Evaluation of Spontaneous DNA Damage Using the Alkaline Comet Assay in Lymphocyte Cells of Humans Living in the High Level Natural Radiation Area of Mamuju, Indonesia

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ABSTRACT

To evaluate the biological impacts of high background radiation exposures that are represented by spontaneous deoxyribonucleic acid (DNA) damage, an evaluation on lymphocyte cells from residents of Mamuju, West Sulawesi, Indonesia was tested. The mean annual dose received by individuals in this area is about 10.40 mSv. Of the 177 adult subjects studied, 102 were from high-level natural radiation areas of Mamuju and 75 subjects were from a nearby normal-level natural radiation area. Both areas are similar in living situations. DNA strand breaks and other parameters of study and control group were determined using a standardized comet assay. Our results showed that chronic low-level natural radiation had resulted in significantly higher ($p<0.001$) DNA damage based on the three parameters of the assay (tail length, tail DNA, and tail moment) compared to those of control. There was a positive correlation between the level of DNA damage and age, where people aged 40 years and older had a higher level of DNA damage than those under 40 year. The level of DNA damage was also found to be higher in females compared to that of males. It was concluded that chronic exposure to natural radiation in Mamuju had induced spontaneous DNA damage in human cells after long-term exposure which was dependent on age and sex.

1. INTRODUCTION

Natural radiation has participated to shaping the present form of human life and is still the largest contributor to the average dose received by the general population. The global mean annual dose from natural sources as background is 2.4 mSv, of which a part of that dose is an external exposure from terrestrial sources such as natural deposits of uranium, potassium, and thorium in soil, water, and vegetation (UNSCEAR, 2000). As in some other countries, Mamuju in the West Sulawesi Province of Indonesia has high level natural radiation areas (HLNRA) with a potential public annual effective dose higher than normal. HLNRA are areas that have radiation doses greater than the worldwide average background dose for a human being, whereas normal level natural radiation areas (NLNRA) have radiation doses at similar levels with the worldwide average background dose (Hosoda et al., 2021). The existence of davidite, thorianite, gummite, and autonite in alkaline volcanic rock in the Mamuju area have produced high natural radioactivity (Syaeful et al., 2014). In a recent survey, an average radioactivity of 32 mSv per year, which is about 13 times higher than the global mean, was detected (Nugraha et al., 2021). Therefore, deep radiobiological assessments are needed in this unique location.

A multi-dimension study with quite complete data allowing better evaluation of health effects related to chronically low-dose-rate radiation exposure has been conducted, and it can be used as the main input in future epidemiology studies (Nugraha et al., 2021; Hosoda et al., 2021). However, the previous study did not cover the natural radiation effects at the

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molecular level, which needs to be examined in more detail. Preliminary research also revealed an insignificant difference in DNA damage between studied inhabitants and control samples based on the observation of all comet parameters (Rahardjo et al., 2017). These results should be validated in a larger study using a higher number of samples. Here we attempted to evaluate natural radiation doses that may induce molecular or genetic changes in the living bodies of local community members. Moreover, the basic mechanisms that trigger the cellular responses to low doses of ionizing radiation remain unclear.

Human organisms are highly sensitive to ionizing radiation, of which this physical insult has a genotoxic effect on the DNA molecule (Santivasi and Xia, 2014). The generation of this DNA damage or its inadequate repair seems to be an important initial event in carcinogenesis and other non-communicable diseases (Milic et al., 2021). The health effects of low doses or low dose-rates of ionizing radiation are not so clear. Much evidence shows that long-term exposure to elevated doses of natural radiation may cause a wide variety of DNA damage, including single strand and double strand breaks, base damage and destruction of sugars (Belli and Indovina, 2020), oxidative stress related DNA damage and interstrand crosslinks (Walczak et al., 2020), chromosomal instability (Elbakrawy et al., 2019), and chromatin modification (Kumar et al., 2013). One study showed that the level of DNA damage intensity found to be higher in subjects exposed to ionizing radiation doses compared to controls (Mavragani et al., 2017). DNA damage rate appears to increase with age, indicating a decline of its repair efficiency with age which is a multi-factorial process as revealed in some studies (Piperakis et al., 2009; Harris et al., 1986). A small variation of DNA damage by age was reported in some datasets (Milic et al., 2021). Although studies by Milic et al. (2021) revealed no effects of sex on DNA alteration, the relationship between the occurrence of DNA damage and sex as a biological variable needs to be clarified to expand our understanding of its importance.

