Assessment of DNA damage in lymphocytes of Mamuju (a high background radiation area) inhabitants using alkaline single cell gel electrophoresis

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Abstract: Mamuju in Indonesia has a highest average dose rate and considered as a high background radiation area (HBRA) in Indonesia. Here in this research a preliminary study to evaluate DNA damage using the alkaline comet assay in inhabitants of Takandeang village, Mamuju, was conducted. Blood samples from 55 healthy donors living in Takandeang village and 22 control samples were obtained and processed into the assay. Results showed that there were insignificant differences of comet tail length (TL) and tail moment (TM) between Takandeang village inhabitants and control samples ($p = 0.578$, 0.174). Regression analysis revealed that the DNA damage was 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 Takandeang inhabitants. Results found in this research should be validating in a larger study using more samples from Mamuju.

Keywords: comet assay; DNA damage; high background radiation area; lymphocytes; Mamuju.

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1 Introduction

The major component of radiation exposure in daily life for the general population is natural radiation that can come from outer space as a cosmic radiation and those that occur in the soil, air, water or building materials (terrestrial radiation) (Balachandar et al.,

2011; Tavakoli et al., 2012; Pashazadeh et al., 2014). Natural radionuclides in soil generate an important component of the background radiation exposure of the population in the world. Some radionuclides are detectable in soil and a natural environment radioactivity also the associated external exposure due to gamma radiation depends on the geological and geographical conditions and has different levels in the soils of each area in the world (Gahrouei et al., 2013). There are several high level natural radiation areas throughout the world, including those in Iran, Brazil, China, and India (Balachandar et al., 2011).

Studies conducted by Syaeful et al. (2014) showed that Mamuju has the highest average dose rate in Sulawesi and even Indonesia, which can achieve 2800 nSv/h. Another study conducted by Alatas et al. (2012) that evaluated the cytogenetics response in Mamuju inhabitants showed there was an insignificant difference in the frequency of chromosome aberrations in the lymphocytes of people who live in Mamuju area and those who live in normal natural radiation area. Until now chromosomal aberrations (CA) in human peripheral lymphocytes were considered as a valuable biomarker of ionising radiation exposure (IAEA, 2011).

A wide variety of biomarkers were used for the detection of DNA damage as early radiation effects. These include chromosome damage measured by unstable or stable chromosome aberration (CA). Another biomarker is comet assay that has shown to be sensitive and rapid in the detection of breaks in the DNA strands of individual cells induce by radiation (Masoomi et al., 2006). The alkaline comet assay has been used for several years for the quantisation of DNA damage as an early biological effect of DNA damaging agents in environmental and occupational settings. The sensitivity of comet assay is also higher compared to other biomarkers of DNA damage like micronucleus assay (Mohammadi et al., 2006).

To our knowledge there has been no report on the level of spontaneous DNA damage in peripheral blood lymphocytes of healthy inhabitants in Mamuju. Therefore we conducted a preliminary study in healthy individuals from Mamuju and subjects from a nearby normal level natural radiation area to evaluate DNA damage using the alkaline comet assay.

2 Material and methods

2.1 Gamma dose rate measurement

The gamma dose rate measurement was conducted based on the Syarbaini and Setiawan (2015) publication. In brief, the gamma dose rate was measured at one metre above the ground by means of a portable gamma spectrometer (Model GR-130 Mini Spec, produced by Exploranium Company, Canada). The instrument was calibrated by a secondary standard dosimetry laboratory under the National Nuclear Energy Agency of Indonesia.

2.2 Study populations and blood sampling

Fifty five healthy adult subjects from Takandeang village in Mamuju, West Sulawesi and twenty two healthy adult subjects from normal background radiation area were included in this study. Peripheral blood samples were collected by venipuncture using heparinised

vacutainer tubes (BD Vacutainer systems). The study was approved by Ethics Committee of the National Institute for Health Research and Development, Indonesian Ministry of Health, number LB.02.01/5.2.KE.051/2015 date of 29 January 2015. All procedures performed in this study were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all donors. A detailed questionnaire was used to obtain information on age and occupation.

2.3 Alkaline single-cell gel electrophoresis (comet) assay

The alkaline comet assay was performed according to the protocol described below.

2.3.1 Lymphocytes isolation

Lymphocytes were isolated from whole blood using Histopaque 1077 (Sigma). Isolated cells were washed three times with ice-cold phosphate-buffered saline (pH 7.5) and suspended at a concentration of 10^4 cells/ml in RPMI-1640 medium (Gibco). The viability of cells was tested using trypan blue (Sigma).

