Capability of Gamma Irradiated *Plasmodium berghei* to Infect Mouse After Cryopreservation and Storage in Ultra-Low Temperature and Study on its Protein Profile

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**Abstract** – Rodent malaria species *Plasmodium berghei* have been widely used to validate vaccine development including its storage. Therefore, the effects of cryopreservation and irradiation of *P. berghei* towards its infectivenss and protein profiling were studied. A group of mouse was injected intraperitonealy with 0, 150, 175 and 200 Gy gamma irradiated *P. berghei* that had been cryopreserved for 1 month. As comparison, the infectiveness of irradiated parasites stored in freezer (-20°C) and ultra low temperature (-80°C) for the same time was also evaluated. The capability of parasite to infect mouse was determined by counting the parasites number in red blood cells drawn from tail by making thin blood smear stained with Giemsa. Protein profile in the blood was analyzed with SDS-PAGE method. It was found that the storage did alter the ability of the irradiated parasites to induce infection in the recipient animals. After one month observation, it was demonstrated that all mice from different treatment groups showed negative parasitemia in their blood. This might have been caused by the death of the parasites or the erythrocytes, where the parasites were hosted, due to physical damage after irradiation and very low temperature storage. However, non irradiated parasites (control) were still infective in mouse even though it required longer to establish an infection. It is suggested that the condition of the sample prior to preservation was another factor that need to be considered. Further analysis demonstrated that the protein profile was maintained throughout the storage time. It was concluded that the storage influenced the performance characteristics of vaccine materials for immunization.

**Keywords** – Cryopreservation, Gamma Irradiation, Malaria Vaccine, *Plasmodium Berghei* Storage.

**I. INTRODUCTION**

Malaria causes an estimated 500 million clinical cases and up to 2.7 million deaths annually, and is highly endemic in Sub-Saharan Africa, Southeast Asia, and South America [1,2]. A vaccine against malaria offers the best hope for wiping out the disease. Many research laboratories around the world have been conducting studies to find vaccine candidates, several of which are in clinical trials on human in Africa. Most of these focus mainly on *P. falciparum*, as the most severe form of malaria [3,4]. Over the years, numerous attempts have been made to develop a functional vaccine against malaria using gamma rays to attenuate parasites [5]. Using an avian *Plasmodium* system, Mulligan et al. [6] found that immunization with irradiated sporozoites could prime the immune system to target normal sporozoites, thus preventing erythrocyte infection. Since then, irradiated sporozoites have been proved to be potent vaccines in all *Plasmodium* systems [7-11].

A number of strategies have also been adopted in the pursuit of making a successful malaria vaccine including the knowledge of vaccine or vaccine materials storage [12]. Particular attention has been paid to the use of freeze drying and the protective matrices used to improve microbial cell viability after drying. There are limited data available regarding this approach to cryopreservation of malaria parasites, mainly after irradiation as an agent to attenuation. Therefore biological characterization of malaria parasites is a subject of particular interest to laboratory-based scientist. In relation to this, the cryopreservation of malaria parasites deserve attention, in order to ensure the supply of well defined and viable isolates to interested research workers [13].

Cryopreservation techniques have been used successfully for many years in the preservation of isolated tissue cells and pathogens. However, there has been insufficient work to elucidate the optimum conditions for reliable and reproducible cryopreservation of malaria parasites and its irradiated form to make a vaccine. It is important that a high degree of parasite recovery is attained, since it has been demonstrated that low survival rates after freezing and thawing may represent a selective event, which may favour mutant subpopulations [14]. A study of the currently available techniques for the preservation of *P. falciparum* demonstrates that glycerol technique allows cryopreservation of both culture-adapted parasites and those present in infected blood taken directly from the host [15].

This report presents the experimental result on the infectiveness of parasites and protein profile in mouse blood after being irradiated and stored in liquid nitrogen, ultra-low temperature storage and freezer.

**II. MATERIALS AND METHODS**

This study was conducted at the Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia from December 2013 till February 2014.

2.1. **Parasites.**

Mouse blood infected with *P. berghei* of ANKA strain was obtained from Malaria Group, Eijkman Institute for Molecular Biology and/or National Institute of Health.
Research and Development, Indonesian Ministry of Health.

2.2 Mice and Ethics.

Male Swiss Webster mice purchased from SEAMEO-TROPMED Center, Regional Center for Community Nutrition University of Indonesia were used in this study. Four weeks old mice, which were almost 20 g in weight at the start of the experiment, were housed at room temperature (20-23°C), with unlimited access to rodent food and tap water in animal facility and handled according to the institutional guidelines. All procedures were reviewed and approved by the Animal Care and Use Committee National Institute of Health Research and Development, the Indonesian Ministry of Health.