There are several methods that can be utilized to search for the molecular basis of genotoxic agent effects, including exposure to radiation (Møller et al., 2021). One of them is the alkaline comet test, that can be used to measure DNA damage microscopically and is an important tool in *in vivo* and *in vitro* studies on the population due to radiation mutagen exposure. Several studies have used the comet technique to evaluate DNA damage in human (Møller et al., 2021; Kumar et al., 2015; Kumar et al., 2011), root cells of *Allium cepa* seeds (Saghirzadeh et al., 2008) or adult male albino rats (El-Marakby et al., 2021) exposed to high natural radiation. The assay was also used to measure the DNA damage in residents exposed to high concentrations of radon (Walczak et al., 2020); and DNA damage and its repair after a dose challenge (Dicu et al., 2022). This technique is relatively simple, visual, and sensitive for detecting DNA instability, even in early damage, without the requirement for cell culture (Møller et al., 2021; Gonzalez and Plasencia, 2017; Gradzka and Iwanenko, 2005; Olive et al., 1990). While some molecular studies conducted in high background radiation areas (HBRA) have shown significantly increased frequencies of DNA damage Geetha and Sreedharan (2016), other investigations failed to find a significant difference.

A previous study revealed that DNA damage observed in the blood obtained from residents living in Botteng Village with a comet assay demonstrated a difference, but not significant, between the study and the control group. This is predicted due to the limited number of subjects involved. The objective of the present work was to determine potential effects induced by exposure to high naturally occurring radiation doses on the population of inhabitants of Mamuju, in West Sulawesi, Indonesia, with a higher number of participants and a wider area than in the previous study. It was demonstrated that exposures in these areas cause genotoxicity as measured with a standard technique.

2. METHODOLOGY

2.1 Study site

Mamuju as a study area is located at 1º38′110″-2º54′552″ South Latitude and 11º54′47″-13º5′35″ East Longitude and is the largest district in West Sulawesi Province with an area of 5,056.19 km^2, consisting of 11 sub-districts with 99 villages. The concentration of radon, a naturally occurring radioactive gas, that was measured in this area was between 184 and 380 Bq/m^3 (Syaifudin et al., 2018), which may cause external and internal radiological hazards to the local population.

The study was conducted in four villages of Mamuju, located in an area with a reddish yellow color shown in Figure 1, which shows a highly natural radiation area based on the radioactivity measurement using a portable gamma spectrometer of Exploranium GR-135 Plus (Iskandar et al., 2010). The results of an initial survey conducted in several villages in Mamuju show that the range level of gamma radiation in this

area is 700-1,000 nSv/h with a mean annual exposure of 6.15±0.81 mSv, whereas in the control area it is 2.02±0.03. However, a follow-up survey conducted with a more precise and sophisticated measurement tool in 2020 revealed that annual exposure in that study area was 32 mSv (Nugraha et al., 2021). One near-by village served as a control, shown in yellow in Figure 1.

2.2 Subjects

One hundred and two volunteers, with ages from 14 to 85 years old, who were residents of one of four studied villages (Botteng, Botteng Utara, Binanga, and Tampa Padang Villages) with high background radiation (exposed group, HLNRA) and 75 volunteers from one village (Topoyo) with normal or lower background radiation (control group, NLNRA), took part in the study. In more detail, the study group consisted of 32 people from Botteng, 20 from Binanga, 26 from Botteng Utara, and 24 from Tampa Padang. All subjects were explained the purpose of the study, signed a consent form, and answered a questionnaire before blood sampling. The interview contained some questions about their diet and the history of any illness they may had. The study's ethics approval was granted by the Indonesian Ministry of Health with the number LB.02.01/02/KE.070/2019 (date of March 14, 2019). The study group area had a similar living situation to the control group.

