THE USE OF MICRONUCLEUS ASSAY ON SWISS-WEBSTER MICE (*Mus musculus*) BONE MARROW FOR THE MUTAGENICITY TEST OF γ-IRRADIATION

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ABSTRACT

THE USE OF MICRONUCLEUS ASSAY ON SWISS-WEBSTER MICE (*Mus musculus*) BONE MARROW FOR THE MUTAGENICITY TEST OF γ-IRRADIATION. Ionizing radiation is a potentially chromosomal damaging agent. The induction of chromosomal damage as well as the incidence of cell cycle disturbances may depend on the dose of irradiation. One of the indication of chromosomal damage is the formation of micronucleus (MN) during the anaphase of mitosis. This study deals with the MN assay on femur bone marrow polychromatic erythrocyte (PCE) cells of Swiss-Webster mice, for the mutagenicity test of γ-irradiation. The study was conducted on five groups of mice (each group consist of five mice) that were irradiated at the doses of 0; 0,2; 0,4; 0,6 and 0,8 Gy respectively. One day after irradiation, the mice were killed by cervical dislocation. Furthermore the femur bone marrow was taken, the cells were then prepared by smear technique onto slides followed by Giemsa staining. The MN in PCE cells or MNPCE were examined microscopically by the magnification of 1000 and counted for every 1000 cells in each mice. The results showed that the MNPCE frequencies on the treatment groups were significantly higher than that of the control ($P < 0,05$). Further evaluation indicated that the MNPCE frequencies increased with the increase of irradiation dose.

Key words: micronucleus, bone marrow, polychromatic erythrocyte, γ-irradiation, Swiss-Webster mice.

INTRODUCTION

The basic indicator of ionizing radiation effects at the subcellular level is DNA damage in molecular structure which may caused either by direct or by indirect exposure [1]. Therefore the common end-points is chromosomal damage or chromosomal aberration, that might lead to mutation and give rise to cancer. One of the methods for chromosomal damage determination is micronucleus (MN) assay. The MN is formed during anaphase transition, originated from chromosome fragments that are not included in the main daughter nucleus during mitosis or cell division [2]. During mitosis, the genetic material replicates and then divided equally between the two daughter cells. If the process is disrupted, or the chromosomes are broken or damaged by chemicals or radiation, then the distribution of genetic material between the two daughter nuclei during cell division may be affected and pieces or entire chromosome may fail to be included in either of two daughter nuclei. When this situation occurs, the genetic material that is not incorporated into a new nucleus may form its own

"micronucleus" with the size of about 1/20 – 1/6 in diameter of the own cell, which is clearly visible under microscope examination. Firstly the MN assay is widely used for evaluating the potential of chemicals that induced chromosomal aberration [3,4]. This mutagenicity test is also used by US Environmental Protection Agency (US.EPA) as a reference method to evaluate the toxicity or genotoxicity of xenobiotic [5].

A study on mutagenic effect of *in vivo* irradiation on animal trial by using a simple method needs to be done, to figure out such effect on human. The simple method is MN assay on polychromatic erythrocyte (PCE) cells of mice bone marrow. Erythrocytes arise from stem cells in the bone marrow and are produced by a series of division in a precursor cell population. The constant, rapid turnover of precursor cells makes erythrocytes an ideal cell type for a MN test. Another unique feature of the erythrocyte is that immediately after formation of the fully differentiated erythrocyte, the nucleus is pushed out of the cell: erythrocytes are the only mammalian cell type that does not contain a nucleus, and therefore, the differentiated erythrocyte cannot further be divided. Thus the bone marrow stem cells are continuously producing new erythrocyte to replace the one that eventually die, and hence in the bone marrow remain two types of erythrocyte the immature one or newly form erythrocyte called as polychromatic erythrocyte (PCE) and the mature one called as normochromatic erythrocyte (NCE). If the stem cell is damage by irradiation and a MN is formed as a consequence of this damage, the MN remains in the cell after the main nucleus has been pushed out and is very easy to observe microscopically [6]. This article reported the use of MN assay of femur bone marrow PCE cells of irradiated and non-irradiated Swiss-Webster mice, to assess the mutagenic effect of gamma irradiation.

