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Gamma-ray irradiation inhibits *Plasmodium falciparum* multiplication in *in vitro* culture supplemented with tritium-labeled hypoxanthine

HARRY NUGROHO EKO SURNIYANTORO, DARLINA, SITI NURHAYATI, DEVITA TETRIANA, MUKH SYAIFUDIN♥

Nuclear Medicine Technique and Radiation Biology Division, Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency. Lebakbulus, Cilandak, Jakarta 12440, Indonesia. Tel.: +62-21-7513906, Fax.: +62-21-7657950, ♥email: mukh_syaifudin@batan.go.id.

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Abstract. *Surniyantoro HNE, Darlina, Nurhayati S, Tetriana D, Syaifudin M. 2016. Gamma-ray irradiation inhibits* Plasmodium falciparum *multiplication in* in vitro *culture supplemented with tritium-labeled hypoxanthine. Nusantara Bioscience 8: 8-13.* Malaria remains a major public health threat in the world. Therefore an attempt to create malaria vaccine for supporting the control of disease was taken by attenuating parasites with gamma rays and it was proven effective based on microscopic observation. Objective of this research was to assess the effectiveness of gamma rays to attenuate malaria parasites based on isotopic method. A laboratory strain of *P. falciparum* (3D7) was in vitro cultured with standard procedure and it was irradiated with gamma rays at doses of 150-250 Gy and unirradiated parasites served as control. Twenty four hours after 1-2 µCi of ^{3}H-hypoxanthine was added into culture 100 µl of medium was taken and was repeated at various times, then hypoxanthine incorporation was measured with beta counter. Microscopic observation of parasitemia in culture was also done. The results showed that there was a fluctuation in multiplication of parasites post-irradiation mainly in higher dose (more than150 Gy). Irradiated of parasites were more active in incorporate with purine precursor up to 48 hours. Parasites returned to their highest activity at 116 hours after hypoxanthine addition. No significant difference was found among doses of irradiation with p of 0.05. This was quite different from the finding from microscopic observation. It was known that dose of 150 Gy was the most effective dose for inhibiting of the parasite multiplication where some factors affecting these facts.

Keywords: malaria, *P. falciparum*, vaccine, gamma rays, microscopic, ^{3}H-hypoxanthine
Abbreviations: DNA: deoxyribonucleic acid; LSC: liquid scintillation counter; PBS: phosphate-buffered saline; RBCs: red blood cells; pLDH: parasite lactate dehydrogenase; HRP2: histidine-rich protein 2.

INTRODUCTION

Malaria remains a major public health threat in the developing world, with most deaths attributed to *P. falciparum* infection (Snow et al. 2005; Greenwood et al. 2008). Despite several efforts by local and national authorities have been done to control malaria incidence, it remains one of the major diseases causing death in Indonesia (Asih et al. 2009). Several factors may contribute to this situation, such as uncontrolled use of anti-malarial drugs, the spread of anti-malarial drug resistance, and inadequate vector control measures (Syafruddin et al. 2006). Therefore, we were attempted to create malaria vaccine for supporting the government program in controlling the disease by attenuating parasites with gamma rays as vaccine materials candidate by emphasizing on the determination of optimal dose and dose rate of irradiation (Syaifudin et al. 2011, 2013).

Efforts to develop effective vaccines have been done for more than six decades, being the irradiated sporozoite vaccine as the most excellent model (Hill 2011; Oakley et al. 2013). These attenuated sporozoites can invade the liver cells but undergo arrested development and it expresses antigens that can induce a protective immune response (Roestenberg et al. 2013). The most advanced recombinant vaccine such as RTS-S, which is made from the circumsporozoite protein, conferred significant but limited protection against infection in young children and infants (Aponte et al. 2007; Hoffman et al. 2010; Epstein et al. 2011).