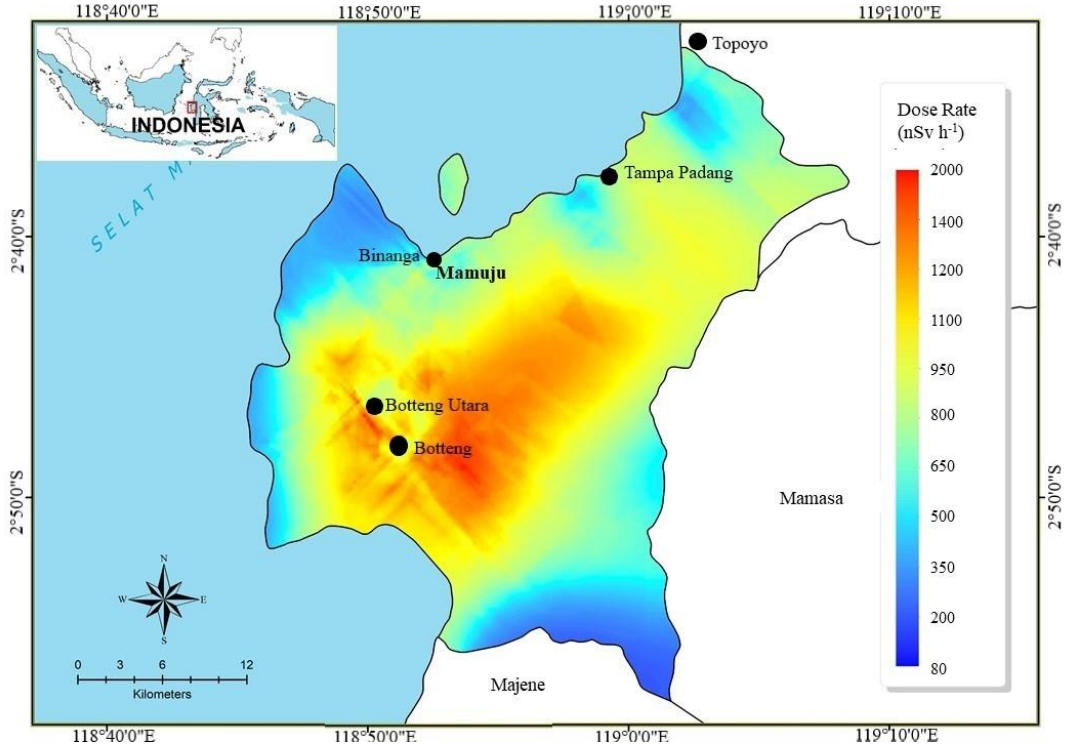

Figure 1. Study area of Mamuju, West Sulawesi Province (Botteng Utara, Botteng, Tampa Padang, and Binanga) and control area (Topoyo). The map of Indonesia, Sulawesi Island, with boxed area and scaled radioactivity level is shown.

2.3 Blood collection

Blood samples were collected from the antecubital vein (~5 mL) of all healthy adult subjects of the study and control areas via venipuncture using a 21-gauge needle and syringe and immediately transferred into heparinized vacutainer tubes (BD Vacutainer systems). After that, the samples were sent to the laboratory in Jakarta as quickly as possible by placing them in proper storage to minimize deterioration. Blood was then processed for the isolation of lymphocytes for the comet assay.

2.4 Isolation of lymphocytes

The isolation of lymphocytes procedure was done according to Bonassi et al. (2021). Briefly, 2 mL of blood samples were mixed with 2 mL of freshly prepared phosphate buffer saline (PBS) solution. Three milliliters of this mixed solution were added very slowly and layered on into a tube containing 3 mL of Histopaque (Sigma), and then the tube was centrifuged at 1,500 rpm for 30 min, after which four layers were formed (PBS, plasma, buffy coat, and blood cells). After transferring buffy coat containing lymphocyte cells to new tube, 5 mL of PBS was added

and shaken until homogeneous, then the tube was centrifuged at 1,000 rpm for 15 min. After supernatant was discarded, the pellet was resuspended by adding 75 µL RPMI and then stored in the freezer.

2.5 The comet assay

The procedure was carried out according to Lu et al. (2017). Briefly, preparation of the slide/preparate was carried out by making 3 layers of gel as a sandwich. The base layer was made of 1% normal melting point (NMP) agarose. The second layer was made of 10 µL of isolated cells (40×10^3 cells/mL) suspended in 90 µl of 0.5% low melting point (LMP) agarose. The third layer was made by dripping 75 µL 0.5% LMP agarose just above the second gel layer, and covering the sandwich with a cover glass.