MATERIALS AND METHODS

Materials

The chemicals used in this study were pro analyzed grade purchased from E. Merck, consist of methanol, ethanol and Giemsa dye-solution; while physiological solution (NaCl 0,9%, pyrogen free) was obtained from IPHA Laboratories.

Twenty five male Swiss-Webster mice (*Mus musculus*) three months old with the body weight of about 25 – 30 grams were used throughout the experiment. The mice were divided into five groups (each group consist of five mice).

Irradiation Facility

Radiation treatment was done using a ^{60}Co gamma source with the activity of 20.46 mCi, available at Nuclear Technology Center for Material and Radiometry-BATAN, Bandung.

Methods

Irradiation. The four treatment groups of mice were irradiated with the doses of 0.2; 0.4; 0.6; 0.8 Gy respectively using a ^{60}Co gamma source at a dose rate of 0.3 Gy/h. The treatment groups of mice were prepared for irradiation as follows: One group (five mice) were taken, each of them was then put in a special cage. The cages were arranged in the irradiation facility in a circle position in such a way in order the final distance of each cage/mice to the source was about 2 cm. According to the dose rate calculation, in this position the dose rate would be 0.3 Gy/h. Thus the irradiation time for the doses of 0.2; 0.4; 0.6; and 0.8 Gy were 40, 80, 120, and 160 minutes respectively.

Femur bone marrow preparation. One day (24 hours) after irradiation the mice were killed by cervical dislocation. The both femurs (left and right) were taken, then the bone marrow was flushed from the femur using pasteur pipette filled by 1,0 mL physiological solution. The obtained bone marrow solution was then centrifuged at 1,000 rpm during 5 minutes. The supernatant was discarded, and the precipitate was taken for cells' examination.

Slides preparation and staining. The cells were spread onto slides, air dried, fixed and stained with Giemsa-methanol as follows: the slides were immersed in absolute methanol for 10 minutes, then immersed again in 20% (v/v) Giemsa solution for 30 minutes. Finally the slides were washed with tap water, and dried at room temperature for microscopic examination.

Micronucleus examination. The MN in PCE cells or MNPCE on the slides were examined microscopically by the magnification of 1,000, according to Schmid [7] method. The frequency of MNPCE were counted for every 1,000 PCE cells in each mice.

RESULTS AND DISCUSSION

The MN is formed during the anaphase transition of mitosis. It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event), which do not integrate in the daughter nuclei during cell division, as illustrated by Fenech, M. et al. [9] in Figure 1.

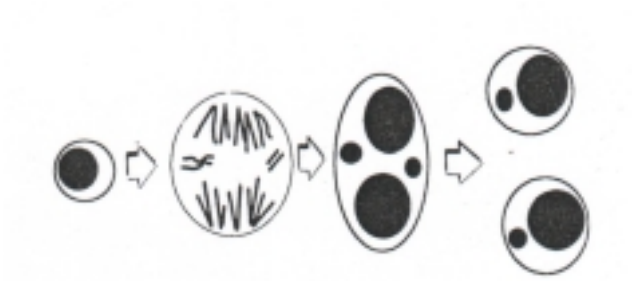

Figure 1. The illustration of MN formation from lagging whole chromosome and acentric chromosome fragments in dividing cell at anaphase.

Based on the cell cycle and maturation times of the erythrocyte, harvesting of the bone marrow usually occurs 24 hours after the final treatment. At that time, about 50% of the erythrocytes in the bone marrow are immature appear as polychromatic erythrocyte (PCE) and that is the cell type that was checked for the formation of MN. Therefore, in this study the mice were killed 24 hours after irradiation, for further preparation.

In our preliminary experiment [8], the bone marrow was isolated from non-irradiated mice, followed by slides preparation, using the method mentioned above. After Giemsa staining, the slides were examined under a microscope by the magnifincation of 1000. The PCE cells appear as round shape cell blue-purple in colour, while the colour of mature erythrocytes or normochromatic erythrocytes (NCE) are pale purple, as illustrated in Figure 2.