2.3. Parasitized blood production.

P. berghei was stored as frozen stabilate. Before use, the stabilate was thawed and then injected into a source mouse to generate an inoculum for the experiments. Proliferation of the parasite was done in vivo by injecting parasitized blood intraperitoneally into 3-4 mice in Biomedical Laboratory of Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia.

2.4. Gamma irradiation.

Blood from the source mouse was diluted with phosphate buffered saline (PBS) to prepare aliquots of the inoculum. In the first series of experiments, the blood was diluted 1:6 in PBS upon collection from the source mouse. The number of red blood cells (RBCs) per milliliter was determined by using a hemocytometer. The number of RBCs and the percent parasitemia were used to calculate the final dilution necessary to obtain 1 × 10⁶ parasitized red blood cells (pRBCs). The blood was irradiated with gamma ray at doses of 0, 150, 175 and 200 Gy from Co-60 source using IRPASENA irradiator with dose rate of 380 Gy/hour as measured with Frick dosimeter at the Center for Application of Isotope and Radiation, National Nuclear Energy Agency of Indonesia.

2.5. Cryopreservation and Storage in Ultra-Low Temperature.

The irradiated mouse bloods were supplemented with equal volume of cryoprotectant (Alsever’s buffer solution which containing sorbitol, 0.9% NaCl, 99% glycerol and added with sodium citrate and glucose) in an equal volume and then stored either in liquid nitrogen (-196°C, cryopreserved), ultra-low temperature storage (-80°C) or freezer (-20°C) for 1 month.

2.6. Treatment of mouse.

In total of 36 mice were randomly divided into three groups, each consisting of 12 mice. Each group was divided into 4 subgroups; 0 (as a control), 150, 175 and 200 Gy. Each consisted of 3 mice. Mice in the first subgroup were injected with unirradiated(0 Gy) malaria parasites, whereas the second, third and fourth subgroups mice were injected with malaria parasites that had been irradiated with 150 Gy, 175 and 200 Gy, respectively. Standard inoculum of about 1 × 10⁶ P. berghei/ml (ANKA strain) aerythroctic stage were injected intraperitoneally into the mice on the first day (day 0).

2.7. Parasite growth observation.

The growth of parasites in the blood was checked every other day starting from the third day after infection by Yokoo’s thin blood smear. A drop of blood was collected from the mice by venesection of the tail and transferred onto a standard microscope slide and air-dried before fixing the films in methanol for 5 seconds. They were stained with fresh Giemsasolution (10% v/v in distilled water). The stained blood films were observed under a standard light microscope using 100x objective lens with immersion oil. Infected and uninfected RBCs in different fields of view were identified and counted. A total of at least 2000 RBCs were counted per slide. The percentage parasitemia was then determined.

2.8. Protein profiling with SDS-PAGE.

The procedure was done according to protocol published elsewhere (Manna, 2006). Ten microliter acetone was added to 5μl samples of parasitized blood that had been irradiated with 0, 150, 175 and 200 Gy gamma rays and stored at low temperature, then sonicated for 15 minutes. Twenty five microliter Laemli buffer was then added to the solution, before being warmed in boiling water for 5 minutes, and centrifuged at 8000 rpm for 5 minutes. A total volume of 15 protein extract solution as supernatant was loaded into each well of 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out at 120 V until the bromophenol blue dye reaches 1 cm below the top of the gel. As a molecular weight marker, Broad range SDS-PAGE Standards (Biorad) was used. After stained with Commassie R-250 for overnight, gel was immersed in fixative solution for 2 hours and then destained for another 2 hours. The appeared protein bands on the gel was analyzed with GelDoc Imaging System (Biorad).

III. RESULTS AND DISCUSSION

In this experiment we focused on the parasitaemia levels as a sign of infectiveness of parasites in the mouse body after one month storage in very low temperature. The infectiveness were taken as the percentage of pRBCs measured. The results shows that all mice that injected with 1 month cryopreserved-irradiated parasites did not get infected with parasites and survived for more than 40 days. So injection of parasites that had been irradiated with 150, 175 and 200 Gy of gamma rays and stored at ultra low temperature produced no detectable parasitemia in the recipients. However, all mouse injected with non irradiated cryopreserved parasites developed rapidly increasing parasitaemia and died on the 20th-28th day after inoculation (maximum parasitaemia up to around 75%), showing the high influence of irradiation to the parasite infectiveness after stored in very low temperature (Fig. 1).

