

In Vitro Infectivity Study of Cryopreserved Irradiated Intraerythrocytic Form of *Plasmodium falciparum*

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ABSTRACT

In control human malaria infection studies using irradiated *Plasmodium falciparum*, the cell bank of irradiated *P. falciparum* infected erythrocytes is needed. The cell banking methods represent an obvious way to obtain suitable material for blood stage *Plasmodium*. In a cell bank development of irradiated *Plasmodium* infected erythrocytes, the ability to cryopreserve procedure of *Plasmodium* is important to recover the infectivity of irradiated *Plasmodium*. This study aims at evaluating the *in vitro* infectivity of cryopreserved irradiated intra-erythrocytic form of *P. falciparum*. A protein profile investigation using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of cryopreserved irradiated *P. falciparum* also conducted in this study to know the cryopreserved effect on the protein of irradiated *P. falciparum*. *Plasmodium falciparum* of 3D7 strain in human erythrocytes was maintained in *in vitro* continuous culture. When the percentage of parasites was 10–20 %, the culture was harvested and irradiated with gamma rays at a dose of 175 Gy. Irradiated *P. falciparum* then was mixed with cryopreserved solution and stored in -80 °C for one hour before transferred into liquid nitrogen for 20, 40 and 60 days. After being stored the irradiated *P. falciparum* was thawed and cultured for 20 days. The percentage of parasitaemia was enumerated by examining Giemsa stained thin blood films prepared for 20 days after initiation of culture. Results showed that storage time significantly ($p < 0.05$) influence the percentage of parasitaemia. The cooling procedure and cryopreservation media may affect this study results. It also showed that there was insignificant difference of irradiated *P. falciparum* protein profile in all storage times. Overall it can be assumed that the irradiated *P. falciparum* still kept their infectivity after stored in liquid nitrogen for 60 days. Further study using different cooling procedure and different formula of cryopreservation media with a longer storage time should be conduct to validate this study results.

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INTRODUCTION

Until now, malaria remains a global public health problem in the tropical world. It has estimated that 198 million cases of malaria infection occur globally and there were 584,000 deaths in 2013 cause by the disease [1]. Malaria disease in human can due to infection of one from five *Plasmodium* species that were *Plasmodium*

falciparum, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. *Plasmodium falciparum* is responsible for 92 % of deaths in human because of malaria disease [2].

Developing an effective vaccine for *P. falciparum* is needed for use in malaria endemic populations. Unfortunately, there were no licensed malaria vaccines and candidates have consistently produced a protective level of efficacy [2,3]. There were different targets in malaria cycle for developing malaria vaccines. Pre-erythrocytic

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vaccine aims to prevent sporozoites entry and develop in the liver, while asexual blood-stage vaccine prevents disease by targeting merozoite invasion and intra-erythrocytic development; and the transmission-blocking vaccine targets on the sexual and sporogonic stages to prevent parasite development in the mosquito [2].

Eventhough developing a vaccine using ionizing radiation has some advantage because it can remove chemical contaminants and penetrate pathogens to damage the DNA, but it has not been pursued avidly over the past 20 years. There are two reasons why radiation technology was no longer used in vaccine development. First, the development of new radiation techniques has been considered impractical or difficult due to issues accessing the radiation equipment. Second, it has been thought that modern subunit vaccines would provide a solution, as they can be developed more easily [3].

Our previous study showed that Gamma ray irradiation at doses 150 and 175 Gy were effectively suppress the growth of *P. falciparum* [4]. Our study also revealed that at least 150 Gy radiation dose was needed to weakened the malaria parasites [5]. Until now a controlled human malaria infection (CHMI) studies have been used as a powerful tool to evaluate malaria vaccine since the mid-1980s [6]. A cell bank of irradiated *Plasmodium* infected erythrocytes is need in malaria vaccine development to control human malaria infections. The cell banking methods represent an obvious ways to obtain suitable material for blood stage *Plasmodium* [7]. In a cell bank development of irradiated *Plasmodium* infected erythrocytes, the ability to cryopreserve and recover infectivity of *Plasmodium* is an essential procedure.

