Chapter 4

Essentiality of de novo heme biosynthesis in the sexual and liver stages of malaria parasite
4.1 Introduction

Transmission of malaria parasite from a mammalian host to mosquito vectors requires parasite differentiation into male and female gametocytes, which gets ingested as part of the blood meal by female mosquitoes thereby initiating the sexual phase development in the mosquito midgut. Male gametocytes divide rapidly into a number of motile flagellated microgametes each of which can fertilize a female macrogamete to form a zygote. The parasite then becomes a motile ookinete, penetrating the mosquito gut wall and encysting as a rounded oocyst. The parasite multiplies asexually within this to form many hundreds of motile sporozoites (a process known as sporogony). Mature sporozoites escape through the oocyst wall into the insect’s blood cavity (haemocoel) and thence to the salivary glands, penetrating their walls to reach the mosquito’s stored saliva in readiness for transmission where the sporozoites are injected with saliva into the skin of a vertebrate, by a feeding Anopheles mosquito. These sporozoites enter the bloodstream or lymphatic and circulate to infect the liver in mammalian host (Bannister et.al, 2009). Towards the global fight to control malaria, targeting the sexual stages of malaria parasites to interfere with and (or) block and parasite’s development within mosquitoes, have been as important targets for disrupting the malaria transmission (Kang et al., 2013). Several genes have been implicated to be essential for development of sexual stages in the mosquitoes and liver stage development in mammalian host (detailed under section 1.5.1 and 1.5.3). The Plasmodium genome-coded heme biosynthetic enzymes differ significantly from the mammalian host (human) counterparts in terms of biochemical properties and localization and possess several unique features (reviewed by Padmanaban et al., 2013; Nagaraj et al., 2013b) (detailed under section 1.9.4.1).

Heme is indispensable for parasite growth as it is an essential cofactor for cytochromes in the mitochondrial electron transport chain (Fry et al., 1992; Srivastava et al., 1997). In addition to the possibility of acquiring heme from haemoglobin to make cytochromes in the blood stages, there is also a suggestion that Plasmodium may be able to scavenge heme in the liver stages as well, as is the case with organisms infecting nucleated cells such as T. cruzi, Leishmania and M. tuberculosis (van Dooren et al., 2012). Results from Chapter 3 have shown that the de novo heme biosynthetic pathway is non-essential in the asexual stage development of malaria parasites. Therefore, understanding the role of de novo heme in sexual stages and liver stage is important and may reveal several targets for therapeutic interventions (Padmanaban et al., 2013; van Dooren et al., 2012). In the present study, the
ability of gene knockouts for parasite-encoded heme biosynthetic enzymes ($Pb$ALAS and $Pb$FC) to undergo sexual stage development was assessed by examining the formation of ookinetes at 24h post feeding, oocysts on day 10 and sporozoites on day 19-21. Further, ALA the product of ALAS, the first enzyme of this pathway was provided in feeding solution to the parasite infected mosquitoes to examine whether ALA supplementation could rescue the growth defect of the parasite if any, in $Pb$ALASKO infected mosquitoes. The liver stage development of the knockout parasites was examined by injecting the sporozoites intravenously and monitoring the appearance of asexual stage parasite by preparing Giemsa smear.

4.2 Materials and Methods

4.2.1 Maintenance and propagation of Plasmodium berghei

*In vivo* maintenance and propagation of *P. berghei* wild-type and $Pb$ALAS and $Pb$FC knockout parasites were carried out as described in chapter 3.

4.2.2 Drugs used during *P. berghei* growth and maintenance

Sulfadiazine: Sulfadiazine selectively kills asexual stage of the malaria parasite while the mature gametocytes remain unaffected. Thus, to obtain relatively pure preparations of fully mature gametocytes for *in vitro* ookinete culture, the infected mice were administered with 30mg/L Sulfadiazine (Sigma-Aldrich) in drinking water to the mice when the parasiteemia reached ~5-6% and were subsequently bled after two days of Sulfadiazine treatment.

