [27]. The MN test has intensively been used to assess the genotoxic effects of chemicals, which has a sensitivity of 74.2%, lower than that of the comet assay, as reported by A. Zeller *et al*. [28].

TABLE I
THE FREQUENCY OF MN, NPB AND NBUD IN LYMPHOCYTE OF STUDY AREA OF TRANSMIGRATION VILLAGE OF BOTTENG AND MAMUJU CITY AS HBRA AND TOPOYO AREA AS CONTROL SAMPLES.

Group	Age (years)	No. samples	Mean Age ± SD	No. binucleated cells counted for MN	Total MN (frequency ± SD)	No. NPB (frequency)	No. NBUD (frequency)
HBRA	14 – 73	36	38.11 ± 14.98	36,091	586 (0.0162 ± 0.0042)	7 (0.00019)	22 (0.00061)
Control	20 – 68	15	39.73 ± 14.78	15,000	338 (0.0225 ± 0.0042)	2 (0.00013)	18 (0.00120)

TABLE II
THE NUMBER OF MN IN FEMALE AND MALE AND AGE GROUP OF RESPONDENTS LIVING IN TRANSMIGRATION VILLAGE OF BOTTENG AND MAMUJU CITY AS HBRA.

Sex or Age group	Number of sample	Mean Age ± SD (Range, Years)	MN frequency ± SD	NPB frequency	NBUD frequency
Female	17	39.03 ± 13.99 (14 – 77)	0.0171 ± 0.0038	0.000235	0.000469
Male	19	39.48 ± 14.44 (20 – 68)	0.0155 ± 0.0046	0.000157	0.000735
>=40 y.o.	12	54.50 ± 11.02 (44 – 73)	0.0173 ± 0.0046	0.000167	0.000583
< 40 y.o.	24	29.92 ± 8.56 (14 – 40)	0.0158 ± 0.0040	0.000208	0.000623

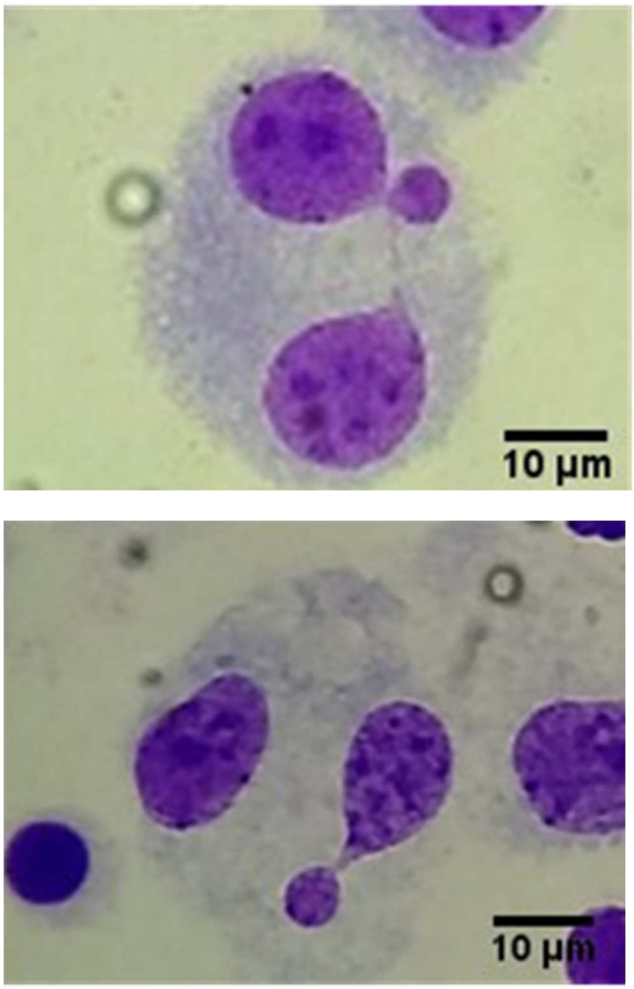

Fig. 1 A microscopic image of Giemsa stained NPB and NBUD (upper) and NBUD (lower) in a sample obtained from residents living in the high background area of Transmigration Village of Mamuju District in West Sulawesi.