Cell lysis was carried out by inserting the slide into a staining jar, then the lysis solution was poured into it until the slide was completely submerged. After that, the slide that was submerged in the jar was stored in the refrigerator at 4ºC for 1 h. Alkaline electrophoresis was carried out by placing the slide into an electrophoresis tank, about 1.2 L of DNA unwinding solution was poured slowly until the slide was completely submerged. After that, the electrophoresis voltage was adjusted at a voltage of 25 volts and 300 A and stored in a refrigerator at 4ºC for 40 min. Duplicate slides were prepared and run together for every sample and about 10 cells were observed for every sample. The neutralization process was carried out by immersing the slide in a Tris-base solution containing pH 7.5 at room temperature for 5 min (washing was carried out 3 times). After that, the slide was fixed by immersing it in a 100% ethanol solution for 3 sec. After being fixed the slide was placed in a desiccator for overnight. To observe DNA damage, staining was done by dripping 75 µL of ethidium bromide (EtBr) dye on the slide, then covering it with cover glass. Observations were made in a dark room using a fluorescent microscope at a wavelength of 510-560 nm at a magnification of 400X. All steps of the comet assay were conducted in the dark room. An example of a microscopic view of comet assay results with different levels of DNA damage shown as the length of the tail is presented in Figure 2.

2.6 Statistical analysis

The statistical analysis was performed basically as described in the previous article by Rahardjo et al. (2017). Data analysis was done using SPSS 22.0. Statistical significance of the difference between normal and high background radiation group means was performed by t-test with $p<0.001$ considered to have a statistically significant difference. For all potential confounding factors (age group and sex as presented in Table 1) which have been reported to influence the parameters investigated, the same statistical program was applied.

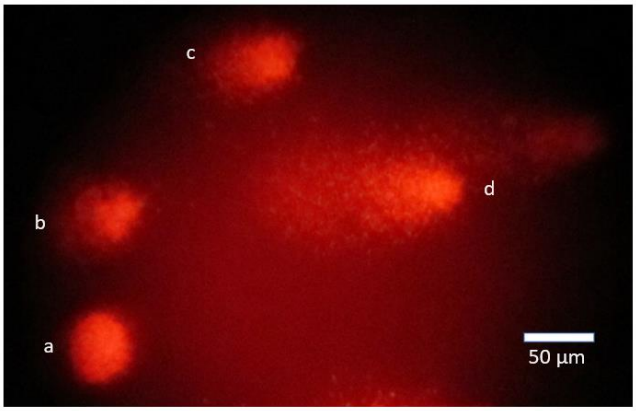

Figure 2. Microscopic view of comet assay results with four types of intact (a), slightly damaged (b), moderately damaged (c), and highly damaged (d) cells 1,000 times magnification

3. RESULTS AND DISCUSSION

3.1 Annual dose of radioactivity

Annual mean radioactivity in HLNRA of the entire Mamuju was reported between 17 and 115 mSv/year, with an average of 32 mSv/year, whereas in NLNRA it was reported at 2.46 mSv/year, indicating that HLNRA has radioactivity 13 times higher (Nugraha et al., 2021). Recently, a paper reported that the results of measurements on ambient dose equivalent rates in the HLNRA for residential houses indoors was 551 nSv/h (4.83 mSv/year); whereas the rates for outdoors were 613 nSv/h (5.37 mSv/year). The measurement result of ambient dose equivalent rates in NLNRA or the control area for indoors was 81 nSv/h (0.71 mSv/year) and the rates outdoors had a geometric mean of 71 nSv/h (0.62 mSv/year) (Hosoda et al., 2021). Previous research found that the annual effective dose received by Botteng Utara residents from terrestrial gamma rays was lower (10.40-18.62 mSv/year) (Nurokhim et al., 2020).

3.2 Alkaline comet assay

Results for all parameters (TL, TDNA, and TM) of the alkaline comet assay in the present study are presented in Table 1. These results showed that the spontaneous level of DNA damage in the inhabitants of HBRA was higher than that of the inhabitants of NBRA. It is also known that there is a statistically

significant increase in the mean values for all three parameters of the standard comet assay ($p<0.001$) in the sample of the study area in contrast to the control sample (Figure 3). The mean values of TL, TDNA, and TM in the HLNRA were 36.60 µm, 9.51%, and 4.81 µm, respectively, whereas in the NLNRA samples they were 21.71 µm, 7.57%, and 2.66 µm, respectively.