4.2.3 In vitro ookinete formation in *P. berghei* Wild type and Knockout parasites

*In vitro* ookinete formation in *P. berghei* wild-type and $Pb$ALAS and $Pb$FC knockout parasites was analysed by injecting $2 \times 10^7$ parasites in phenylhydrazine-treated mice, followed by sulfadiazine treatment for two days to remove asexual stages (described under section 4.2.2) and mice were anesthetized with ketamine/xylazine and the infected blood containing only gametocytes was collected through cardiac puncture and passed through CF-11 cellulose (Sigma) columns equilibrated with RPMI 1640 medium to remove leukocytes, the gametocyte-infected blood was diluted with nine volumes of ookinete culture medium RPMI 1640 containing L-Glutamine, 25mMHEPE (Gibco), 25mM Sodium bicarbonate and supplemented with 20% Fetal bovine serum (FBS) (Invitrogen), pH 8.0 at 19°C for 19-21 h (Biller et al., 2004). Thin smear was prepared by taking a known volume of cultured blood,
fixed with methanol and stained with Giemsa reagent and observed under 100X objective to quantify the in vitro formed ookinetes.

4.2.4 Exflagellation assay
Exflagellation is a process of conversion of male gametocytes into male gametes and is an important measure of functional maturity of the gametocytes. The exflagellation assay was carried out as described (Shimizu et al., 2010) with some modifications. In brief, one or two drops of blood were taken from the tail vein of each P. bergehi infected mouse in approximately 1ml of exflagellation medium (RPMI 1640 containing 25mM HEPES, 25mM sodium bicarbonate, 20% FBS, pH 8.0) and incubated at 19°C. The number of exflagellating male gametocytes was counted after 10-15 mins by placing ~10µl of culture into hemocytometer and observed under 40X objective.

4.2.5 Rearing of Anopheles stephensi mosquitoes
Anopheles stephensi mosquitoes were reared under standard insectary conditions maintained at 27°C and 75-80% humidity with a 12h light and dark photo-period as described (Benedict, 1997; MR4, Methods in Anopheles Research, 2010). Larvae were reared on yeast tablets at a fixed density of one larva per ml. Upon maturation, the pupae were segregated for adult emergence. The emerged adult mosquitoes were fed on filter-sterilized 10% glucose solution containing 0.05% paraminobenzoic acid (PABA). The Adult mosquitoes were maintained as F1 generations. For egg production, adult female mosquitoes were allowed to take blood feeding on pathogen free healthy mice anesthetized with ketamine/xylazine. For infectivity studies, the >3 day old mosquitoes were allowed to feed on filter-sterilized 10% glucose solution containing 0.05% PABA and antibiotics (1% Penicillin-streptomycin cocktail and 40µg/ml gentamycin) for 3-4 days and used for infection with Plasmodium spp.

4.2.6 Observation of P. bergehi infection in An. Stephensi
4.2.6.1 Determination of ookinete
To study the in vivo ookinete in Plasmodium, fully engorged mosquitoes 20 h post feeding of the infectious blood meal are anesthetized at 4°C and transferred onto a microscope slide containing one or two drops of ookinete medium or PBS. Visualizing under the dissection microscope, hold the thorax of mosquito with a fine pointed forceps and gently pull the penultimate abdominal segment with 20G needle to remove blood bolus. Gently rupture and
blood-filled midgut into the drop of PBS or ookinete medium and make homogeneous suspension to transfer into a microfuge tube and centrifuged at 2000xg for 3 min to remove the supernatant retaining a smaller volume to prepare thin smear, fixed with methanol and stained with Giemsa reagent and observed the under 100x objective lens. The morphology of normal ookinetes appears as banana shaped bodies.

4.2.6.2 Determination of Oocyst

Mature oocyst stages of *Plasmodium* can be observed from day 10 post feeding of infectious blood meal, till day 14 in the midgut of infected mosquito. In brief, on day 10 post feeding, the mosquitoes are anesthetized at 4°C and transferred onto a microscope slide containing one or two drops of PBS. Visualizing under the dissection microscope, hold the thorax of mosquito with a fine pointed forceps and gently pull the penultimate abdominal segment with 20G needle to remove the mid gut. Mature oocyst appears like a distinct rounded structure on the midgut surface under 10X or 40X objective lens. However, for determination of oocyst number infected midgut can be stained with 0.5% mercurochrome prepared in PBS for 3 min and washed twice with PBS and observed under microscope at 10X and or 40X objectives lens.