The detection of parasites by light microscopy of Giemsa-stained thick and thin films remains the standard laboratory method for the diagnosis of malaria (Moody 2000; Coleman et al. 2002). Microscopy can detect up to 0.001% parasitemia and identify the plasmodia in 98% of the cases (Murray et al. 2008). However, this technique requires highly skilled personnel and its information is limited when parasite levels are very low or when parasite morphology is altered (Oyedeji et al. 2007; Prescott et al. 2012). The procedure is also not simple and time-consuming, requiring large amount of samples to be stained quickly to maintain its reliability (Bates et al. 2004). Therefore a sensitive assay to monitor *P. falciparum* inhibition based on ^{3}H-hypoxanthine, a nucleic acid precursor, incorporation into DNA had been developed (Raabe et al. 2009; Sanz et al. 2012) that appears suitable in the reproducible determination of irradiation dose of gamma rays in inactivating parasites. Moreover, the isotopic tests are more informative than the microscopy tests, due to its capability in reflecting the arrest of metabolic activity of the parasites before they disappear (Wein et al. 2010). The objective of the research is to assess the effectiveness of gamma rays in inhibiting the

development of *P. falciparum* based on the incorporation of ^{3}H-hypoxanthine.

MATERIALS AND METHODS

Parasites. A laboratory strain of *P. falciparum* (3D7) in the form of infected blood was obtained from Malaria Group, Eijkman Institute for Molecular Biology in Jakarta and continuously in vitro cultured with standard procedure.

Preparation of culture medium. RPMI-1640 medium (Sigma, St Louis, MO) was complemented with 2 g/L $NaHCO_3$, 5.958 g/L HEPES, 1 ml/L gentamicin, 13.6 mg/L hypoxanthine and 4.5 g/L D-glucose in deionizing tissue grade water. Culture medium was sterilized by filtering through a 0.45 µm pore filter unit (Nalgene, NY) and kept at 4ºC. Culture medium was further supplemented with Albumax II (Gibco, New Zealand) on a daily basis. For this, a 5% (m/v) stock solution was prepared from 10 g of Albumax II powder in 200 mL of RPMI 1640, stirred at 37ºC until dissolved completely, sterilized, and stored at 4ºC until further use. The parasitemia and stage distribution of the parasites were assessed microscopically on a thin blood film stained with Giemsa's solution. Parasitemia was expressed as the number of erythrocytes containing asexual stages of *P. falciparum* per 1,000 erythrocytes under oil emersion at a 1,000x magnification.

Red blood cell preparation. O blood of human type was collected in acid citrate dextrose. Buffy coat and plasma were removed after centrifugation at 2,500 rpm for 5 minutes. Packed cells were washed three times in RPMI-1640, centrifuged at 2,000 rpm for 5 minutes and resuspended at a 50% hematocrit in culture medium. The erythrocyte suspension was washed daily with 50 ml freshly prepared complete culture medium. The rest of the culture medium was replaced with 200 ml of freshly medium once or twice a day depending on the total number of parasites (density).

Plasmodium falciparum cultures. Parasites, obtained from continuous stock cultures, were subcultured in plates. *P. falciparum* 3D7 strain parasites were maintained in vitro in complete medium (RPMI 1640 medium [Sigma-Aldrich] supplemented with 2 mM L-glutamine, 10 µg/ml hypoxanthine, 20 mM HEPES (pH 7.4), 25 mM $NaHCO_3$, 20 mM glucose, 1 mg/L streptomycin penicillin, and 0.25% of Albumax II [Invitrogen, Carlsbad, CA]) at 4% hematocrit of O^{+}human RBCs (Interstate Blood Bank, Inc., Memphis, TN). Parasites were tightly synchronized by a series of treatments with 5% D-sorbitol (Sigma-Aldrich) and parallel cultures were harvested at 2, 8, 14, 20, 26, 32, 40 and 48 h of the asexual cycle. RBCs were lysed by treatment with 0.15% of saponin in PBS for 10 min, and intact parasites were pelleted by centrifugation then washed and stored at -80°C until it was used.