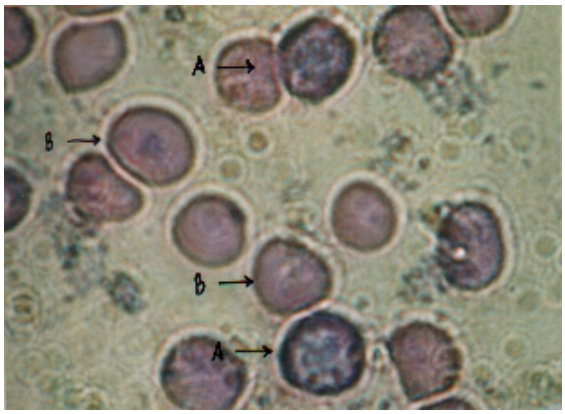

Figure 2. Microscopic examination by magnification of 1000 of A: polychromatic erythrocyte (PCE) and B: normochromatic erythrocyte (NCE), isolated from Swiss-Webster mice femur bone marrow, and stained using 20% (v/v) Giemsa dye solution.

In the attempt for getting better illustration of the PCE and the MN, in all experiments slides were immersed again in 30%, 50%, 70%, 80% and absolute ethanol during 10 minutes respectively. At the end of each immersing process, the slides were washed with tap water. Finally the slides were immersed in xylol for fixation during 10 minutes, then wash again with tap water, and let them dry at room temperature. The MN in PCE or MNPCE was simple to be detected as round to ellipsoidal spot, dark colour with the size of about 1/20 – 1/6 in diameter compared to the erythrocyte cell, as presented in Figure 3.

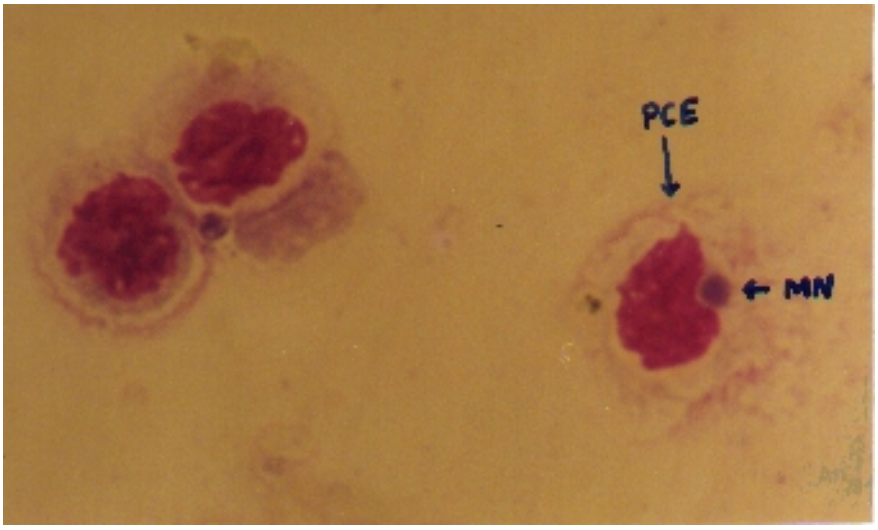

Figure 3. Microscopic examination of NMPCE by the magnification of 1,000 MN is presented as round to ellipsoidal spot.

Based on the microscopic examination and MNPCE counting for every 1000 PCE cells in each mice, the data were summarized in Table 1. Statistical analyses indicated that $F_{calculation} = 30.64$ higher than $F_{table}(0,05) = 2.87$. It revealed irradiation affected the formation of MN significantly, the MNPCE frequencies in the treatment groups were significantly higher than that of the control. Further evaluation (see Table1) indicated that the differences in MNPCE frequencies occured as the results of irradiation with the dosis of above 0.4 Gy, while irradiation at a dose of 0.2 Gy gave no significant effect. As shown from the notation (resulted by Duncan test at $P < 0.05$), also presented in Table 1, it was evident that irradiation effects with the doses between 0.2 and 0.4 Gy as well as between 0.4 and 0.6Gy were not significantly different. The significant differences on irradiation effects occurred using the doses between 0.2 and 0.6 Gy; 0.4 and 0.8; 0.6 and 0.8 Gy respectively. It is also important to note that in non-irradiated mice the frequency of MN was not originally zero, but found to be 2.2 ± 0.45. It means

that the formation of MN may occur spontaneously in nature, that was also in accordance to the findings of Fenech, M., et al. [9] and Hadiansyah, C [10].

