Effects of the antimalarial drugs ferroquine and artesunate on *Plasmodium yoelii yoelii* gametocytegenesis and vectorial transmission

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Abstract

Chemistry still has a role in the management of malaria, alongside the mosquito netting soaked in insecticide that is used increasingly, as we continue to await the long anticipated vaccine. During its cycle, the hematozoon parasite develops through three major periods. The first, malarial infection, corresponds to the intrahepatic development of infective forms from the mosquito vector; this period is not sensitive to treatment and is often asymptomatic. The period of erythrocytic schizogony is the most urgent, and treatment activity is primordial. Finally, the phase of sexual reproduction, when gametocytes develop within the erythrocytes ensures the perpetuation of the species when these reach the blood-feeding female anopheles mosquitoes. The aim of our work was to study the effect on gametocytes of drugs known to be effective on the asexual blood forms of the protozoan and thus the potential repercussions on malaria transmission. This experimental study was conducted with an animal model whose parasite cycle and modes of transmission are close to those of human malaria: *Plasmodium yoelii*, maintained on Swiss mice, with the *Anopheles stephensi* vector (maintained in an animal facility at the National Museum of Natural History in Paris). Two drugs were tested: ferroquine (a chloroquine derivative with a ferrocene molecule at the lateral carbon chain that restores its efficacy against chloroquine-resistant strains) and artesunate (a derivative of artemisinin, from *ginghao*, a Chinese plant also known as *artemisia annua*, or sweet wormwood), a treatment of choice in the combined therapies recommended by WHO. The efficacy of these drugs, prescribed at doses subcurative for the asexual forms, were tested against gametocyte production, quantitatively by counting them in the blood and qualitatively by counting the quantity of oocysts developed on the mosquito’s midgut, which are indicators of gametocyte activity. The mice that were parasite-infected and then treated served as their own controls: lots of 30 mosquitoes fed on each mouse before treatment and then 90 minutes and 5 hours after treatment. Quantitatively, the comparison of the blood parasite level and the gametocyte index shows that treated mice had a higher level of circulating gametocytes than untreated parasite infested mice, regardless of drug or dose (5 or 10 mg/kg). For artesunate at 5 mg/kg, we noted that the blood gametocyte level was almost double that of the controls. On the other hand, qualitatively, the first results obtained with optical and electronic microscopy showed morphologic alterations of the circulating gametocytes (pigment clumping and lateralisation within red blood cells) and reduced infectivity of the gametocytes for the mosquitoes that fed at 1 and 5 hours after treatment. We were able to demonstrate statistically that the infectivity of gametocytes, measured by the quantity of oocysts counted in the mosquito midgut, was reduced by 70% for those treated with ferroquine and by 85% for those from mice treated by artesunate. Complementary studies will seek to specify the populations (age) of gametocytes damaged by treatment and the importance and nature of their morphologic alterations.

Key words: artesunate; ferroquine; gametocyte; *Plasmodium yoelii yoelii*; vectorial transmission.

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Résumé

Actions de la ferroquine et de l’artésunate sur la gamétocytagénèse et la transmission vectorielle de Plasmodium yoelii yoelii

La chimie trouve encore sa place dans la prise en charge du paludisme entre la vaccination très attendue et la moustiquaire imprégnée d’insecticides de plus en plus utilisée. Au cours de son cycle, le hématozoaire évolue en trois grandes périodes : l’imaludation correspondant au développement intrahépatique des formes infectantes venues du moustique vecteur, cette période est peu sensible aux thérapeutiques mais souvent asymptomatique, la période de la multiplication intra-érythrocytaire est la plus bruyante et l’action thérapeutique primordiale, enfin, la phase sexuée du parasite où les gamétocytes développés dans les érythrocytes assureront la pérennité de l’espèce chez le moustique anophèle femelle hématophaghe.


La souris parasitée et traitée est son propre témoin : des lots de 30 moustiques se gorgent sur elle avant le traitement et une heure 30 et cinq heures à la suite de la prise de thérapeutique. Quantitativement, la comparaison de l’indice gamétocytaire, de la parasitémie et de l’index gamétocytaire montre, chez les souris traitées comparées aux souris parasitées non traitées, une augmentation des gamétocytes circulants, quel que soit le médicament ou la dose (5 ou 10 mg/kg). Pour l’artésunate à 5 mg/kg, on a pu noter que la gamétocytagénie était pratiquement double de celle des témoins. En revanche, qualitativement, les premiers résultats obtenus en microscope optique et électronique montrent des altérations morphologiques des gamétocytes circulants (amas de pigments, latéralisation des gamétocytes dans l’hémocytoblaste) et une moindre infectivité des gamétocytes pour les moustiques gorgés une et cinq heures après la prise de thérapeutique. Nous avons statistiquement pu démontrer que l’infectivité des gamétocytes, mesurée par la quantité d’oocystes comptés sur l’estomac du moustique, était respectivement diminuée de 70 % pour celles traitées par la ferroquine et de 85 % pour ceux issus de souris traitées par l’artésunate. Des études complémentaires chercheront à préciser les populations (âge) de gamétocytes atteintes par les thérapeutiques et l’importance et la nature des altérations morphologiques des gamétocytes traités.