These results indicate that, after being irradiated with gamma rays, blood stage parasites (merozoites) stored by freeze drying or in the frozen state were not as effective as freshly isolated merozoites when used for vaccination. With both freshly isolated and stored parasites (without irradiation) all animals became infected with different
prepatent period. Without storage, the experiments provide an evidences that a 175 Gy gamma irradiation is able to abolish the replication of erythrocytic forms of ANKA strain of *P. berghei*, probably by inactivating their infectivity up to 4 weeks after injection.

Virtually all *P. berghei* infected mice develop petechiae and become moribund on day 10 or 11 of infection. Previous studies have shown that the ANKA strain of *P. berghei* causes a rodent form of cerebral malaria and leads to death at relatively low parasitemias. Our irradiated-stored parasited mice died 26 - 29 days after infection with high parasite densities, indicating that the Swiss Webster mice we used in our experiments were susceptible to cerebral malaria. In contrast, mice injected with unirradiated parasites had similar activity levels and weight gain to those of the untreated controls. Example of microscopic view of the parasites in mouse blood was presented in Fig. 2.

The same tendency of parasitemia as above was found in mice injected with parasites that had been irradiated and stored in freezer (-20°C) for a month, where only unirradiated parasites developed in the mouse body by infecting the erythrocytes (Fig. 3). Irradiated parasites could never infected erythrocytes, presumably because of the death of parasites due to irradiation and freezing. Both nitrogen and freezer storages effectively maintained the infectiveness pattern of the parasites but a slightly longer prepatent period was observed in parasites that had been stored in liquid nitrogen compared to those that had been stored in freezer.

As in case of liquid nitrogen and freezer storages, non irradiated parasites that had been stored in ultra low temperature storage (-80°C) were also still infective in mouse, whereas irradiated parasites, as well as those that had been stored in nitrogen and freezer, did not cause any infection in mouse RBCs. This might be due to the death of parasites or the damage of host RBCs (Fig. 4). No data was available for 175 Gy irradiated blood samples that may be due to technical error in the storage of pRBCs, so the data for low temperature storage were only of 0, 150 and 200 Gy. We predicted that 175 Gy irradiated blood injection would not infect the mouse and be similar with the results of other doses of irradiation.
The additional finding of this study was profile pattern of plasmodial proteins that was not so different between cryopreserved (after freezing) and non cryopreserved (before freezing) (Fig 5). The new and light bands at about 38 kDa were found in samples after being stored at low temperature that may be as a response to the temperature alteration such as heat shock proteins. Indeed, during cryopreservation, major proteins were not altered with the temperature. Thus, cryopreservation of parasites did not alter their molecular pattern as determined by SDS-PAGE. In this research irradiation alone or in combination with storage at low temperature did not affects the protein profile where there is no additional bands formed after irradiation with 150 and 175 Gy, even among stress-related or heat shock proteins such as HSP70 which was slightly increased as found in another our experiment [16]. The expression was similarly regulated, depending on the growth phase of the parasites.

Immunization programs are among the most cost-effective ways to prevent malaria disease and continue to savemillions of lives annually and have improved the quality of life of tens of millions of individuals in many tropical areas [17]. The success of this program depends heavily upon the high immunization coverage of the target group, including proper storage and handling of vaccines or its material. The practicality of vaccination is enhanced by the finding that freeze-dried parasites constitute an effective vaccine [18]. However there is a limited data or even no data on irradiated one.

During the freezing process, water in an organism or samples crystallizes to ice. This may in itself have implications for the stability of the protein(s) present, which may be destabilized and denatured because of the change in ionic strength. Some proteins may be satisfactorily immobilized in the lyophilized state but undergo changes that result in aggregation on reconstitution and its infectiveness capability [19]. Most pathogens do not multiply at freezing temperature and many of them perish because their enzymes do not work properly to maintain normal cell activity. Also, pathogens need water to grow and freezing turns the available water into solid ice crystals. One investigation found out that the slower the freezing process the larger the crystals become and the more cells they damage [20].

This research was done based on the fact that vaccines or vaccine materials are also sensitive biological substances that can lose their potency and effectiveness if they are exposed to temperatures (heat and/or cold) outside the required temperature range. Vaccines may not appear frozen but may have been damaged at these temperatures. Failure to adhere to cold chain requirements may reduce vaccine potency, resulting in lack of protection against vaccine preventable diseases and/or increased local reactions after administration of vaccine. Vaccines are also fragile because if exposed to temperatures outside the recommended ranges it can have reduced potency and protection [21]. Specifically in malaria cases, after freeze-thawing, the RBCs lyse more rapidly because changes in the cell wall due to dissociation and denaturation of lipoproteins resulting in osmotic fragility are probable [22]. If unirradiated, the in vivo qualities of the RBCs are well preserved. Cryopreserved RBCs were capable of reinitiating sodium and potassium transport activities with a facility equal to that of nonfrozen cells, indicating that the cryoprocessing is protective of enzyme systems essential for this mechanism [23]. Irradiation seems to alter this capability.