The correlation of irradiation dose and MNPCE frequency are shown in Figure 4. Using regression and correlation analyses, the dose-respons relationship equation was obtained as follows: $Y_{micronucleus} = 1.64 + 6.7\ X$. Meanwhile, correlation coefficient of $r = 0.846$, indicated that there was strong correlation between irradiation dose and MNPCE frequencies. It was clear that the MNPCE frequencies increased with the increase of irradiation dose.

This method supposed to be worthwhile for cytogenetic risk assessment of chemicals or other xenobiotic as compared to irradiation. Such comparison studies will lead to more understanding whether radiation do not always more harmful than chemicals. Unfortunatelly MN assay on bone marrow is difficult to adopt for human. Nowadays, the lymphocyte cell is one of the cell types that has been choosen for measuring DNA damage in human by MN assay. However, it is also impossible to carried out *in vivo* irradiation experiment to human, even though one of the final objective of MN assay may be addressed for predicting the dose of irradiation accepted for instant by operator in nuclear accident. Apparently, an approach utilizing animal trial such as mice is still needed. This study worth to be continued to get more understanding about the actual different effects between *in vivo* and *in vitro* irradiation, to make further extrapolation using the same approach to human lymphocyte cells.

CONCLUSION

Based on the obtained data, it could be concluded that:

1. MN assay on Swiss-Webster mice bone marrow for the examination of chromosomal damage caused by γ - irradiation, was very simple and quick method. This method did not require any special reagent or expensive chemicals, thus relatively cheap as well.
2. MN assay on Swiss-Webster mice bone marrow found to be usefull for the mutagenicity test of γ -irradiation with the doses of above 0.4 Gy. In general this method could be used as biological marker in risk assessment of γ - irradiation.
3. There was very distinct correlation between irradiation dose and MNPCE frequencies, the MNPCE increased with the increase of irradiation dose.

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Table 1. MNPCE frequencies of non-irradiated and irradiated Swiss-Webster mice bone marrow. The doses of irradiation were 0.2; 0.4; 0.6 and 0.8 Gy. The frequencies of MNPCE was counted for every1000 PCE cells in each mice. The mean of MNPCE frequencies were obtained from 5 mice. The notation of a, b, c and d was obtained using Duncan test at $P < 0.05$, which indicated that the effect of irradiation at the doses with the same notation was not significantly different one to another.

No	Doses (Gy)	MNPCE Frequencies (mean and deviation standard)	Notation (Duncan test P<0,05)
1	0	2.2 ± 0.45	a
2	0.2	3.1 ± 0.87	ab
3	0.4	3.8 ± 0.45	bc
4	0.6	4.4 ± 1.14	c
5	0.8	8.2 ± 1.48	d

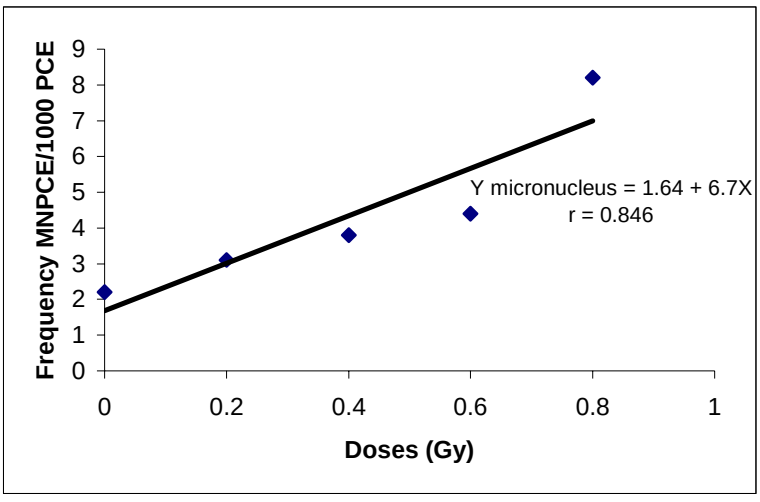

Figure 4. Linear regression of MNPCE frequencies upon γ - irradiation with the doses of 0; 0.2; 0.4; 0.6 and 0.8 Gy.