Mots clés : artésunate ; ferroquine ; gamétocyte ; Plasmodium yoelii yoelii ; transmission vectorielle.

For curative and prophylactic treatment drugs have a primary role in the fight against malaria for individuals and groups, including mosquito nets impregnated with insecticides [1] and intermittent treatment for pregnant women and young children until vaccination becomes available. The priority in the choice of drugs to be developed depends on their efficacy in clinical emergencies, including the development of asexual forms of erythrocytes. On the other hand, it is necessary to pursue research into the efficacy of drugs on other parasitic forms, in particular intrahepatic asexual forms and sexual expression ensuring the transmission and durable maturation of plasmodial species in the mosquito, i.e. gametocytes.

Many studies undertaken to study transmission have therefore depended on breaking the parasite cycle by using antimalarials at subcurative doses, which increases the damage to parasites producing the gametocytes. Some of these studies have demonstrated that subcurative doses increase the gametocytic index (number of gametocytes/number of parasites). Buckling et al. [2]
reported that gametocytogenesis in mice infected with *Plasmodium chabaudi* 4-6 days post-treatment with a subcurative dose of chloroquine (12 mg/kg) was 2.5 times higher than that of the control group. However, other studies on human Plasmodia *Plasmodium falciparum* and *Plasmodium vivax* and a murine Plasmodium, *Plasmodium vinckei petteri*, demonstrated no increase in gametocytogenesis following treatment with chloroquine or quinine [3-6]. Various studies have been performed to study the infectivity of gametocytes in mosquitoes following treatment with subcurative doses. A significant decrease in infectivity of gametocytes of *P. v. petteri* was reported in mosquitoes fed on mice one hr (H1) post-treatment [3] and five hours (H5) later in *Plasmodium cynomolgi* treated with CDRI 80/53 (elubaquine: a new 8-aminoquinoline analogue of primaquine). Oocyst development was inhibited at a dose of 3.75 mg/kg of CDRI80/53, indicating a gametocytocidal action of the compound [7]. Ramkaran and Peters [8] reported enhancement of the short-term effects (H12 post-treatment) of chloroquine on the infectivity of gametocytes with the chloroquine-resistant strain of *Plasmodium berghei* (NK65) and with the chloroquine-sensitive strain of *P. chabaudi* [9], whereas no enhancement of infectivity was found for gametocytes of a drug-sensitive strain of *P. v. petteri* [3].

Our aims were to measure the eventual effectiveness of known treatments and of those under development on gametocytogenesis and to follow the effects on transmission by measuring the infectivity of gametocytes in mosquitoes by enumeration of the number of oocysts found in the mosquito stomach.

**Aims of study**

The aims of the study were to evaluate quantitatively and qualitatively the effects of subcurative doses of ferroquine and artesunate on the gametocytogenesis of rodent *Plasmodium yoelii yoelii* at H0, H1h30, H5, H24 and H48 post-treatment, and transmission to mosquitoes at H0, H1h30 and H5 after drug administration.

**Materials and methods**

**Biological material (figure 1)**

**Parasites**

We used *P. y. yoelii* [10], strain 17X, isolated from the rodent *Thamnomys rutilans* in the Central African Republic in 1969 and cloned (clone 1.1), for its high production of gametocytes. It was maintained in Alseveer’s solution at - 80 °C by the Laboratory of Comparative Parasitology of the Museum National d’Histoire Naturelle (Paris).

**Rodents (mice)**

Experiments were carried out on female Swiss mice, 25 g body weight (Janvier laboratory). The mice were maintained in cages in an animal research facility and fed a special diet.

**Vector**

*Anopheles stephensi* of Indian origin was used as vector; the complete cycle in this mosquito has been maintained continuously in the insectarium of the Muséum national d’histoire naturelle de Paris at 23 °C and relative humidity of 70-80%. All mosquitoes used in the experiments were 5-7 days old.