2.3.2 Slides preparation

Two microscope slides were used for each subject. The slides were coded and then coated with 1% normal melting point agarose. Twenty microliters of lymphocyte suspension containing about 1×10^4 cells were mixed with 150 µl of 2% low melting point agarose at 37°C. Seventy five microliters of this mixture were applied as one drop in the slide. In total, each slide had two drops. Each drop was covered with a 22 × 22 mm coverslip and allowed to solidify for about 10 min at 4°C.

2.3.3 Lysis

The coverslips were gently removed when the gel had solidified. Slides then were immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma and 1% Triton X-100, pH 10) and maintained for 1 h at 4°C in darkness.

2.3.4 Alkali unwinding and electrophoresis

The slides (two from each subject) were randomly distributed on a horizontal electrophoresis unit. Twelve slides were used at a time. The unit was filled with freshly prepared ice-cold electrophoresis buffer (100 mM NaOH; 1 mM Na2EDTA; 10 mM Tris-basa, pH >13) to a level approximately 0.5 cm above the slides. The slides were incubated for 40 min to allow DNA unwinding. Electrophoresis was carried out at 0.7 V/cm and 300 mA (Biorad model 1000/500) for 30 min at 40°C and protected from exposure to room light. Subsequently, slides were washed with neutralisation cold buffer (0.4 M Tris-HCl, pH 7.5) in a staining jar, three times, 5 min in each wash. Each gel was fixed in ethanol and stained with 20 µg/ml ethidium bromide then covered with a coverslip.

2.3.5 Acquisition and analysis of images

The coded slides were examined using a fluorescence microscope (Nikon 50i) equipped with a monochrome CCD camera connected to Cytovision 3.1 Software. About 50 randomly chosen images were acquired from two independent gels at a magnification of 200×. The DNA damage was estimated in terms of comet tail length (TL, calculated as the distance between the x coordinates of the rightmost point of the tail and rightmost point of the head) and comet tail moment (TM), as the mean of 50 comet measured using CASP software in each subjects (Końca et al., 2003). TM was calculated as the product of the TL.

2.3.6 Statistical analysis

Data analysis was done using the SPSS 22.0. Statistical significance of the difference between normal and high background radiation group means was performed by *t*-test with $p < 0.05$ were considered to have a statistically significant different.

3 Results

The terrestrial gamma dose rate of Takandeang village ranged from 600 to 2200 nSv h^{-1} with a mean of 1141 nSv h^{-1}. The data of TL and TM from normal and high background radiation groups were showed in Table 1. *T*-test analysis showed there was insignificant difference in TL and TM between normal and high background radiation groups ($p = 0.578$, 0.174). Figure 1 showed that the comet appearance between two groups did not differing.

Table 1 TL and TM measurements from samples in normal background radiation area and high background radiation area

Characteristics	*Normal background radiation area*	*High background radiation area*
Number of samples	22	55
Age (Years)	40.18 ± 15.429 (18–72)	42.53 ± 12.953 (16–70)
Smokers	7	13
Comet Parameters		
Tail Length (TL)	14.6323 ± 2.91933	15.1304 ± 3.75041
Tail Moment (TM)	1.4597 ± 0.30496	1.6055 ± 0.45897

3.1 Tail length

The tail length range in Takandeang village inhabitants was 9.04 to 23.16, with mean value 15.1304 pixels. In the control group the tail length range was 7.90 to 19.66 with mean value 14.6323 pixels. Statistical analysis (*t*-test) showed that the difference was not significant ($p = 0.578$). When all samples (control and HBRA groups) were classified into three different groups based on age (Group I: < 30 years, Group II: 30–50 years, Group III: > 50 years) and a *t*-test analysis was applied in each group, the differences of all groups were also not significant ($p = 0.379$; $p = 0.307$; $p = 0.215$). A regression

analysis in Takandeang inhabitants showed a significant (p = 0.02) negative correlation between TL and age (y =18.97–0.09 × Age). In contrast a regression analysis in control group showed a positive correlation between TL and age (y =13.43+ 0.03 × Age), even though the correlation was not significant (p = 0.483).