4.2.6.3 Determination of Sporozoite

Anesthetize >19 Day infected mosquitoes and transfer on to microscope slide containing one or two drops of PBS (DMEM medium can be if the sporozoites being used for infection studies). Visualizing under the dissection microscope, hold the thorax of the mosquito with a fine pointed forceps and gently tease the head away from the thorax with 20G needle, the tissue that emerges from the neck will contain both sets of salivary glands that appear as opalescent or transparent trilobed ‘bunches-of-grapes’. Gently tease the glands apart with a needle, or place a cover slip which will rupture and glands and release the sporozoites that can be observed under the 40X objective lens. Sporozoites are straight or sinuous wormlike cells and were virtually free of microscopically visible mosquito debris, and sporozoites were viable as judged by their motility when observed under the microscope.

4.2.7 Isolation of sporozoite and counting

The salivary glands from infected mosquitoes are dissected out as described in section (4.2.6.3) and transferred into DMEM medium in a microfuge tube and gently vortexed for few mins to facilitate the rupture and release of sporozoites. The suspension is then passed
through the column packed with sterile glass wool and flow collected which contains only sporozoites in DMEM medium. 10µl of this flow through containing sporozoites is added on to counting chamber in hemocytometer and counted for the number of sporozoites present in 0.25 X 0.25 mm (0.0625 mm$^2$) area which represent the volume of 6.25 nl of sample.

4.2.8 P. berghei infection studies in Anopheles stephensi

P. berghei infection studies in A. stephensi mosquitoes were carried out as described elsewhere (Nacer et al., 2008; Ellekvist et al., 2008). In brief, antibiotic-treated adult female mosquitoes of 5-7 days old, starved for 12 h, were allowed to feed on anesthetized P. berghei infected mice with 8-12% parasitemia showing 2-4 exflagellation centres per field. The fully engorged mosquitoes were then separated and maintained at 19°C with 70-80% relative humidity. At 20 h post feeding, the mosquito midguts were dissected to remove the blood bolus and ookinete numbers were quantified as described (Shimizu et al., 2010). On day 10 post feeding, 0.5% Mercurochrome staining was carried out for the dissected midguts to determine the number of oocysts formed (Usui et al., 2011), followed by the dissection of salivary glands on day 19 to examine and count the number of sporozoites present (Touray et al., 1992). To supplement the PbALASKO infected mosquitoes, routine feeding was carried out with sugar solution containing 0.1% ALA from 20 h post feeding until the dissection of salivary glands on day 19. To supplement PbFCKO-infected mosquitoes, blood feeding was given to the mosquitoes in six day interval from the day of infection till the sporozoite analysis, besides the routine feeding with sugar solution.

4.2.9 Sporozoite infections in mice

The ability of the sporozoites to develop asexual stage infections was studied by allowing the mosquitoes infected with P. berghei wild-type and knockout parasites to feed for 15-20 min on 6-8 weeks old Swiss mice (30 mosquitoes/ mouse) anesthetized with ketamine/xylazine. The development of asexual stage parasites was monitored by examining the Giemsa stained blood smears from day 5 post infection. To inject $10^4$ sporozoites intravenously in mice, salivary gland extracts of the infected mosquitoes were prepared and sporozoites were counted as described (Touray et al., 1992). ALA supplement in mice was carried out immediately after sporozoite infection and continued for 7 days by including 0.1% ALA in drinking water.
4.2.10 Statistical analysis

Statistical analysis was performed using unpaired t-test of Excel software with two-tailed distribution and unequal sample variance. P values of < 0.05 were considered as significant. Graphs were prepared using Sigmaplot 10.0. Error bars given in the figures represent the standard deviations.

