The infectiveness of gamma ray irradiated *Plasmodium berghei* in pregnant mouse and its progenies

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**ABSTRACT**

Malaria is an important cause of severe anemia in pregnant women, and by this mechanism malaria causes around 10,000 maternal deaths each year. In the world, research on the malaria vaccine in pregnancy has also been relatively neglected. In this paper, we studied the effects of gamma irradiated *P. berghei* injection to pregnant mouse and its progenies. On the day 9 of pregnancy, a number of 150, 175 and 200Gy irradiated parasites in blood were injected intraperitoneally to mouse. The monitoring of parasitemia in blood of pregnant and progenies were started 3 days after infection and 2 weeks after delivery, respectively. The number, body weight and survival times of progenies were recorded. Results showed that non irradiated parasites injection produced less progenies than those of irradiated parasites. There were no parasites in blood of progenies of mother injected with non irradiated and irradiated infected bloods. Peak of parasitemia and survival were similar in infected pregnant and non pregnant mice, although development of parasitemia was slightly accelerated in pregnant mice. We concluded that there were no effects of parasite injection to number, body weight and survival time of progenies, suggesting no transmission of parasites from mother to fetus. It is may be due to low number of parasite injected or disappearance of parasites in early age of progenies.

**Key words:** Irradiation vaccine, malaria, parasitemia, *P. berghei*, pregnancy

**INTRODUCTION**

Each year, 25–30 million women become pregnant in malaria-endemic areas of Africa, and similar numbers are exposed to malaria in Asia, Oceania, and South America [1]. In recent years, the burden of malaria in pregnancy in these areas has been estimated with some degree of confidence [2,3]. However, some questions about the burden of mortality and morbidity attributable to malaria in pregnancy remain unresolved. Malaria is an important cause of severe anemia in pregnant women worldwide, and by this mechanism malaria causes an estimated 10,000 maternal deaths each year globally [4]. Moreover, malaria infections result in 75,000–200,000 low birth weight babies each year, due to combinations of preterm delivery and fetal growth restriction [1,5]. Malaria also spares none but affects mostly young children, travelers and pregnant women living in endemic area. Maternal malaria has deleterious effect on developing fetus such as low birth weight, fetal death or abortion [6,7]. Thus, pregnant women, especially primigravidae, are a priority group for vaccination.
Vaccines provide a potentially important way of preventing malaria in pregnancy. Any highly effective, pre-erythrocytic vaccine that induces protection and is not strain specific should protect against malaria in pregnancy. Thus, once it has been established that a pre-erythrocytic vaccine can induce a substantial and sustained level of protection in non-pregnant adults or children, a trial to determine its ability to protect against malaria in pregnancy would be justified [2].

Attenuated parasite vaccines have long been an interest for malaria [8], and so far the major efforts to develop such whole organism vaccines have focused on generating attenuated sporozoites by radiation disruption methods [9,10]. In comparison, less research has been done on live vaccines against the malaria blood stages which are responsible for the clinical symptoms of the disease [8,11]. Attenuated blood-stage vaccines produced by radiation [12,13], have demonstrated effectiveness for protection against parasitemia and symptoms of severe malaria.

In the current study, we investigate the effectiveness of radiation-attenuated blood-stage parasites for protection against parasitemia and severe disease in experimental models of malaria in pregnancy. Mouse malaria models have clear advantages for the study of pregnancy-associated malaria (PAM) pathology due to the relative short gestational period that allows a reasonable experimental time frame and to the availability of a wide variety of immunological and genetic tools. This rodent is also the most accessible and therefore is the best-studied experimental malaria model system. A number of studies of malaria in pregnant animals are available, including a recent study advocating murine P. berghei infection as a useful model of PAM [14-16]. However, the report on the protective capability of irradiated vaccine was not explored extensively, and there are no such studies on immunity and susceptibility to infection in progenies available in PAM.

**MATERIALS AND METHODS**

**Animals, parasites and infection**

Swiss Webster mice of 4 weeks old at the starting experiment were maintained under standard condition (25±3°C, 45-65% humidity). The animals had access to modified standard mouse feed and water ad libitum. All the animals were acclimatized to laboratory condition for 5-7 days before commencement of the experiment. All procedures were in accordance with National Regulations on Animal Experimentation and Welfare, authorized by the Indonesian Ministry of Health’s Animal Welfare Committee. Infected blood containing P. berghei of ANKA strain was obtained from donor mouse with 20 to 40% parasitemia. Blood for inoculation was obtained by cardiac bleeding using 3% sodium citrate as an anticoagulant. Experimental mice received one inoculum of 0.2 ml of P. berghei which was applied intraperitoneally (IP).