Here in this study the evaluation of *in vitro* infectivity of cryopreserved irradiated intra-erythrocytic form of *P. falciparum* was conducted. A protein profile investigation of cryopreserved irradiated intra-erythrocytic form of *P. falciparum* also conducted in this study to know the cryopreserved effect on the protein of irradiated *P. falciparum*.

EXPERIMENTAL METHODS

Culture of *P. falciparum* parasites

P. falciparum (3D7) were thawed and initially grown in a 5 % suspension of purified human O+ RBCs in RPMI 1640 medium, 2 g/L sodium bicarbonate, 25 mM Hepes (pH 7.4), and 10 mg/L gentamicin at 37 °C. Parasites were counted and diluted with uninfected RBCs in culture medium to

50 % late stage parasitaemia at a 1 % hematocrit unless specified otherwise. Parasite development in five different plates was monitored by daily microscopic evaluation of Giemsa-stained thin smears.

Irradiation treatment of parasites and storage

Asynchronous growing *P. falciparum* at ~10-20 % parasitaemia were harvested and resuspended to 50 % hematocrit. The parasites were immediately exposed to a dose of 175 Gy of Co⁶⁰ source in Multi Purpose Panoramic Irradiator (IRPASENA), Center for Application of Isotope and Radiation, BATAN at room temperature with a high dose rate (380.4 Gy/hour). Irradiated parasites then were mixed with cryopreserved solution at the same volume and stored in -80 °C for one hour then transferred to liquid nitrogen for 20, 40 and 60 days.

Irradiated *P. falciparum* infectivity evaluation

After being stored in liquid nitrogen, the irradiated *P. falciparum* were thawed and cultured in RPMI 1640 medium. The effect of cryopreservation on irradiated *P. falciparum* infectivity was evaluated in asynchronous cultures. Percentage of parasitaemia (parasitized RBCs/total RBCs × 100) was enumerated by examining Giemsa stained thin blood films prepared on days 2 until 20 days with 2 days interval after initiation of culture.

Protein profile study by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of the post-irradiated culture for each storage time was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % acrylamide for the running gel of 30 minutes. A detail protocol of SDS-PAGE can be found in our previous publication [8-10]. The molecular weight marker was run on the first well of the gel. The gels were stained with Coomassie Brilliant Blue G for overnight, then was immersed in fixative solution for 2 hours and then destained for another 2 hours. The appeared protein bands on the gel was analyzed, and pictured under GelDoc Imaging system.

Statistical analysis

The one-way ANOVA test was applied to determine the different of parasitaemia percentage mean in all storage times groups. T-test analysis was used to compare the infectivity of irradiated and unirradiated parasitaemia that was stored for 60 days. All statistical tests were conducted using SPSS 22.0 statistical software and levels of significance were set at 0.05.

RESULTS AND DISCUSSION

Result showed that there was an indication of downward trend in percentage of parasitaemia when the storage times were increased. As can be seen in Fig. 1, that the lowest percentage of parasitaemia was found in the experimental group that stored for 60 days. The statistical analysis revealed that the difference in mean percentage of parasitaemia in all storage times was statistically significant ($p < 0.01$). In this study, we also compared the percentage of parasitaemia between irradiated and unirradiated *P. falciparum* after being stored for 60 days. A *t*-test analysis showed that the difference was significant ($p < 0.01$) (Fig. 2).

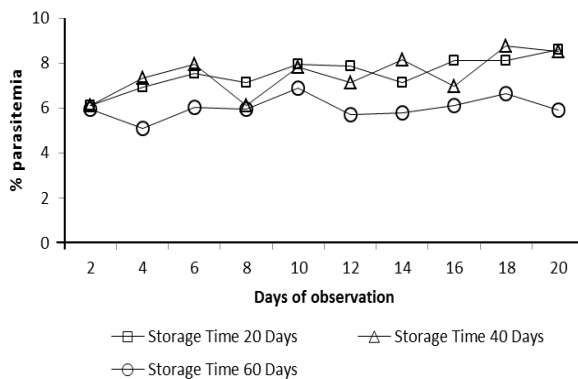


Fig. 1. The percentage of parasitaemia in culture of irradiated *P. falciparum* at dose of 175 Gy that stored for 20, 40 and 60 days.