In vitro *growth inhibition assay*. Triplicate in vitro cultures of asynchronous parasites at 0.5-1.1% initial parasitemia were used. Culture of *P. falciparum* in an appropriate-sterile container was irradiated with gamma rays from ^{60}Co source of Irradiator Facilities in The Center for Application of Isotope and Radiation, National Nuclear Energy Agency (BATAN) at doses of 150, 175, 200, 225 and 250 Gy (dose rate of 380 Gy/h). Unirradiated (0 Gy) parasites served as control. After 1-2 µCi of ^{3}H-hypoxanthine (Amersham Biosciences, France) was added to each medium, 100 µL of culture medium was taken at 3, 6, 24, 30, 48, 72, 96, 102, 120, 144, 168 and 192 hours and was put in 12 mL LSC bottle containing 2 mL deionized water. After added with 10 mL of cocktail solution (LLT Ultima Gold™, PE) the bottle was counted in LSC (1220 Ultralow Level, Perkin Elmer) for 1 h to determine the activity of tritium that demonstrating hypoxanthine incorporation. Standard and blank solutions were included in every serial counting. The net incorporation of radioactivity was obtained after subtraction of the non-specific incorporation measured in uninfected erythrocytes and expressed as a percentage of the incorporation by the positive controls. The culture was also taken for microscopic observation by making Giemsa stained thin blood smear.

Statistical analysis. The statistical significance of the differences in the irradiation doses and time of culture taking was determined by two-tailed and unpaired Student's *t*-test with probability (*P*) values of 0.05 considered significant.

RESULTS AND DISCUSSION

In this research, we evaluate the growth of *P. falciparum* in vitro culture after irradiation, where ^{3}H-hypoxanthine was added to parasite cultures and radioisotope incorporation was measured with a counter. When culture parameters were carefully controlled ^{3}H-hypoxanthine incorporation was proportional to the number of parasitized erythrocytes present.

Our experiment showed that there was a slight increase in the number of parasites in incorporating hypoxanthine until 20 hours post inoculation of ^{3}H-hypoxanthine that was seen by a reduction of tritium activities in medium of culture for all doses of irradiation. This finding implies that exposure to ionizing radiation has no highly profound effect on the viability and further replication of parasite cells in earlier time of culture. After 20 hours, there was a very high reduction of hypoxanthine incorporation by parasites except for dose of 150 Gy suggesting that this is the most effective dose in suppressing the growth of parasites. It was supported by the lowest activity of tritium in the medium which was in opposition to the culture or parasites number (Figure 1). This case is supported by the low and no parasitemia seen in microscopic observation. However, statistical analysis showed that there is no significant difference between doses of irradiation as well as among hours of sampling after inoculation of ^{3}H-hypoxanthine ($p=0.05$).

Microscopy is the gold standard for malaria diagnosis, it is cost-effectively, simple to use and familiar to most laboratorians. But if it was not done well, it may cause poor reproducibility, variable sensitivity, and unacceptably high false-positive rates (Bashir et al. 2013). However, here we still use this method to confirm the isotopic results.

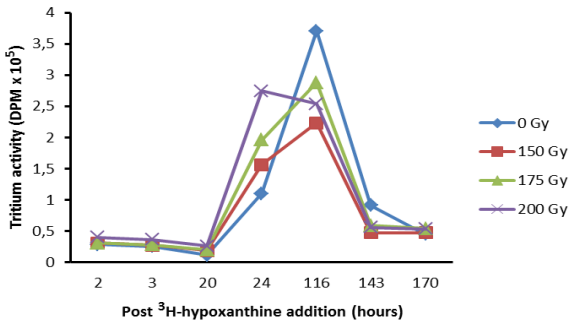

Figure 1. Tritium activity in the medium (not incorporated into parasites) that oppositely correlated with the viability of *P. falciparum* at hours post-^{3}H-hypoxanthine addition