**Gamma irradiation**

To attenuate parasite, freshly harvested P. berghei-infected bloods were diluted in citrate phosphate dextrose (CPD) as anti-coagulant to a concentration of about 10^7 parasitized red blood cells (pRBC) per milliliter. One milliliter aliquots of parasites were then immediately exposed to gamma rays of a Cesium-137 source for various time periods at room temperature in IRPASENA Irradiator of the Center for Application of Isotope and Radiation Technology, National Nuclear Energy Agency. Radiation doses used were 150, 175 and 200 Gy and the dose rate was 380.2 Gy/hour.

**Parasitemia, body weight and survival in offspring**

A group of females were put to mate and detection of the vaginal plug and measurement of body weight were jointly used to time gestation, as previously described [17]. The day of vaginal plug detection was considered as gestational day one (D1). Pregnant females were subjected to injection of non irradiated and irradiated parasites at D9. Pregnancy was confirmed between G10 and G13 when the animals had an average increase of 3–4 g in body weight. At delivery, the number of live newborns was registered. Newborns parasitemia, body weight and survival time were recorded started at 2 weeks after birth and followed up to day 60. Non-infected injected pregnant females were used as controls.

**Parasitemia observations**

For parasitemia the blood of pregnant mouse and their progenies was used to make thin blood smear on slides, directly from each mouse’s tail snip, then air-dried and used for the RBC counts. The dry smear was fixed with absolute methanol and stained with 10% Giemsa for 15 – 20 min. Tap water was used to flood off the stain and the slide air-dried. The thin blood film was first examined using the x10 objective and then the monolayer portion
examined with x100 (oil immersion) objective. The percentage parasitemia was taken at each follow-up day that is day 14 and thereafter.

RESULTS

We evaluated the effects of irradiated malaria parasites in pregnancy on survival and growth of progenies at organogenesis stages (D9) by analyzing progenies from 14 pregnant females infected at D9 and from 5 non-infected pregnant females. Table 1 shows that the survival time of pregnant mouse injected with irradiated parasites was higher than those of non irradiated parasites injected mouse. They were survived for more than 40 days. The percentage of parasitemia in pregnant mouse injected with 150 and 175 Gy irradiated parasites was much lower than non irradiated one on days after injection. The highest percentage (1.0-2.0%) was observed in the early days post injection and then decreased to 0% and mice were succumbed to infection (Figure 1).

Table 1. The survival time and number of progenies delivered by irradiated *P. berghei* infected pregnant mouse.

<table>
<thead>
<tr>
<th>No. pregnant mouse</th>
<th>Dose of Radiation (Gy)</th>
<th>Parental Survival time (day)</th>
<th>Progeny No. delivered (mean)</th>
<th>No. infected (percentage)</th>
<th>Survival time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>10 – 29</td>
<td>3 – 4 (3.5)</td>
<td>0 (0)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>&gt;40</td>
<td>8 – 10 (9)</td>
<td>0 (0)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>&gt;40</td>
<td>6 – 7 (6.5)</td>
<td>0 (0)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>&gt;40</td>
<td>4-7 (5.3)</td>
<td>0 (0)</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

The number of progenies delivered was irradiation dose dependence. The number was decreased with the increasing dose of radiation given to the injected parasites into D9 of pregnant mouse. Dose of 150 Gy was more effective in protecting the pregnant mouse by maintaining the higher number of progenies delivered and more stable body weight compared to doses of 175 and 200 Gy. This certainly related to the protection of pregnant mice from maternal anemia, low birth weight, and decreased litter size. This was supported by macroscopic (physical) observation conducted in other research on malaria vaccine that showed that liver and spleen of mouse injected with 150 Gy irradiated parasites were fresher compared to those of 175 Gy and 200 Gy [18]. Higher dose of irradiation results in darker color of liver and spleen due to the higher contents of digested hemoglobin by the parasites. The body weight of 0 Gy irradiated parasites injected mouse was sometimes decreased that might be due to the lost or death of fetus.

![Figure 1. The percentage of parasitemia of pregnant mouse post injection with gamma irradiated *P. berghei* infected mouse blood.](image)

Two weeks after birth there was no parasites found in the blood of progenies of mother injected with 150, 175 or 200 Gy irradiated infected blood. No significant difference found between these doses. The results also showed that there was no effect of irradiated parasite to the number and survival time of progenies. It is predicted that may be due to lower number of parasites injected into mouse. Moreover the observation of parasitemia in progenies was started two weeks after their birth that means that parasites already disappeared in early age of progenies.

