Alkaline Comet Assay as a Predictor of DNA Damage in Medical Radiation Workers

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Abstract. The study was aimed to examine the deoxyribonucleic acid (DNA) damage level between medical and non-medical radiation workers using the comet assay method. The radiation-exposed group (29 subjects) and the control group (29 subjects) were chosen from the administration staff. The assessment of single-strand DNA damage hosted by peripheral blood lymphocye was performed using the alkaline comet assay, the tail length and long-tailed DNA were measured to detect the DNA damage. An independent sample t-test was used to assess the statistical difference between groups. The results showed that comet tail length mean measured in the exposed worker's group was 25.57±4.4 µm whereas the mean percentage of long-tailed DNA was 6.97±1.53. In the control group, the mean tail length was 23.15±7.57 µm and the long-tailed nucleus percentage was 5.65±1.99. Mean values for the tail length measured were no significantly higher in the exposed group compared to the control group (P=0.15), whereas the percentage of long-tailed nuclei was significantly higher in the exposed group compared to the control group (P=0.007) . The observed single-strand DNA can be measured by using the alkaline comet assay. The DNA damage can be influenced by radiation exposure in medical radiation workers.

1. Introduction
There has been a lot of ionizing radiation used in medicine (radiotherapy and radiodiagnosis), industries, and research for more than a century after the discovery of X-rays by Roentgen in 1895 [1]. The safety side always takes precedence in the use and application of radiation, especially gamma radiation exposure. The gamma rays can penetrate into the nucleus and head to deoxribonucleic acid (DNA) found in the nucleus. This will cause ionization of the nucleotide and sugar bases, so cause the DNA damage [2]. Radiotherapy is known to play a role in treating several cancers such as prostate cancer, lung cancer, bone cancer, skin cancer, bladder cancer and breast cancer [3-8]. Radiotherapy has a detrimental effect on patients as well as radiation workers. The levels of responses to radiation-induced DNA damage depend on many factors, such as lifestyle, genetic predisposition, inflammatory responses, age, oxidative stress, and gene variants [9]. Therefore, it is important to analyze the adverse effect of radiation on DNA damage based on comet assay measurement.

Ionizing radiation causes DNA double-strand break directly, but apart from that base damage due to indirect effects is also induced. This radiation causes the formation of reactive oxygen species (ROS) which are indirectly involved in DNA damage. This ROS produces apurinic/apyrimidinic (abasic) sites in DNA [10,11]. When DNA is damaged, the cell repair machine is activated and stops the cell cycle at special control checkpoints to repair DNA damage and prevent cycle continuation. It is known that the intrinsic radiosensitivity of tumor cells is strongly influenced by the ability of DSB cell repair [12].
tumor cells are able to repair radiation damage efficiently, resistance to radiation develops, allowing cells to survive and replicate. If the damage is still not repaired, this mechanism induces cell death or programmed apoptosis to prevent the accumulation of mutations in daughter cells [13,14]. Genetic variants are one of the factors that influence the emergence of cancer and play an important role in repairing DNA damage due to radiation exposure. Several previous studies have shown that genetic polymorphisms of XRCC1 exon 6 and 10 had a significant correlation to the frequency of micronuclei among radiation workers and various cancers patients [15-17].

The alkaline comet assay is a very fast, flexible and sensitive assay that can be adapted for various exposure conditions and applies to almost all types of tissue or cells. This makes it the ideal choice for safety testing of new or previously tested products that require fast results, special exposure conditions, and/or assessment of target organs that are not compatible with other in vivo tests. The alkaline version can detect direct effects of DNA damage from radical-forming chemicals, alkylating agents, stirring chemicals, various metals, and UV or ionizing radiation. It can also detect DNA strand damage that is indirectly caused by postinjury processes such as repairing excision and inflammation [18,19]. The purpose of this study was to analyze the DNA damage among radiation workers using alkaline comet assay.

2. Materials and Methods

2.1 Blood sampling
This research was a case-control study. Participants consisted of 29 radiation workers in the Radiology and Radiotherapy installation at Ulin General Hospitals, Banjarmasin, South Kalimantan, Indonesia as a case group and 29 non-radiation workers as a control group who had never been occupationally exposed to ionizing radiation. Questionnaires were given to the participants to find out complete information about gender, age, and history of the disease ever suffered. Each of the participants was briefed about the protocol, with specific information about the comet assay, the aim of the study and signed informed consent. Blood samples (10 mL) were obtained from participants (case and controls) and taken to the Molecular Radiobiology laboratory, Center for Technology of Safety Radiation and Metrology, National Nuclear Energy Agency of Indonesia, Jakarta for further analysis.