Figure 1 Comet appearance from PBL of Takandeang village inhabitants (left) and control group (right)

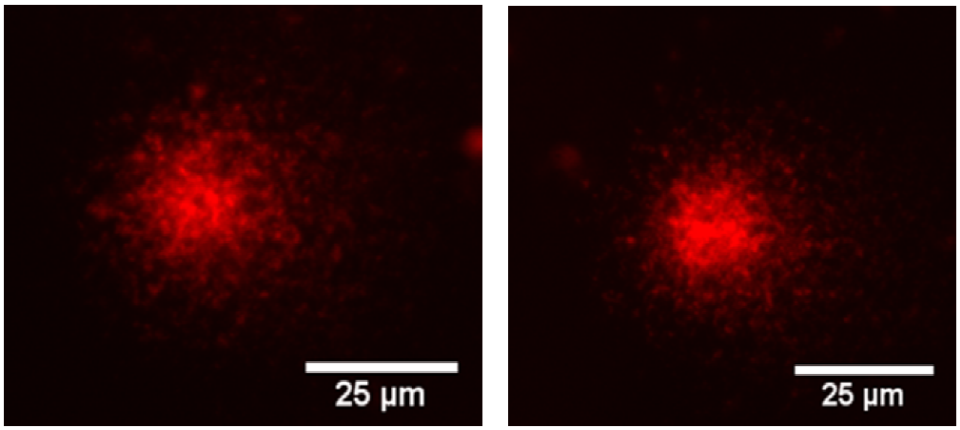
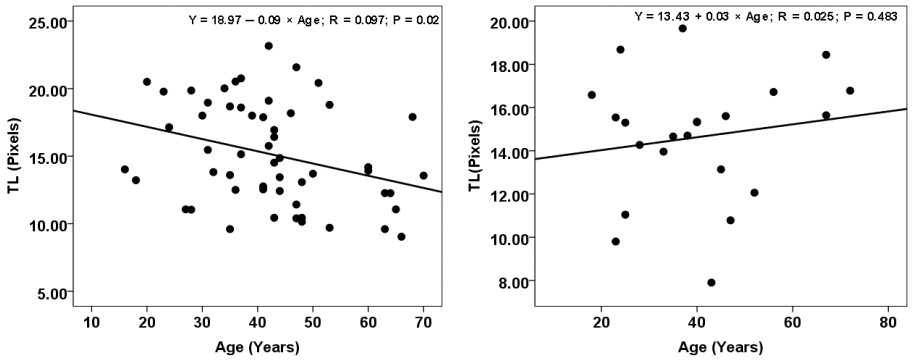


Figure 2 Regression analyses showing the negative correlation between TL and age in Takandeang village inhabitants and positive correlation in control samples

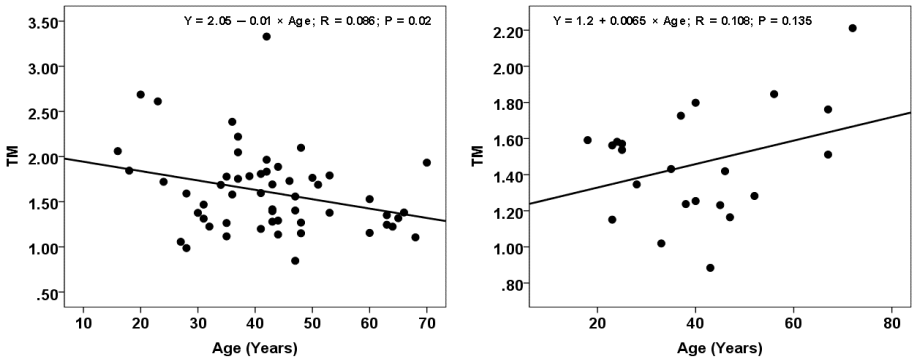


3.2 Tail moment

The TM in Takandeang village inhabitants was 0.85 to 3.31, with mean value 1.6055. In control group the tail length range was 0.88 to 2.21 with mean value 1.4597. Statistical analysis (t-test) showed that the difference was not significant (p = 0.174). When all samples (control and HBRA groups) were classified into three different groups based on age (Group I: < 30 years, Group II: 30-50 years, Group III: > 50 years) and a t-test analysis was applied in each group, the different of all groups were also not significant (p = 0.238; p = 0.052; p = 0.071). A regression analysis in Takandeang inhabitants

showed a significant ($p = 0.02$) negative correlation between TM and age ($y = 2.05–0.01 \times$ Age). In contrast a regression analysis in control group showed a positive correlation between TM and age ($y = 1.2+ 0.0065 \times$ Age), even though the correlation was not significant ($p = 0.135$).