CBMN technique is suggested as a reliable method for determining chromosomal damages caused by cytotoxic agents of many chemical and radioactive compounds [21]. However, it has some weaknesses, such as inter-individual variability of MN measurement and the need for a laboratory capable of performing cell cultures. Another different weakness is that the technique is laborious and subject to scorer bias and fatigue, resulting in inter-and intra-scorer variability [5], [10], [29]. Therefore, flow cytometry techniques [10] and *in vitro* 3D tissue models [29] in genotoxicity testing have been proposed.

MN has been utilized broadly in cytotoxicity tests due to its effectively evaluated indicator of DNA insult, but very little is known about their affiliation with other types of cell damage such as NPB and NBUD. These three alterations are important because of the distinguished mechanisms from which each comes. In the last several years, generally, the studies with CBMN assay have focused only MN frequencies and have not mentioned bridges (NPB) or buds (NBUD) [6]. Our study conducted here found an extremely low frequency of NPBs. To the best of our knowledge, this is the first paper described the NPB and NBUD frequencies in lymphocyte of residents staying in the high natural radiation area.

Cheong *et al.* [6] revealed that common chances proportions for MN and NPB were impressively bigger than unity, demonstrating that the nearness of one or extra MN in a cell forces a major chance of getting one or more NPB in that same cell, and contrariwise. This strength association means that the occurrence of MN and NPB did not depend on radiation dose. However, there is no association between MN and NBUD and between NPB and NBUD due to heterogeneity in the odds ratios, so that its induction depends on the radiation dose.

TABLE III
POISSON REGRESSION ANALYSIS OF GENDER AND AGE ON THE FREQUENCIES OF MICRONUCLEI, NUCLEOPLASMIC BRIDGES AND NUCLEAR BUDS

Confounding factors	MN frequencies		NPBs frequencies		NBUDs frequencies	
	IRR (95% CI)	P value	IRR (95% CI)	P value	IRR (95% CI)	P value
All						
Gender (0,1)	0.998 (0.995-1.001)	0.138	0.430 (0.105-1.761)	0.24	1.504 (0.783-2.887)	0.22
Age (years)	1.00 (1.00-1.00)	0.336	1.019 (0.976-1.063)	0.39	1.011 (0.99-1.032)	0.32
Controls						
Gender (0,1)	0.999 (0.995-1.003)	0.71	0.700 (0.515-0.953)	0.02*	1.256 (0.545-2.896)	0.59
Age (years)	1.00 (1.00-1.00)	0.56	1.011 (1.00-1.021)	0.06	1.047 (1.017-1.078)	0.002*
HBRAs						
Gender (0,1)	0.998 (0.996-1.001)	0.17	0.648 (0.143-2.941)	0.57	1.644 (0.684-3.952)	0.27
Age (years)	1.00 (1.00-1.00)	0.18	1.008 (0.960-1.059)	0.75	0.989 (0.959-1.019)	0.45

IRR: Incidence Rate Ratio; Gender: 0, Males; 1, Females; MN: micronuclei; NPBs: nucleoplasmic bridges; NBUDs: nuclear buds
*. The mean difference is significant at the P-value < 0.05.

Zhang *et al*. [30] reported that the diesel engine exhaust posed workers showed considerably higher MN, NPB, and NBUD frequencies than control. MN frequencies have increased by 23.99%, and NPB and NBUD due to this stimulant, showing that diesel engine exhaust level is related to this biomarker frequencies. At the same time, Tian *et al*. [31] studied the relationship between NPB and a relatively low dose of ^{60}Co γ-rays in human peripheral blood lymphocytes. The lowest observable radiation doses of MN were 0.08, whereas and NPB were 0.08 Gy. It was concluded that NPBs positively correlated with the relatively low dose radiation.