This study was conducted to observe the effects of irradiation as an agent used to attenuate parasites on cryosurvival of P. berghei. Here, we used Alsever’s solution as a cryoprotectant that did not effectively protect the parasites from damage. Other scientists used a final concentration of 20% glycerol as cryoprotectant and storage in aluminum flask at -150°C. Other cryoprotectants include dimethylsulfoxide, glycerol, blood serum or serum albumin, skimmed milk, peptone, yeast extract, saccharose, glucose, methanol, polyvinylpyrrolidone, sorbitol, and malt extract with their own specificity [24,25]. Ten percent glycerol or 10% dimethyl sulphoxide in RPMI 1640 growth medium had also been used and found equally effective as cryopreservatives [26].

In this experiment, studies had been done to determine if irradiation or freezing of the parasitized blood provided the best immunogenic response, the latter being preferable from a vaccine production perspective. One of the advantages of live, attenuated vaccines, and a reason to re-investigate the potential of attenuation by irradiation, is their potent immunogenicity since the organisms are still able to replicate and behave in a similar manner to a natural infection, thereby stimulating the immune system to secrete the immunoregulatory products and induce the cellular activation that would normally occur [27]. However, so far the work in malaria vaccine research have met only limited success, in marked contrast to other recent studies where irradiated organisms have behaved in a manner that enables them to be more effective immunogens that can sometimes elicit substantial cross-protection.

Fig.5. Protein profile of irradiated parasites of P. berghei with SDS-PAGE before and after stored in low temperatures. New bands are signed by arrow. M is protein marker.
Different from our results above, Orijih et al. [28] proved that irradiated sporozoites (sexual stage of parasites which not needs RBCs as host) of P. falciparum (PfSPZ) and freezing them either at -75°C or in liquid nitrogen did not appreciably alter their ability to induce protective immunity in the recipient animals of UI mouse after a challenge. The highest protection (80-100%) was induced with sporozoites maintained in 10% serum and stored at -75 °C. Whereas Ruben et al. [29] had demonstrated that cryopreservation probably caused approximately a 6.4-fold loss of infectivity of sporozoites and concluded that the impact of cryopreservation on PfSPZ might be cumulative. For rodent malaria, P. berghei sporozoites frozen in different solution consisting MEM medium enriched with 10% hydroxyethyl starch and 50% normal mouse serum, could retained their 0.5% infectivity to hepatoma cells culture, whereas it retained to 6.8% before freezing. This indicates that frozen-thawed sporozoites may be used in in vitro investigations of the exoenzythrocytic malarial parasite meaning that they may be frozen and preserved for later use if there are large numbers of sporozoites [30].

Data from studies in mice with other rodent malaria parasite show that administration of purified cryopreserved P. yoelii SPZ required ~23 times more PySPZ administered ID than IV to achieve 80% infection rates. Thus, cryopreservation may also be responsible for reduced infectivity as proven in this research. Rodent model in vivo data, however, suggest that a ~7-fold loss in infectivity caused by cryopreservation is more likely [31].

Different from the aforementioned finding, in this experiment all irradiated erythrocytic parasites were dead after storage in liquid nitrogen and low or ultra low temperature. It is predicted that this is due to the fact that irradiation causes immediate haemolysis of the red cells, which had their cell membrane damaged because of the treatment, causing ions to freely diffuse across the membrane, hence an osmotic lysis and a leakage of potassium the supernatant [32]. Surprisingly, however in a different experiment, red cells irradiated with 50 Gy of X rays and stored up to 4 weeks in CPD did not show any difference with the controls [33].

Recently, it was revealed that the irradiation modified the haemoglobin conformation due to alterations in oxidative stress in the erythrocytes [34]. Alterations of the erythrocyte membrane ionic permeability and increases in lipid peroxidation or proteolysis are events that can be triggered by an increase in oxidative stress, and these events have been well described. Therefore, an investigation of the effects of irradiation and storage time on the integrity and function of the erythrocytes, as done in this research, is relevant to ensure that there is no negative effect on the infectiveness of irradiated parasites.

IV. CONCLUSION

Based on the results above it can be concluded that there was no difference in the infectiveness found between parasites irradiated with different doses (150, 175 and 200 Gy) of gamma rays and stored. This results is, however, different with non irradiated parasites, showing that irradiation clearly lowering the capacity of parasites life or killing them after which they were stored in very low temperature. Studies of protein profile showed that the molecular weight pattern was maintained throughout the storage time.

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