Figure 3 Regression analyses showing the negative correlation between TM and age in Takandeang village inhabitants and positive correlation in control samples



3.3 The correlation between sex and smoking habit on the level of DNA damage in control and Takandeang village inhabitants

Statistical analysis using t-test showed that there were no effects of sex and smoking habit on the level of DNA damage in control and Takandeang village inhabitants ($p = 0.426$; $p = 0.229$). The mean values of TL and TM in females were 14.67 pixels and 1.5062, while in males were 15.3145 pixels and 1.6231. The mean values of TL and TM in smokers were 16.0205 pixels and 1.621680, while in non-smoker they were 14.6258 pixels and 1.5436.

4 Discussion

Here in this study, we evaluated the DNA damage using comet assay in Takandeang village inhabitants. Other assays also can be used to evaluate the DNA damage like chromosome aberrations, micronuclei, and γ-H2AX. Compared with other assays the alkaline comet assay has an advantage because it can be used to detect the DNA damage after very low doses of radiation exposure (Kumar et al., 2012). Our study showed that the DNA damage in Takandeang village inhabitants was higher than in control samples. The mean tail length in Takandeang village inhabitants was 15.1304 pixels. This value was equal to 4.96 μm, since using Set Scale function in ImageJ software we found that 3.05 pixels is equal to 1 μm. A study by Balachandar et al. (2011) in Kanyakumari, Tirunelveli, and Thoothukudi districts inhabitants also showed quite similar results to our study. Using a comet assay they found that the mean TL and TM in the three districts inhabitants were 3.76 μm and 0.27 μm.

A higher incidence of DNA damage in Takandeang village inhabitants compared to control samples found in this study probably correlated with the cumulative exposure of background radiation as stated by Mohammadi et al. (2006), who also found a similar trend that DNA damage in Ramsar (Iran) inhabitants was higher compared to control samples. Mohammadi et al. (2006) also stated that people living in high background radiation areas can be more susceptible to DNA damage and thus possess a more pronounced DNA repair system. This is probably the reason why a significant negative correlation of DNA damage with age in Takandeang inhabitants was found in this study. A possible explanation of this finding was the older people in Takandeang village have a more effective DNA repair system compared to a younger people.

An insignificant difference of DNA damage also found when comparing males and females in all samples. However the DNA damage was in males was higher compared to females. This finding was in a good agreement with another study that found a higher DNA damage in females compared to males (Kopjar et al., 2006). Kopjar et al. (2006) stated that the higher of DNA damage in males was mainly caused by smoking habit. As can be seen in this study that smokers have a higher DNA damage, even though the difference was not statistically significant. Many studies revealed that smoking habit can increased the DNA damage in blood (Betti et al., 1994; Betti et al., 1995; Dhawan et al., 2001; Kopjar et al., 2006; Söylemez et al., 2011). Smoking can induce DNA damage by generating free radicals (Söylemez et al., 2011).

Even though the DNA damage in smokers was higher compared to non-smokers, the difference was not statistically different. Factors that possibly made the DNA damage in smokers not significantly higher included the caffeine intake. It was very common in Indonesia that smokers also have high caffeine intakes. Some studies showed that caffeine intake can decrease DNA damage and maintain DNA integrity (Bichler et al., 2007; Mišík et al., 2010; Bakuradze et al., 2014a; Bakuradze et al., 2014b). Kumar et al. (2001) stated that caffeine can reduce DNA strand breaks induced by radiation and effectively protects the DNA by the scavenging of radiation-derived primary as well as secondary reactive oxygen species. Another study by Devasagayam et al. (1996) also revealed that caffeine has antioxidant abilities against oxidative damage induced by reactive oxygen species. It is possible that the caffeine intake in Takandeang inhabitants reduced the DNA damage induced by natural radiation exposure. A further examination should be conducted to verify this result.

Overall, based on this study, it can be concluded that the DNA damage level in Takandeang inhabitants did not differ from the control group. It is possible that adaptive response might be induced in Takandeang inhabitants. Radio-adaptive response is a biological process induced by low dose ionising irradiation that elicits cellular resistance to the genotoxic effects of subsequent irradiation. Until now the molecular mechanism of radio-adaptive response still remains unknown. Further research using more samples from Takandeang should be conducted to investigate the possible radio-adaptive response in Takandeang inhabitants.

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