Table 1. The characteristics of subjects, its age and sex groups, and parameters of DNA damage (Tail length (TL), TDNA, and tail moment) were measured with the comet assay in people of the studied area (HLNRA) and its matched control (NLNRA).

Characteristics	NLNRA mean±SD (range)	HLNRA mean±SD (range)
Subjects		
Number of samples	75	102
Age (years)	40.37±13.14 (20-79)	41.61±13.06 (19-79)
Age and sex groups		
<40 year	40	58
≥40 year	35	44
Male	36	51
Female	39	51
Comet parameters		
TL (µm)	21.71±11.03 (7.76-68.68)	36.60±10.50 (18.32-58.71)
TDNA (%)	7.57±3.04 (3.89-19.65)	9.50±3.40 (3.92-18.81)
TM (µm)	2.66±2.12 (0.75-13.75)	4.81±2.64 (1.45-11.94)

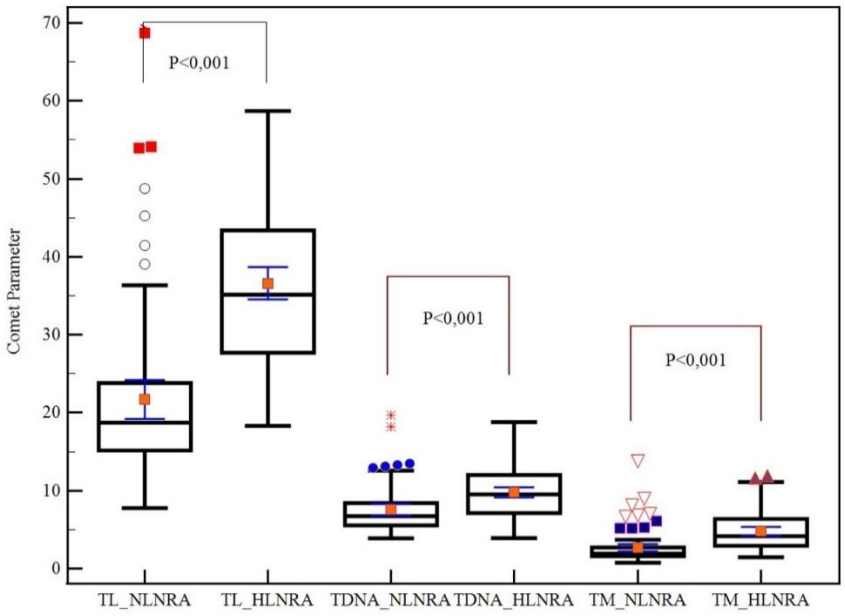

Figure 3. Graph of parameters of DNA damage (tail length (TL) (2 left graphs), tail DNA (TDNA) (2 graphs in the middle) and tail moment (TM) (2 right graphs)) measured with a comet assay on samples of NLNRA and HLNRA of Mamuju. A significant difference was shown between NLNRA and HLNRA ($p<0.001$).

Of the four HLNRA villages, Binanga had the lowest comet test value of all the parameters observed, and the comet test results in this village were not significantly different from NLNRA. It is contradicted by the fact that the reported effective dose of environmental radioactivity in Binanga village was 5.22 (2.71-6.01 mSv/year), almost similar with other villages of HLNRA. Low DNA damage in this village is probably related to the high level of nutrition status of the respondents. The highest comet test value was found in Tampa Padang Village and was higher than NLNRA. The TL values of comet measurements in three HLNRA areas (Botteng, Botteng Utara, Tampa Padang) were significantly higher than NLNRA (39.7 vs 21.71) ($p<0.05$), as were the values of TM and TDNA parameters. Among the villages in the exposed area under study, Tampa Padang village has the highest values of all comet parameters except for TM, where Botteng Utara is the highest value (Figure 4).