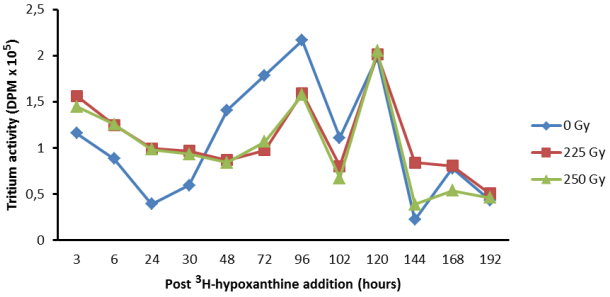

Figure 2. Tritium activities in the culture medium of irradiated *P. falciparum* at hours post-^{3}H-hypoxanthine addition

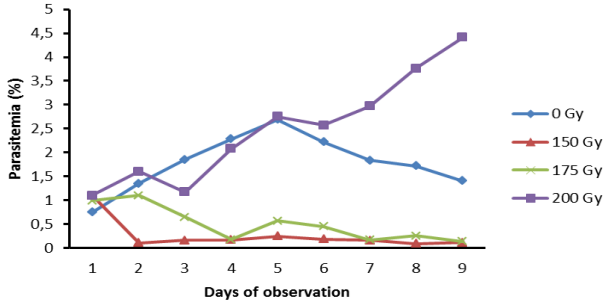

Figure 3. Parasitemia of *P. falciparum* culture at days post-irradiation with gamma rays based on microscopic observation

Other result was showed in Figure 2 where there was a fluctuation in tritium activities that related to the multiplication of parasites post-irradiation, mainly in higher dose (225 and 250 Gy). Similar with previous results, there was an increasing number of hypoxanthine incorporation by parasites up to the first of 24 hours culture. No difference was found in attenuating the parasites between these two doses. This may be because in this experiment the parasites were not synchronized before addition of ^{3}H-hypoxanthine. Irradiated parasites were more active in incorporate purine with precursor of hypoxanthine up to 48 hours. Parasites returned to their highest activity at 116 hours. This result showed quite different from the finding from microscopic observation where irradiation dose of 150 Gy was the most effective dose in inhibiting the parasite multiplication. In murine models, it was known that asexual blood-stage parasites exposed to high doses of irradiation at 150 Gy failed to cause blood-stage parasite infection, but not too high exposure that may cause the death of parasites.

Our experiment found that based on microscopic evaluation on the parasite density of *P. falciparum* after irradiation at doses of 150 Gy and 175 Gy both were effectively suppress the growth of parasites (Figure 3). This is similar with statement by other researchers that to ensure that the parasites are sufficiently weakened for the vaccine, yet remain alive, they must be exposed to a radiation dose of at least 150 Gy, but not much more (Syaifudin et al. 2011). There is no or very low number of parasites seen in the blood smear. The complete data on the parasitemia observation under microscopic up to day 8 post inoculation of ^{3}H-hypoxanthine that presented in the graph was seen in Figure 4.

Based on microscopic observations, in untreated control (0 Gy) cultures, parasitemia increased from 0.51% to 1.35% and 2.28%, after 24 and 72 hours, respectively and then dropped to 1.84% and 1.41% after 6 and 8 days of culture, respectively, and therefore this is an indication of overgrowth culture of *P. falciparum*.