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Body weights of pregnant mice were recorded daily. Mice exhibited an increase in body weight during the pregnancy that certainly due to the fetal development. High reduction in body weight in mice was due to delivery on day 21 of pregnancy (13 days post injection). It means that there is no effect of parasite injection on the longevity of pregnancy. However, the injection of irradiated parasites on day 9 of pregnancy affected the body weight of mouse. Non irradiated parasites injection was adversely affected the fetal growth. Their body weights were lower than those of injected with irradiated parasite. Of five pregnant mice progressed to delivery, only three was survived up to day 18 post injection (Table 1). Two other mice did not deliver progenies due to fetal loss that had occurred at least several days earlier before delivery. Fetal loss is one of the most severe and understudied consequences of malarial infection in pregnant women living in areas of low endemicity.

For non irradiated parasites, peak parasitemia following inoculation with parasite-infected murine erythrocytes and survival were similar in infected pregnant and non pregnant mice, although development of parasitemia and anemia was slightly accelerated in pregnant mice. Pregnant mice confer an increased susceptibility to malaria and showed that pregnant mice experienced slightly faster increase in parasitemia as compared to non-pregnant females. Parasitemia in pregnant mice was 63.9% on day 23 post-infection as compared to 57.1% in non-pregnant mice (Figure 2).

The survival to infection was reduced in pregnant mice, with all deaths occurring between day 1 (survived 10 days) and day 20 (survived 29 days) post-infection on D9 (data not shown). In contrast, the majority of non-pregnant infected females survived until day 30 post-infection. Average survival time for pregnant and non-pregnant infected mice was 17.5 and 22.5 days, respectively. Some experiments found the mouse survived until more than 30 days. These results suggest that, similarly to humans, pregnant mice show increased susceptibility to malaria infection which may affect their progeny or compromise pregnancy. Figure 2 shows the increased malaria susceptibility in pregnant mice infected with *P. berghei* of ANKA strain and data were obtained from two mice survived until more than 20 days.

In Figure 2, plots represent cumulative results of three independent experiments in a total of 5 pregnant and 3 non-pregnant females. Parasitemia curves were data points represent mean. From day 3 post-infection onwards parasitemia was higher in pregnant females. Survival curves up to 10 days after infection show that survival time of pregnant female mice are lower than in controls. It should be noted that non-pregnant females died at a later stage with hyperparasitemia.

Figure 3 shows the microscopic observation of parasitemia in blood of mouse injected with non irradiated parasites 5 days after injection where high density of parasites (13.2%) was found. This is significantly different with 150 Gy irradiated parasites of *P. berghei* ANKA strain injected mouse.
Figure 3. Microscopic observation of parasitemia in blood of mouse injected with non irradiated (left) and 150 Gy irradiated (right) parasites of \textit{P. berghei} ANKA strain 5 days after injection. The observation was with 1000 times magnification.

**DISCUSSION**

Pregnant women are susceptible to malaria during pregnancy. \textit{P. falciparum}, which sequesters in the placenta, causes the greatest disease, contributing significantly to maternal and infant mortality [19]. The severity of PAM has been associated with the ability of parasitized erythrocytes to bind to a sugar present in the placenta, chondroitin sulfate A (CSA). After several pregnancies, women acquire protective antibodies that block CSA-binding. In the present study, we show the features of irradiated \textit{P. berghei} injected during pregnancy that is consistent with the current understanding of PAM pathogenesis and immunity. In the mouse model, pregnancy-associated \textit{P. berghei}-infection has adverse consequences for the pregnant mice and their offspring.

Early studies on the interrelationship of malaria and pregnancy in mice used \textit{Plasmodium berghei}. These studies, also in our study, reported a more severe clinical course in pregnant animals, with maternal mortality, fetal loss, and reduced litter size. However, Poovassery, J. and Moore, J.M [20] stated that this model is not suitable to study the development of early maternal antimalarial immune responses or the impact of malarial infection on early pregnancy, because the infections were initiated on day 7 of pregnancy and were lethal to the mother. In a series of papers published in the '80s, Van Zon and coworkers [16] developed a mouse model to study the impact of pregnancy on immunity to \textit{P. berghei} infection. Importantly, they used the model to demonstrate pregnancy-related recrudescences accompanied by severe clinical symptoms in mice with preexisting acquired protective immunity. However research on malaria vaccine for pregnancy created with irradiation is very rare.