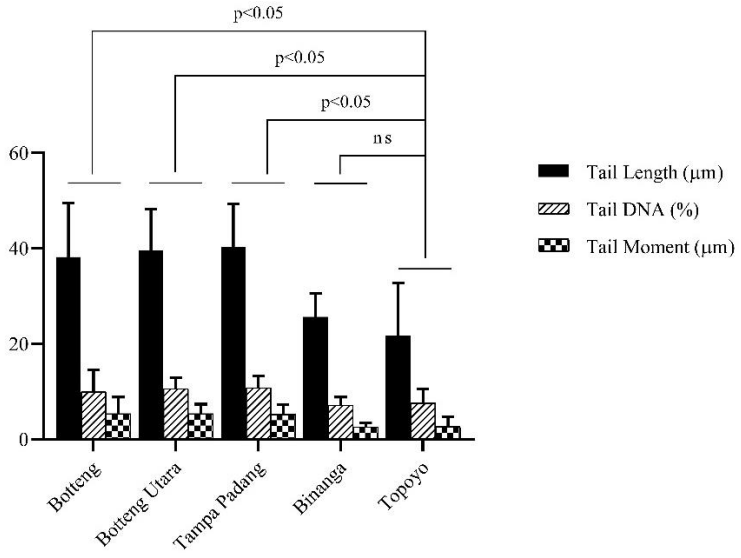

Figure 4. Measurement of all comet assay parameters (TL, TDNA, and TM) among a study group of 4 villages in HLNRA (Botteng, Botteng Utara, Tampa Padang, and Binanga) compared to a control group of 1 village in NLNRA (Topoyo). Ns denotes a non-significant difference.

From the tables and graphs, it is shown that all parameters of the Comet assay in the NLNRA sample have a wider range of values compared to HNLRA (for example 7.76-68.68 μm vs. 18.32-58.71 μm in TL). It means there is a larger variety of subjects in NLNRA. This suggests that many factors contribute to DNA damage in humans other than radiation. Endogenous and exogenous factors can cause changes in DNA structure. Endogenous factors come from inside the cell, such as reactive oxygen species (ROS) produced by mitochondria (Madamanchi and Runge, 2007). Under normal circumstances, low ROS production can be neutralized by antioxidants (Ozougwu, 2016). Exogenous factors come from outside the cell, namely cytotoxic and genotoxic inducing agents such as ionizing radiation, ultraviolet (UV) rays, and harmful chemical compounds (Desouky et al., 2015). In addition, the other factor is each individual's response to different cytotoxic-inducing agents.

Previous research by Rahardjo et al. (2017) showed that there were insignificant differences in comet tail length (TL) and tail moment (TM) between Takandeang inhabitants, an adjacent village of Botteng, and control samples (p=0.578). Regression analysis revealed that DNA damage increased with age in control samples, even though the correlation was not significant (p>0.05). In contrast, a significant negative correlation (p=0.02) was observed in the studied inhabitants. Therefore, results found in this research should be validated in a larger study using more samples from Mamuju. However, unlike in a previous study where the TL, %T, and TM values from HLNRA samples were lower compared to the normal background radiation area as control (Rahardjo et al., 2017), which is also found in the research by Kumar et al. (2015), where the results are inconsistent or conflicting. This fact indicates that there are inconsistent or controversial outputs or results among different research projects at the same study area, which are influenced by several factors such as sample size collected and preservation, as well as the entire process from collection to analysis, technique and condition used, parameters and cell types tested, sampling location that related to effective dose of radiation, individual's response and adaptation, and so on.

3.3 Relationship between DNA damage and age

As seen in Figure 5, DNA damage represented by TL in the people of both NLNRA and HLNRA showed that people aged 40 years and over had higher damage than those under 40 years, but statistically, there was no significant difference found (p>0.001) between both age groups. However, it is very clear trend that mean value of DNA damage in the HLNRA group was higher than that of the NLNRA group. We suspect that natural radiation has an effect on increasing DNA damage in HLNRA. This assumption is strengthened by references which state that the slightest exposure to natural radiation received by the population will result in DNA changes and carry a risk of cancer (Mortazavi and Mozdarani, 2013). Meanwhile, because each individual had the ability to carry out an adaptive response systemically expressed after radiation exposure, the value of DNA damage

that was not statistically significant different between both groups was suspected (Shimura and Kojima, 2018). Here TL is considered as a sign and information of how high the DNA damage level are where they migrate away from the undamaged DNA-containing nucleoid body, resembling the structure of a comet. Thus, the percentage of DNA in the tail is directly proportional to the percentage of DNA damage that has occurred in a particular cell (Møller et al., 2014).