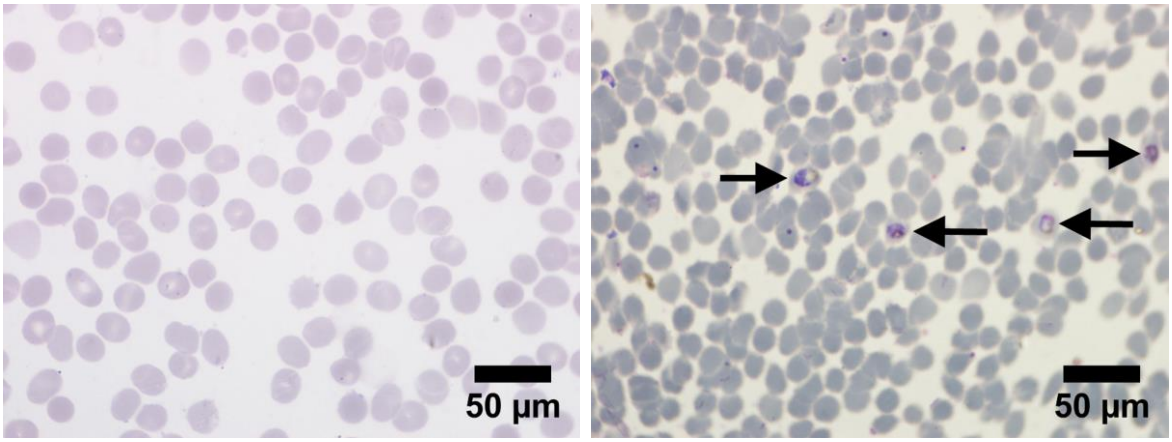

Figure 4. Microscopic view of Giemsa stained thin blood smear of *P. falciparum* culture post gamma irradiation at dose of 150 (left) showing no parasitized RBCs and at dose of 175 Gy (right) that showing some parasitized RBCs (arrows) at day 8 post inoculation.

After 8 days of continuous culture, parasitemia in untreated samples continued to drop and clearly distinguishable from that of treated cultures. Treatment with gamma-irradiation resulted in a dose-dependent decrease or increase in parasite growth and survival that was most evident at 48 and 72 hours after treatment except for 150 Gy. In cultures treated with 150 Gy of gamma-irradiation, parasitemia remained low (0.09-0.25%) and relatively unchanged during the experiment, whereas parasite cultures treated with 175 Gy has fluctuated after the treatment. The detrimental effect of gamma-irradiation on parasite survival was even more dramatic at irradiation dose of 200 Gy where parasites were growth up to more than 4% at the end of experiment.

Eradication of malaria will depend on an effective vaccine that prevents parasite infection. The development of an effective vaccine remains highly desirable. Since last 15 years, considerable progress has occurred in the development of malaria vaccine (Hoffman et al. 2010). However, malaria vaccine development is very difficult to obtain due to several problems such as antigenic diversity, complicated identification of a useful target for vaccine development, and continuously parasite changes during their life even while in the human host (Kalra et al. 2006). Population structure of *P. falciparum* in each location will have impacts on clonal diversity. Based on these facts, the development of malaria vaccine should be conducted regionally, not globally. Therefore it seems that we should develop our own malaria vaccine that is specific for Indonesian or at least Asian populations (Syaifudin et al. 2011).

Scientists in many parts of the world have created harmless versions of malaria-causing parasite as vaccine materials by applying nuclear technology. Different with our results, experiments conducted by Ferreira-da-Cruz et al. (1997) provide evidence that a 200 Gy gamma-irradiation is able to abolish the original replication of erythrocytic forms of the Palo Alto *P. falciparum* strain, probably by inactivating their infectivity. According to their data 100 or 150 Gy irradiation doses were enable to inactivate the parasite, despite the reduction of parasitemia, suggesting the existence of heterogeneous plasmodial populations as concerns susceptibility to irradiation and pointing to the possibility that the same dose could inactivate parasites from other *P. falciparum* strains.

Here precursor incorporation based assay to evaluate viability of parasites post-irradiation was developed. ^{3}H-hypoxanthine precursors incorporation is widely used in vitro drug sensitivity test (isotopic tests) (Ancelin et al. 2003), whereas others are done by CYBR green I-based fluorescence assay (Bacon et al. 2007), the pLDH assay (Barends et al. 2007), and a HRP2 assay (Noedl et al. 2007) in drug sensitivity test. To our opinion, this is the first report on the ^{3}H-hypoxanthine precursors incorporation as the basis for evaluating the most effective dose of gamma irradiation to create vaccine materials.