2.2 Comet assay
DNA damage evaluation was carried out by the Comet assay technique under suitable alkaline conditions by the Singh method [20]. A total of 10 µl lymphocyte samples were added with 70 µl of low melting point agarose (LMP) from Sigma. A total of 70 µl of the sample mixture dripped on glass preparations that have been coated with normal melting agarose (Sigma). The preparations are soaked for 1 hour at 4°C in a lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, adjusted to pH 10 with NaOH (Sigma) and added 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Sigma). After cell lysis, glass preparations are placed in a position horizontal in an electrophoresis tank filled with electrophoresis buffer (300 mM NaOH / 1 mM EDTA, pH 13) and allowed to stand for 20 minutes. Then the process is carried out electrophoresis at 25 V, 300 mA for 20 minutes. After electrophoresis, the sample neutralized in a neutral solution (PBS, pH 7.4, 3 times, 5 minutes). Samples were fixed with methanol later stained with ethidium bromide (2 g/mL). Procedures of isolation and Comet assay were performed in dark conditions.

2.3 Data analysis
Samples that have been colored were observed using a fluorescence microscope (Nikon). Comet tail length and long-tailed DNA were observed from 50 cells of each treatment using a digital imaging system. Stacked cells are not counted. The Comet image is analyzed digitally with the use CASPLab comet assay software [21]. The statistical analysis of the data was conducted with SPSS version 16 for Windows. All of the data were expressed as mean ± SD. An independent sample T-test was used to test
Comet tail length and long-tailed DNA difference between cases and controls and to test a significant relationship between them. The level of significance was set at P <0.05.

3. Results and Discussion
Peripheral blood cell lymphocytes are known as very sensitive to radiation. The lymphocyte cell radiosensitivity is very affected by the results of DNA damage analysis due to radiation with the comet test. The fragment of damaged-DNA will be released and then migrate to the positive charge pole during the electrophoresis and formed "Comet" [22]. The epifluorescence microscopy visualization of DNA Damage is presented in figure 1, whereas the results of the alkaline comet assay parameters of Comet tail length and long-tailed DNA for each participant of the cases and controls group are presented in figure 2.

![Figure 1. The epifluorescence microscopy visualization of DNA damage.](image)

![Figure 2. (a) Comet tail length mean measured in the exposed workers vs controls group (25.57 vs 23.15, P-value 0.15) (b) Long-tailed DNA mean measured in the exposed workers vs controls group (6.97 vs 5.65, P-value 0.007)](image)

In the present study, the deoxyribonucleic acid (DNA) damage level between medical radiation workers and non-medical radiation workers was investigated. The DNA damage, for medical radiation workers from several hospitals in Indonesia, was performed using the alkaline comet assay method. This study was used totally 58 participants which consisted of 29 radiation workers and 29 controls. The strength and novelty of this study is the investigation of DNA damage as a risk of radiation exposure in radiation workers, linked to the comet tail length and long-tailed DNA as a biological marker of the DNA damage. To our best knowledge, this study has not been done in Indonesia. The results showed that comet
tail length mean measured in the exposed worker's group was 25.57±4.4 µm whereas the mean percentage of long-tailed DNA was 6.97±1.53. In the control group, the mean tail length was 23.15±7.57 µm and the long-tailed nucleus percentage was 5.65±1.99. Mean values for the tail length measured were no significantly higher in the exposed group compared to the control group (P=0.15), whereas the percentage of long-tailed nuclei was significantly higher in the exposed group compared to the control group (P=0.007).

The DNA damage caused by exposure radiation is indicated by an indicator of comet tail length and long-tailed DNA parameters (Figure 2A and 2B, respectively). The benefit of the comet assay is its ability to analyze DNA damage of cells. The accuracy of the assessment depends greatly on comet image data analysis. Comet tail length optimizes the use of comet images and generating accurate quantifications of DNA damage. Long-tailed DNA allows more accurate DNA damage quantification by averaging the intensity of each column of pixels and multiplying it by the distance from the center of the head to that column, and then dividing by total intensity. Alternatively, tail moment calculates the product of a single intensity ratio and the single distance from the center of mass of the tail to the center of mass of the head [23]. Tail length increases due to the DNA damage and other reasons increasing the migration of intact DNA loops. Direct evidence that the increase in comets measured by a neutral comet test may not be treated with DNA damage is provided by different research groups [24,25]. In this study, the increase or decrease in neutral comets was caused by decondensation or condensation, respectively, of intact DNA loops. The increase in tails measured by an alkaline comet test can be caused by an increase in the number of open replicas or transcription units due to the inhibition of their extension but not by DNA damage. Because of the control tail's dependence on cell types (supercoiling level and the DNA loop length, transcription and replication rates), longer or shorter tails can be observed in different cell types.

The rapid and simply comet test techniques are proven can be used for various purposes, such as determine the mutagenicity of environmental agents including ionizing radiation, double-strand break (DSB) DNA, crosslink, apoptosis, and bases damage. Radiation genotoxicity in radiotherapy can also be analyzed with the comet assay techniques, with increasing DSB frequencies of DNA will show increasing speed DNA migration towards the anode in an electrophoresis system [26]. DNA molecules contain electrically charged phosphate groups negative when in an alkaline solution, so the region in the DNA double-strand is experiencing relaxation and containing DSB will migrate towards positive pole (anode) when electrophoresis was carried out. The migration will form a comet tail, while the area is not experiencing sagging will form comet head [27].