It was also reported in some datasets, suggesting a higher number of DNA damage events in the oldest age-classes. The increase in DNA damage with age may be caused by a decrease in DNA repair capacity that results in its accumulation (Singh et al., 1990; Piperakis et al., 2009) and is characterized by reducing physiological integration and function (Fischer and Riddle, 2018). Garm et al. (2013) stated that the improvement of single strand breaks can still be maintained in individuals aged 40-70 years, but experienced a decrease in the improvement of double strand breaks. Li et al. (2016) stated that there is a decrease in the expression of proteins and factors in old age that help improve DSB in each of these pathways. It means that DSB repair decreases in old age.

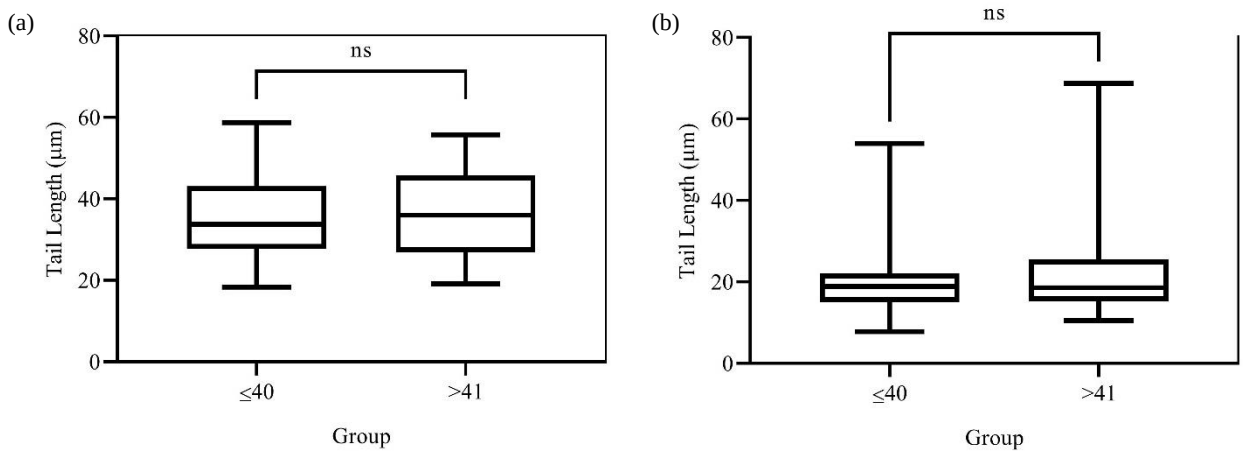

Figure 5. Comparison of DNA damage represented by tail length (TL in µm) of residents according to age group (≤40 and >40) from study area (A, HLNRA) and from control area (B, NLNRA) of Mamuju, West Sulawesi. Ns denotes a non-significant difference.

3.4 Relationship between DNA damage and sex

The relationship between gender (sex) and DNA damage represented by TL in HLNRA residents showed that DNA damage in in females was higher than in that of males, but statistical analysis revealed no significant difference ($p>0.001$). The result is presented in Figure 6. The finding is in contrast with previous study by Ishikawa et al. (2003) which stated that the DNA damage in a male is higher than in a female, which is caused by the characteristics of the Y chromosome in a male which is more fragile than the X chromosome in a female. Another DNA damage biomarker, micronuclei, showed that females had 2.48-fold higher micronuclei values than males (Surniyantoro et al., 2018). This shows that females are more sensitive to radiation exposure than males (Babayan et al., 2018). Different results were shown in the study by Garm et al. (2013), who conducted a study on 200 residents in Denmark, where this study shows that there is no influence of gender on DNA damage. A higher incidence of DNA damage in males compared to females was found by Fischer and Riddle (2018). Besides that, there is another factor such as smoking and drinking habits that should be considered mainly in the male group (Bonassi et al., 2021).

In contrast with our finding, a previous paper by Rahardjo et al. (2017) reported that DNA damage in males was higher compared to that of females, which was in good agreement with another study by Kopjar et al. (2006) and Ishikawa et al. (2003). A higher level of DNA damage is predicted to be caused by smoking habits that generate free radicals.