The incorporation of variously labeled phospholipid precursors in comparison with the incorporation of nucleic acid and protein precursors was also tested by Sanz et al. (2012) to evaluate *P. falciparum* growth in vitro. The precursors, ^{3}H-ethanolamine, ^{3}H-hypoxanthine, ^{3}H-palmitate, ^{14}C-serine, ^{3}H-choline, ^{3}H-inositol, and ^{3}H-isoleucine, were all accurate indicators of parasite growth. Because of its high level of incorporation, ^{3}H-ethanolamine proved to be the best tool for assessing parasite viability. When culture parameters were carefully controlled, ^{3}H-ethanolamine incorporation into phospholipids was proportional to pulse time, precursor concentration, and initial parasitemia and was sensitive to the number of uninfected erythrocytes. The use of ^{3}H-ethanolamine for in vitro antimalarial drug screening is a good alternative method. The major advantage is that the culture medium can be supplemented with hypoxanthine, which results in better parasite growth.

There are several in vitro tests for the assessment of antimalarial activity based upon observation of parasite development in blood cells. These are observation of parasites in thick films under microscope, isotopic assays, quantification of parasite proteins and DNA dye intercalation assays. The standard method for in vitro antimalarial drug screening and or vaccine materials development based on the isotopic assay ([^{3}H]-hypoxanthine incorporation assay) is expensive and utilizes radioactive materials with limited availability, safety, and disposal problems in developing countries. Abiodun et al. (2010) found that the [^{3}H]-hypoxanthine-based assay exhibited the most robust signal-to-noise ratio of 100, compared with signal-to-noise ratios of 7 for SYBR Green and 8 for pico green.

Some factors affecting ^{3}H-hypoxanthine incorporation including initial parasitemia, duration of culture, duration of radioisotope pulse, parasite stage, concentration of uninfected erythrocytes, the use of serum or plasma to supplement growth, and the concentration of a variety of purines in the culture medium (Monatrakul et al. 2010).

Isotopic assays rely on the incorporation of radioactive ^{3}H-hypoxanthine into the parasite DNA. These methods are relatively reliable and objective, but not sufficiently sensitive, and require the use of hazardous radioactive material (Noedl et al. 2003). In this experiment, tritium-labeled hypoxanthine had been used to determine the effectivity of gamma rays in attenuating human parasites. Tritium (^{3}H) is a very low energy emitter that can be used to label proteins, nucleic acids, drugs, and toxins, but requires a tritium-specific film or a tritium-specific phosphor screen. In a liquid scintillation assay, the efficiency is 20-50%, depending on the scintillation cocktail used. However, there is often more than one tritium atom per molecule, for example, tritiated-UTP with carbons 5 and 6 each bonded to a tritium atom. Other isotopes such as ^{14}C, ^{35}S, and ^{33}P have similar emission energies; however, ^{32}P and ^{125}I have higher energy emitters that result in inaccurate measurement (Lüttge 2008).

Because of the weakness of both microscopic and isotopic techniques, molecular technique has also been developed in malaria diagnosis, especially in reference centers. Real-time polymerase chain reaction assays are particularly attractive because of the short turn-over-time and the avoidance of post-PCR contamination (Taylor et al. 2010; Proux et al. 2011). Microarray analysis to elucidate

the molecular mechanism of gamma-radiation-induced attenuation of parasite growth and survival was also used. Their ultra-structural changes within the parasite were also observed by fluorescence and electron microscopy (Oakley et al. 2013).

In conclusion, the use of ^{3}H-hypoxanthine incorporation for vaccine materials development is a good alternative and complement to the method of microscopic observation. No significant difference in parasites growth inhibitory was found among doses of irradiation (p=0.05) in this experiment with precursor incorporation. It was different with the finding from microscopic observation where 150 Gy was the most effective dose in inhibiting the parasite multiplication, and some factors affecting these facts such as dose rate of irradiation and number of parasite being irradiated.

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