4.3 Results

4.3.1 The role of parasite biosynthetic heme in the mosquito stages

To examine the role of parasite-synthesized heme in the mosquito stages, A. stephensi mosquitoes were allowed to feed on mice infected with PbWT and PbKO parasites. Figure 4.1–4.2 shows that both PbWT and PbKO parasites formed ookinetes. No difference was found in the formation of ookinetes in vitro using gametocytes cultures or in vivo using midgut preparations between the WT and KO parasites. In contrast, Figure 4.3 shows a drastic decrease in PbALASKO and PbFCKO oocysts formation in the midgut and absence of PbALASKO and PbFCKO sporozoites in the salivary glands (Figure 4.4). Furthermore, it was examined whether ALA supplement could overcome the block in PbALASKO parasites for which 0.1% ALA was supplemented in feeding solution (PbALASKO(Mq^ALA)). The results obtained indicate that the formation of oocysts and sporozoites were restored (Figure 4.3–4.4). These results reveal that parasite heme synthesis was required for oocyst and sporozoites development in the mosquitoes. In case of PbFCKO, heme supplementation through blood feeding on mice was attempted, but it was not able to rescue the defect. This suggests that the parasite could not acquire heme from the mouse hemoglobin in the mosquito blood meal or from any other mosquito source during the sexual stages of its development.

![Figure 4.1: In vitro ookinete formation in of P. berghei-infected (WT and KOs) mosquitoes. (A) Quantification of ookinetes formed in vitro using gametocyte cultures. The data represent](image-url)
three independent experiments; \( P > 0.05 \). (B) Ookinete formed \textit{in vitro} and stained with Giemsa reagent. Scale bar: 5 µm.

**Figure 4.2**: \textit{In vivo} oocyst formation in \( P. \) \textit{berghei}-infected (WT and KOs) mosquitoes

(A) Quantification of oocystes formed \textit{in vivo}. (B) Ookinete formed \textit{in vivo} and stained with Giemsa reagent. Scale bar: 5 µm. The \textit{in vivo} data are from 30 mosquitoes from 3 different batches; \( P > 0.05 \).

**Figure 4.3**: Oocyst formation in \( P. \) \textit{berghei}-infected (WT and KOs) mosquitoes. (A) Mercurochrome staining of oocysts in the midgut preparations. Arrows indicate oocysts and the magnified images of oocysts are provided in insets. Scale bar: 100 µm. (B) Quantification of oocysts. \( P \) values for \( PbALASKO \) and \( PbFCKO \) with respect to WT are < 0.02. \( P \) value for \( PbALASKO(Mq^{+ALA}) \) with respect to \( PbALASKO \) is < 0.01 and \( PbFCKO(Mq^{+Blood}) \) with respect to \( PbFCKO \) is > 0.05. The data represent 90 mosquitoes from 3 different batches.
Figure 4.4: Sporozoite formation in *P. berghei*-infected (WT and KOs) mosquitoes. (A) Sporozoites in the salivary glands. Magnified images of sporozoites are provided in insets. Scale bar: 50 µm. (B) Quantification of sporozoites. P values for *PbALASKO, PbFCKO, PbALASKO(Mq<sup>ALA</sup>) and PbFCKO(Mq<sup>Blood</sup>)* with respect to WT are < 0.01. The data represent 90 mosquitoes from 3 different batches. UI, uninfected; Mq, mosquitoes; *PbALASKO(Mq<sup>ALA</sup>)* and *PbFCKO(Mq<sup>Blood</sup>), P. berghei* KO parasites from mosquitoes supplemented with ALA and blood feeding, respectively.