Pregnancy modulates the immune system in order to protect the developing fetus from maternal immune attack, many malaria researchers started to see PAM as the inevitable consequence of pregnancy-associated immunosuppression. From this, the development and evaluation of programs to prevent malaria in pregnancy can be facilitated by a better understanding of the pathogenesis of malaria. Malaria in pregnancy is detrimental to the fetus. High grades of fever, placental insufficiency, hypoglycemia, anemia and other complications can all adversely affect the fetus. Both \textit{P. vivax} and \textit{P. falciparum} malaria can pose problems for the fetus, with the latter being more serious. The prenatal and neonatal mortality may vary from 15 to 70%. In one study conducted by Haghdoost et al (2007) it was found that the mortality due to \textit{P. vivax} malaria during pregnancy was 15.7% while that due to \textit{P. falciparum} was 33%. Spontaneous abortion, premature birth, still birth, low birth weight, fetal distress are the different problems observed in the growing fetus. Transplacental spread of the infection to the fetus can result in congenital malaria [21].

Experimental study of malarial infection during pregnancy is particularly problematic, as ethical and logistical constraints limit the longitudinal sampling of pregnant women and the placenta is inaccessible until delivery. An easily manipulable rodent model for malaria in pregnancy would be of great use in overcoming these limitations and improving our understanding of the immunological basis for the poor fetal outcome in non immune pregnant women in areas of low endemicity. Therefore this research is very important and urgently needed.

It is agreed that eradication of malaria is not possible with current tools and that research and development of cost-effective deployable vaccine will be needed to facilitate eradication. Vaccines are often the most cost-effective tools.
for public health and are an area of intensive research. However, there is no effective vaccine that has been introduced into clinical practice [22]. Moreover the mechanisms that control the malaria parasite expansion in pregnancy are still poorly understood and not amenable for study in human subjects. Some important first steps in the development of a vaccine against pregnancy-associated malaria are being done by many researchers. One of them is the determination of efficacy of irradiated vaccine on pregnancy. Vaccination during pregnancy is unlikely to be the most effective way of deploying malaria in pregnancy vaccine because it may be important to protect women from malaria during the first trimester of pregnancy before they present at an antenatal clinic.

Other studies in rats by Bruce-Chwatt, L[23] using the parasite P.berghei established that varying levels of resistance to malaria infection could be transferred from a mother to her offspring during lactation. Two further studies in mice with P. vinckei and in rats with P. berghei also determined that this resistance was due to malaria parasite-specific Abs present in the milk [24]. Although the actual role of maternally derived Abs remains to be established, their presence in the infant and neonate is not disputed. The route of transfer of Abs from mother to offspring differs between humans and mice. Humans acquire their Abs placentally, whereas rodent pups acquire theirs both placentally and in the milk, with the greatest acquisition occurring during the suckling period after birth [25]. Although the route of transfer may differ, the presence of Abs at birth and for a limited duration after birth is similar. This research on the postnatal injection of attenuated parasites is being conducted in our laboratory.

We have studied the effects of gamma attenuated parasites by injecting them on day 9 of pregnancy that is equal or similar with the day 20 of pregnancy in human. This stage is characterized by the formation of most internal organs and external body structures. This stage of pregnancy is the time of organ formation that begins about 3 weeks after human fertilization, when the embryo elongates, first suggesting a human shape. Shortly thereafter, the area that will become the brain and spinal cord (neural tube) begins to develop. The heart and major blood vessels begin to develop by about day 16 or 17. The heart begins to pump fluid through blood vessels by day 20, and the first red blood cells appear the next day. Blood vessels continue to develop in the embryo and placenta [26]. To our knowledge this is the first research that had been done on the protective efficacy of gamma rays attenuated parasites as malaria vaccine in pregnancy.

CONCLUSION

In summary, there were no effects of irradiated parasites of all gamma ray doses on the number, body weight, parasitemia and survival time of progenies. It means that there was no transmission of irradiated parasites from mother to the fetus during pregnancy. It is may be due to low number of parasite injected or disappearance in early age of progenies. In line with our results in the P. berghei model, it might be need to use the higher number or concentration of attenuated parasites injected into the mother that might have an impact in progenies and observed in as early as possible of age of progenies. Pregnant mice show increased susceptibility to malaria infection which may affect their progeny.

REFERENCES