Human and other organism cells are constantly exposed to stress caused by external genotoxic agents and a verity of environmental factors such as high levels of natural radiation that cause cellular components damage (Li and Sancar, 2020). Many methods are available to detect this damage. Single cell gel electrophoresis or comet assay, immunological and PCR-based methods of monitoring DNA damage and its repair at the sub-gene and single nucleotide level are among these methods. Due to its technical simplicity and sensitivity, it is possible that the comet assay is the most useful method

in biomonitoring of the human population suffering from this environmental mutagen (Sykora et al., 2018), including its new Cometchip platform. Here, the potential use of this molecular technique to monitor the effects of natural radiation in the environment has been tried and exploited as conducted by others (Valverde and Rojas, 2009; Rahardjo et al., 2017; Syaifudin et al., 2018; Sykora et al., 2018). It is shown that the comet assay used for evaluation of spontaneous DNA damage induction is a relatively simple and cost-effective test at the single cell level when applied to low level radiation genotoxicity studies. In wider application, induction of endogenous strand breaks, oxidized bases and resistance to H_2O_2-induced damage as well as its mechanisms of action of this genotoxic chemical in cells can also be measured by the comet assay (Martini et al., 2021).

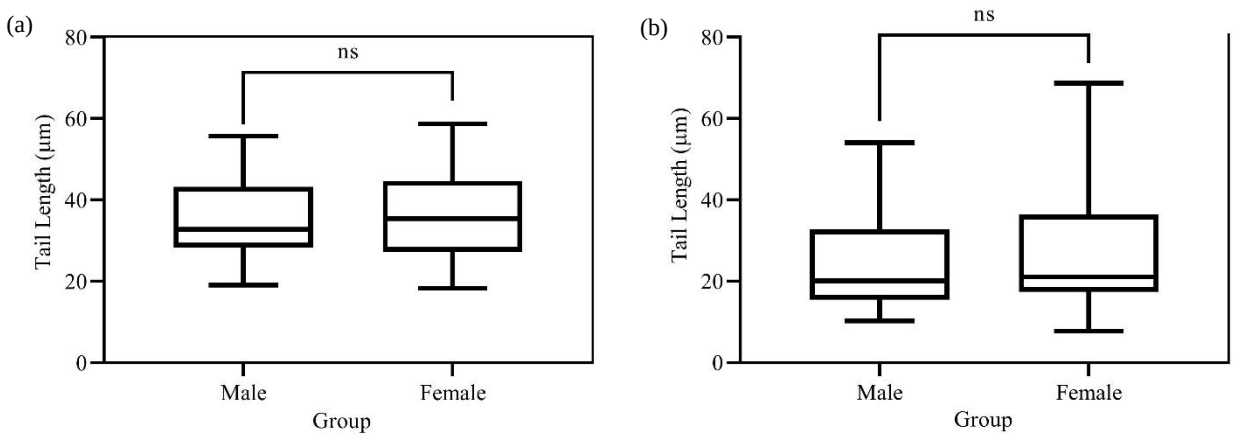

Figure 6. Comparison of DNA damage represented by tail length (TL in µm) of residents according to sex (male and female) from study area (A, HLNRA) and from control area (B, NLNRA) of Mamuju, West Sulawesi. Ns denotes a non-significant difference.

DNA damage, recognized as an important biomarker of cell tumorigenesis, may result in several mechanisms such as unrepaired chromosome telomere damage, causing the activation of signaling events, through which altered cells are predicted to be a more potent driver of aging and disease (Russo et al., 2020; Yousefzadeh et al., 2021). Moreover, another important factor is the presence of an adaptive response of the cell through expression of protective stress response proteins (Nishad et al., 2021) and the process of virtually removal (reduction) of DNA damage, as proposed for the comet assay results, that could not be excluded in the study of DNA damage. Other than environmental situation, some factors that affect DNA damage such as nutrition, lifestyle and occupation must also be considered. To all these individuals, the systemic adaptive responses that may have been prominently expressed at high effective doses of natural radiation also need to be explored.

4. CONCLUSION

Is conclusion, it was revealed that chronical exposure to high natural radiation in Mamuju significantly ($p<0.001$) induced higher DNA damage in local human cells compared to control. This was detected with a validated comet assay where DNA damage was evaluated by three parameters of the assay. The level of DNA damage was influenced by age as well as sex. Humans at age of 40 years and older have a higher level of DNA damage than younger ones; and females have higher DNA damage compared to males. Some confounding factors exist in the inconsistency of these results with others.

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CONFLICT OF INTERREST

We declare no conflicts of interest associated with this publication.

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