In this research, Poisson regression analyses were run to evaluate the impact of confounding components, such as gender and age, on the frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds as biomarkers of genomic instability 3. The outcomes indicated that significant impacts were appeared by gender to the number of NPBs (P=0.02) and age to the number of NBUDs (P=0.002). Both occurred in the control group. By contrast, none of the confounding factors significantly influenced the HBRA group. In controls, male subjects experienced a decrease in NPB to 0.7 times (95% CI, 0.515 to 0.953) compared to female subjects. For the frequencies of NBUDs, every extra one year of age, 1.047 (95% CI, 1.017 to 1.078) times more NBUDs were found, a statistically significant result, P= 0.002.

A previous study by Zeljezic et al. [32] stated that NPB frequency was most affected by smoking habits and age. The effect of gender could not be considered relevant due to the small number of female participants, who expressed that smoking habits and age generally influenced NPB recurrence. The impact of sexual orientation could not be viewed as pertinent because of the modest number of female members [32]. In contrast, Cai *et al*. [33] confirmed that the gender of subjects influences the radiation-prompted levels of NPB and MN. Furthermore, the impact of age on the incited NPB levels demonstrated no specific example and should have been additionally researched [33].

The results showed that frequencies of MN are not related to age or gender. Although in our previous study by Surniyantoro *et al*. [34], the confounding factors, like age, years of employment, and equivalent doses were significantly associated with MN frequencies and every extra one year of age, 1.024 (95% CI, 1.018 to 1.030) times more MN were found in radiation workers who have received a radiation exposure of 0.022 to 0.731 mSv. The gender factor also significantly affects MN frequencies in the population who have never received radiation exposure (control, P<0.001). Females had an increased risk of MN frequencies of 1.455 (95% CI, 1.213 to 1.745) higher than males [34]. This is under our previous study by Syaifudin *et al*. [15], which states that the frequencies of MN are positively correlated with age, where it corresponds to the decreased efficiency of DNA repair, which is typical in older subjects [15]. In this study, the number of female subjects is large enough to be considered relevant to the NPB value.

Based on the evidence that genomic instability has a substantial role in cancer progression, Gashi *et al*. [35] studied the degree of female internal reproductive cervical lesions with MN frequency and a few alternative biomarkers. They showed that MN, NPB, and NBUD in the patient's lymphocytes were significantly higher than controls. This study supports the predictive value of MN, NPB, and NBUD as biological signs of genomic integrity-related cancer. Some other biomarkers such as fused nuclei, horse-shoe nuclei, and circular nuclei might originate from NPB but are not described in this research. Their relationship with ionizing radiation has yet to be established [6]. Other relevant biomarkers that also may urgently be evaluated are apoptotic and necrotic cells [10]. The levels of blood plasma GSH, TAC, and SOD in exposed individuals as an adaptive measure in response to oxidative stress are also options [36].

As evidenced in this research, it is also important to state and recognize that radiation is a weak mutagen and its effects are extremely small to quantify, mainly in humans [37], with the note that individual genes behave differently in response to radiation exposure [38]. On the other hand, as warranted, many published data have demonstrated the potential of radon exposure and its progeny of short-lived nuclides which are found in soils and rocks to induce a significant public health risk [18], [39]. Given these above considerations, this study was conducted to quantify the MN, NPB, and NBUD levels to determine their association with cytogenetic abnormalities and radiation exposure level.

IV. CONCLUSION

The study found a low frequency of MN, NPB, and NBUD cytogenetic biomarkers in the residents living in high natural radiation areas that indicated no negative effects of these environmental insults. It is supported by the fact that MN and

NPB frequencies were lower in the study group compared to the control. None of the confounding factors was significantly influenced in the study group. These evaluated biomarkers create a major challenge to understanding low-dose exposure insults in the area of Mamuju and need additional study to check their relevance.

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