4.3.2 The role of parasite biosynthetic heme in liver stage development

The ability of *PbALASKO(Mq<sup>ALA</sup>)* sporozoites to reinfect mice by measuring the parasitemia in the mice on subsequent days with and without ALA supplement (0.1% in drinking water) was examined. No parasites were detected in the mice infected with *PbALASKO(Mq<sup>ALA</sup>)* sporozoites that did not receive ALA supplement (*PbALASKO(Mq<sup>ALA</sup>−ALA)).* However, parasites were detected in the mice infected with *PbALASKO(Mq<sup>ALA</sup>)* sporozoites that received ALA supplement (*PbALASKO(Mq<sup>ALA</sup>−ALA+ALA)).* The infected animals died after 14-16 days, when the parasitemia levels reached around 60% (Figure 4.5). Mosquitoes infected with *PbALASKO* parasites (without ALA supplement) failed to give rise to blood-stage parasites in mice when they were allowed to feed. This is an additional proof to suggest that the *PbALASKO* parasites did not form sporozoites in the mosquito salivary glands. All the mosquito transmission experiments were reproduced by intravenously injecting the sporozoites obtained from mosquito salivary gland extracts into mice. Thus, the results suggest that parasite heme synthesis is absolutely essential for liver-stage development. These results
discount the suggestion (vanDooren GG *et al.*, 2012) that the parasite may import host synthesized heme during the liver stage.

**Figure 4.5:** Ability of *P. berghei* sporozoites (WT and KOs) to infect mice with and without ALA supplement to the animals. Mosquitoes were allowed to feed on mice (30 mosquitoes/mouse) and parasitemia in blood and mortality of the animals were assessed. The data represent 9 mice each from three different batches. Mq, mosquito; Mi, mice; *PbALASKO*(Mq⁺ALA Mi⁺ALA), *PbALASKO* supplemented with ALA in mosquitoes and mice; *PbALASKO*(Mq⁺ALA Mi⁻ALA), *PbALASKO* supplemented with ALA in mosquitoes but not in mice; *PbFCKO*(Mq⁺Blood), *PbFCKO* supplemented with blood feeding in mosquitoes.

### 4.4 Discussion

The growth pattern of the Knockout parasites in the mosquito stages was striking. While ookinetes formed, oocysts formation decreased substantially, and no sporozoites appeared in the salivary glands. Furthermore, when these mosquitoes fed on mice, we found no intraerythrocytic-stage parasites in the blood of the mice. ALA supplement to the mosquitoes enabled *PbALASKO* to form oocysts and sporozoites. This is clear proof that the *de novo* parasite heme synthesis is required for parasite development in mosquitoes. Hence, inhibitors of heme and porphyrin synthesis, such as diphenyl ether herbicides, can be explored to prevent parasite development in mosquitoes (Jacobs *et al.*, 1992). Equally striking was the
growth pattern of PbALASKO parasites in the liver stage. The sporozoites formed in the mosquitoes with ALA supplement could infect mice only when the mice received ALA supplement. This again shows that parasite de novo heme synthesis is required for development in the liver stage. The liver stage is a major focus of malaria interventions and the role of parasite heme synthesis in liver-stage development needs to be investigated in more detail. Inhibitors of parasite heme synthesis offer newer drug candidates for blocking infection and transmission, since the parasite enzymes involved have unique properties (Nagaraj et al., 2008; 2009; 2010; Padmanaban et al., 2013). Irradiated sporozoites serve as a malaria vaccine candidate (Vanderberg, 2009).

There are several current efforts to design and stabilize irradiated sporozoites for large-scale clinical trials (Vanbuskirk et al., 2009; Roestenberg et al., 2013). Based on the results obtained for PbALASKO(Mq+ALA) sporozoite infections in mice, PbALASKO sporozoites were tested for its potential as genetically attenuated sporozoite vaccine. The biology of parasite heme synthesis may change drastically between the intraerythrocytic stages and the mosquito and liver stages. The malaria parasite essentially depends on glycolysis to generate ATP in the intraerythrocytic stages. Hemoglobin is available as a heme source in addition to parasite-synthesized heme. In the mosquito and liver stages, the parasite depends entirely on its own biosynthetic machinery to provide heme. It is possible that the de novo heme-biosynthetic pathway of the parasite is augmented during the mosquito and liver stages. The ATP synthesized by the ETC may be necessary to provide the energy needed for ookinetes in the mosquito midgut to develop into sporozoites in the mosquito salivary glands. The energy provided by the ETC may also be necessary for the sporozoites to explore the mammalian host from the skin to liver and give rise to merozoites in the hepatocytes.