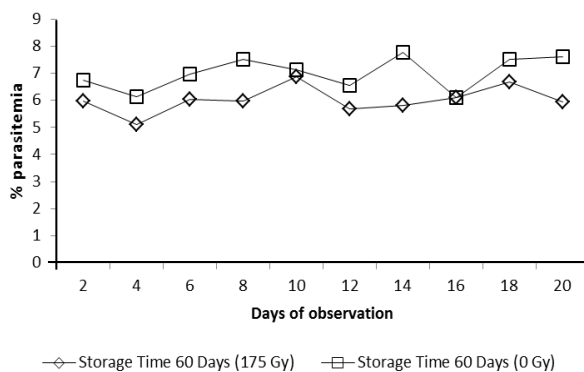


Fig. 2. The percentage of parasitaemia in culture of irradiated and unirradiated *P. falciparum* that stored for 60 days.

Even though the statistical analysis showed that there was a significant downward in percentage of parasitaemia when the storage times were increased, it can be assumed that the irradiated *P. falciparum* still kept their infectivity after being stored in liquid nitrogen for 60 days. This result was in a good agreement with other studies. In 1977 a study by Strome et al. found that the erythrocytic stages of malarial parasites (*P. vallax*) can be stored in the liquid nitrogen for 10 years without significant loss of the infectivity [11]. Another study by Ozbilgin et al. also showed that *P. yoelii* and *P. berghei* can kept their viability and virulence at $-80\text{ }^{\circ}\text{C}$ and $-196\text{ }^{\circ}\text{C}$ between the first and the sixth months of cryopreservation [12].

It is possible that using different technique and media of cryopreservation, the different of parasitaemia percentage in all storage times will be insignificant. Here in this study we transferred the irradiated *P. falciparum* into the liquid nitrogen tank after stored at $-80\text{ }^{\circ}\text{C}$ for one hour. If the storage time was more than one hour, it is possible that the percentage of parasitaemia after stored for 60 days will show insignificant value compare to irradiated *P. falciparum* that store for 20 days.

In this study we used two step cooling procedure. Two step cooling procedures have been found to improve protection against freezing and thawing injury in several types of cell [13]. This because the two step cooling procedure separate the different sets of conditions that lead to cell injury. In this procedure, samples are cooled to a selected temperature ($-80\text{ }^{\circ}\text{C}$ in this study) and are held at that temperature for different lengths of time before being plunged into liquid nitrogen. At the holding temperature, cells may acquire "protection" against damage caused by rapid cooling in liquid nitrogen and subsequent thawing [13].

Even though two step cooling procedures was the most commonly used in *Plasmodium* cryopreservation, the use of direct immersion in liquid nitrogen procedure for irradiated *P. falciparum* cryopreservation should be conduct to evaluate the most effective cryopreservation procedure for irradiated *Plasmodium*. Rapid cooling procedure to $-196\text{ }^{\circ}\text{C}$ considered as the best method for the preservation of erythrocytes and ring forms of the parasite, however it is also destroyed almost large trophozoites and schizonts [13]. In our study observation of parasite growth showed that the stages mostly observed during culture after cryopreservation were the ring forms and the early trophozoites (data not shown), whereas late trophozoites were relatively scarce. It is possible that ring was the mostly stage in irradiated *P. falciparum* before process to cryopreservation and it is also possible that rapid cooling procedure is the best procedure for irradiated *Plasmodium*.

A cryopreservation media used in this study may also affect the infectivity of irradiated *P. falciparum* after storage in liquid nitrogen for 60 days. Here we used a cryopreservation media that consisted of glycerol, NaCl and sorbitol. It is possible that if we use another cryopreservation media formulation like hydroxyethyl starch (HES) mixed with fetal bovine serum (FBS) or dimethyl sulfoxide (DMSO) and sucrose, the different of parasitaemia percentage in all storage times will be insignificant. DMSO and glycerol are the most common used as cryoprotective agents (CPAs) to eliminate ice formation when cooling cells or organs to cryogenic temperatures [14-16]. In DMSO and glycerol the mechanism of protective effect on frozen cells occurs by intracellular permeation. The difference is glycerol permeates more slowly than DMSO [17-19].