Several previous studies stated that radiation exposure could affect to the human tissue, especially on DNA and hematopoietic cells. The development of hematopoietic cells can be disturbed by continuous radiation exposure. Surniyantoro et al. (2019) declare that there is a significant correlation between equivalent dose and red blood cells (RBCs) parameter with a decline in RBCs level of 0.541× 10 6/µL per 1 mSv of radiation dose [28]. Furthermore, the gamma-ray can inhibit Plasmodium multiplication in vitro culture [29], and bring up new mutant variants of the Nix gene on gamma-irradiated Aedes aegypti as a male determination factor [30]. Studies on the relationship of DNA damage and genetic factors are very important, because several diseases are influenced by genetic factors, such as UCP2 gene polymorphism as a risk factor in people with type 2 diabetes [31]. In further studies, it needs to be done examining the DNA repair gene polymorphisms in populations with controlled non-genetic factors, such as lifestyle, environment, and exercise that affect MN frequency as a biomarker of DNA damage.

4. Conclusion
In conclusion, our study reports that radiation-exposed workers have a value for the tail length measured was no significantly higher in the exposed group compared to the control group (P=0.15). Whereas the percentage of long-tailed nuclei was significantly higher in the exposed group compared to the control group (P=0.007). The observed single-strand DNA can be measured by using the alkaline comet assay and the DNA damage can be influenced by radiation exposure in the medical radiation workers.

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References

[1] Timmins JK 2011 Health Phys. 101 562-5
[2] Zhang Y, Guo J, Qi Y, Shao Q and Liang J 2014 J. Radiat. Res. Appl. Sci. 7 274-85
[3] Gay HA and Michalski JM 2018 Mo. Med. 115 146-50
[4] Parashar B, Arora S and Wernicke AG 2013 Semin. Intervent. Radiol. 30 185-90
[5] Felice FD, Piccioli A, Musio D and Tombolini V 2017 Oncotarget 8 25691-9
[6] Manyam BV, Joshi N and Koyfman SA 2017 Appl. Rad. Oncol. 6 6-10
[7] Zhang S, Yu YH, Zhang Y, Qu W and Li J 2015 Am. J. Cancer Res. 5 854-68
[8] Soto GB, Lopez RO and Martinez AR 2015 Genet. Mol. Biol. 38 420-32
[9] Hornhardt S, Robler U, Sauter W et al 2014 DNA Repair 16 54-65.
[10] Redon CE, Nakamura AJ, Zhang YW et al 2010 Clin. Cancer Res. 16 4532-42
[11] Aparicio T, Baer R and Gautier J 2014 DNA Repair 19 169-75
[12] Mladenov E, Magin S, Soni A and Iliakis G 2013 Front. Oncol. 3 1-18
[13] Deckbar D, Jeggo PA and Lobrich M 2011 Crit. Rev. Biochem. Mol. Biol. 46 271-83
[14] Guo GS and Zhang 2011 Int. J. Oral. Sci. 3 176-9
[15] Surniyantoro HNE, Lusiyanti Y, Rahardjo T et al 2018 Atom Indonesia 44 105-11
[16] Surniyantoro HNE, Lusiyanti Y, Rahardjo T et al 2018 Biodiversitas 19 1676-82
[17] Surniyantoro HNE, Rahajeng N, Lusiyanti Y et al 2019 Biodiversitas 20 2128-33
[18] Reus AA, Reisinger K, Downs TR, Carr GJ et al 2013 Mutagenesis 28 709-20
[19] Vasquez MZ 2010 Mutagenesis 25 187-99
[20] Dusinska M and Collins AR 2008 Mutagenesis 23 191-205
[21] Gonzalez JE, Romero I, Barquinero JF and Garcia O 2012 Mutat. Res./Genet. Toxicol. Environ. Mutag. 748 60-64
[22] Collins AR and Azqueta A 2012 Mutat. Res. 736 122-9
[23] Bowden RD, Buckwalter MR, McBride JF et al 2003 Mutat. Res. 537 1-9
[24] Belyaev IY, Eriksson S, Nygren J et al. Biochim. Biophys. Acta. 1428 348-56
[25] Afanasieva K, Zazhytska M and Sivolob A 2010 Electrophoresis 31 512-9
[26] Irina C and Tatiana S 2016 Sovremennye Tehnologii Medicine 8 20-7
[27] Miklos M, Gajski G and Garaj-Vrhovac V 2009 Radiol. Oncol. 43 97-107
[28] Surniyantoro HNE, Rahardjo T, Lusiyanti Y et al 2019 Atom Indonesia 45 123-9
[29] Surniyantoro HNE, Darlina, Nurhayati S et al 2016 Nus. Biosci. 8 8-13
[30] Ernawan B, Surniyantoro HNE, Sugoro I and Tambunan USF 2019 Biodiversitas 20 893-9
[31] Surniyantoro HNE, Sadewa AH and Hastuti P 2018 Kobe J. Med. Sci. 64 64-72