Use of the commercial serum free cryoprotective solution like CryoStor CS2 also possible to kept *P. falciparum* infectivity after cryopreservation. Study by Singh et al. showed that CryoStor CS2 was the best commercial cryoprotective solution for both *P. falciparum* and *P. vivax* sporozoites, whereas Hestar 200 was efficiently only for *P. vivax* sporozoites [20]. Singh et al. study was compared six different commercial cryoprotective media, which were CryoStor CS2, Hestar 200, Voluven, Hetastarch, Glycerolyte and RPMI 1640. From all media it showed that Glycerolyte and RPMI 1640 were two media that gave the lowest viability of *P. falciparum* after cryopreservation. CryoStor CS2 media contain 2 % DMSO, whereas Glycerolyte contain 50-60 % Glycerin (Glycerol). Here in our study we used 40 % Glycerol that similar to a commercial Glycerolyte media used in Singh et al. study [20]. Based on the Singh et al. study, as we already stated before it is possible that using DMSO as cryopreservation media the different of parasitaemia percentage in all storage times will be insignificant.

Cryogenic solutions media usually contain an active ingredient that is responsible for maintaining integrity of cellular membranes. Cryogenic solutions also contain other additives to maintain buffering capacity to minimize the cellular damage. Cryogenic solutions can be divided into two categories, which were protecting the intracellular and extracellular material. Intracellular solutions have low molecular weights and amphipathic properties, permeate cells and are effective in minimizing cell damage in biological systems. Glycerol protects RBCs by limiting the rise in electrolyte concentration that occurs when water is converted to ice and by inhibiting the rate of ice crystal growth. Extracellular solutions operate mainly by forming a shield around the cell to minimize the effects of dehydration during the freezing process [20].

In this study, we also observed the protein profile of irradiated *Plasmodium* using SDS-PAGE. Result showed that the protein profile in irradiated *P. falciparum* stored for 20, 40 and 60 days was similar. It can be seen in fig. 3, the band in lane 2, 3 and 4 were similar. It can be assumed that the storage times did not change the protein of irradiated *Plasmodium*.

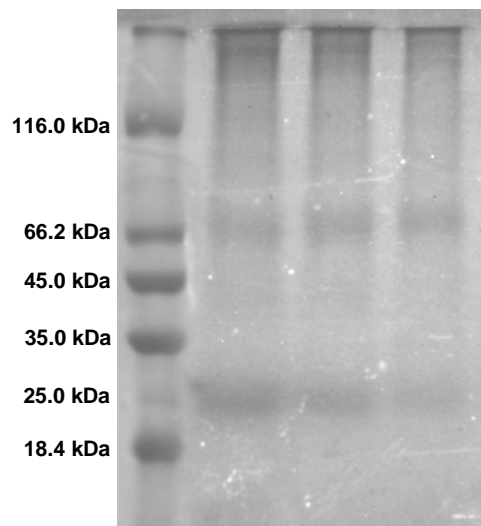


Fig. 3. The protein profile of irradiated *P. falciparum* after stored in liquid nitrogen. Lane 1: Marker, lane 2 : stored for 20 days, lane 3 : stored for 40 days and lanes 4 : stored for 60 days.

CONCLUSION

Various attempts to preserve malarial parasites at low temperatures have been reported since the 1930s. Nevertheless, a specific methodology to preserve irradiated *P. falciparum* has not been established. Here in this study we conduct an evaluation of cryopreserved irradiated *P. falciparum* infectivity. Our study showed that irradiated *P. falciparum* still kept their infectivity after stored in liquid nitrogen for 60 days. The highest percentage of parasitaemia was found in irradiated *P. falciparum* that stored for 20 days compared to 40 and 60 days. Further study using different cryopreservation media and different cooling procedure with a longer cryopreservation time should be conduct in order to find a better cryopreservation protocol for irradiated *P. falciparum*.

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