**Methods**

**Infection of mice**

Throughout the entire experimental setup mice were infected intraperitoneally with 10⁶ parasitized (*P. y. yoelii*) red blood cells (blood was collected from infected mice following parasitemia and smeared as thin blood films on clean microscope slides. Smears were stained with Giemsa stain in phosphate buffer.
Treatment of mice: drugs

The drugs used in the present study were administered at subcutaneous doses, which fail to kill all the parasites, but some surviving parasites may suffer ill effects and be stimulated to produce gametocytes. Subcutaneous therapy is common as partially protective prophylaxis [11]. We studied the effects of two antimalarials, ferroquine and artemunate (Sanofi Aventis), on the development of gametocytes in the bloodstream and their eventual infectivity in the mosquito vector:

- ferroquine is a chloroquine molecule whose side chain has been replaced by a ferrocene nucleus [12]; this ferrocene nucleus facilitates the penetration of the molecule into red blood cells and maintains contact with the parasite, thereby ensuring the toxic action of chloroquine on it. Ferroquine is equally active on CQ-sensitive and CQ-resistant *P. falciparum* laboratory strains and field isolates [13];
- artemunate is a derivative of artemisinin extracted from natural Qinghao (chinese herb: *Artemisia annua*). This strong schizonticide has a short half-life (20-25 minutes). The artemisinin derivatives have a mode of action involving the iron-catalyzed generation of a carbon-centered free radical, followed by the alkylation of malaria-specific proteins [14]. Each drug was diluted in distilled water and injected subcutaneously into mice at doses of 60 μL (5 mg/kg) and 120 μL (10 mg/kg).

**Table 1. Gametocytogenesis experiment: drugs and doses used for each batch.**

<table>
<thead>
<tr>
<th>Batch</th>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 1</td>
<td>Ferroquine</td>
<td>5</td>
</tr>
<tr>
<td>B 2</td>
<td>Ferroquine</td>
<td>10</td>
</tr>
<tr>
<td>B 3</td>
<td>Artesunate</td>
<td>5</td>
</tr>
<tr>
<td>B 4</td>
<td>Artesunate</td>
<td>10</td>
</tr>
<tr>
<td>B 5</td>
<td>Untreated</td>
<td>-</td>
</tr>
</tbody>
</table>

All drugs were administered subcutaneously.

*Figure 2.* Gametocytes in mosquitoes

**Parasite evaluation**

Tail blood was smeared in thin blood films, fixed with methanol and stained with a solution of 10% Giemsa stain in phosphate buffer (pH 7.4) for 45 min. One thousand red blood cells were examined to determine the percentage of infected erythrocytes and subsequent determination of parasitaemia and gametocytaemia as follows:

- parasitaemia (P): number of red blood cells infected (asexual forms) per 100 red blood cells;
- gametocytaemia (G): number of gametocytes (sexual forms) per 100 red blood cells infected;
- gametocytopogenesis index (GI): the gametocytaemia to parasitaemia ratio. Additionally, the morphology of gametocytes was studied through the examination of some elements by optical and electronic microscopy.

**Statistical analysis**

The data were analyzed using the *T* test and Fisher test, the level of significance being set at 0.05.

**Results**

**Effects of ferroquine and artemunate on gametocytaemia and gametocytopogenesis**

Parasitaemia: the effects of ferroquine and artemunate on parasitaemia did not differ between the five batches of mice (B1, B2, B3, B4 and B5) on day 3 post-infection at H0 (*figure 4A*). Parasitaemia reduced one and half hours (H1H30) post-treatment in all batches except for the control batch and the batch treated with 5 mg/kg ferroquine. The reduction in parasitaemia of the three groups was slight except for the group treated with
10 mg/kg ferroquine, which decreased by almost 15% compared to H0 before treatment. The parasitaemia of the control group increased by almost 45%.

Parasitaemia reduced in the four-treatment batches five hours post-treatment (H5), as at H1h30. The reduction was slight, except in the group treated with 10 mg/kg artesunate where it decreased by 14% compared with H0 prior to treatment. The parasitaemia of the control batch increased by 35%. The 10 mg/kg ferroquine dose was thus effective against the asexual stage at H1h30 while artesunate at the same dose was effective at H5.

Gametocyturia: gametocyturia at H1h30 compared with H0 prior to treatment increased in all the batches treated with ferroquine, artesunate (5 mg and 10 mg/kg) and in controls (figure 4B). The increase in gameto-
cyturia was almost two-fold higher compared with H0 at the 5 mg/kg artesunate dose, whereas with the other batches treated with 5 and 10 mg/kg ferroquine and 10 mg/kg artesunate the increase in gameto-
cyturia was approximately half that of H0 in every batch. The increase was significant only with 5 mg/kg artesunate (P < 0.04). In contrast gameto-
cyturia in the controls decreased slightly.

At H5 gametocyturia was increased in all treatment groups, including the control group. The gametocyturia level of the batch treated with 5 mg/kg artesunate increased more than 1.5 fold compared to H0 (significant, P < 0.02), whereas the increase in gameto-
cyturia of the two batches treated with 5 and 10 mg/kg ferroquine was a quarter that at H0. The game-
tocyturia levels of the batches treated with 10 mg/kg artesunate and controls increased slightly. Thus artes-
unate at the dose of 5 mg/kg resulted in more gametocytes compared to other batches.

Gametocytogenesis (table 3) increased overall after H1h30 post-treatment. Gametocytogenesis increased in all the batches except the batch treated with 10 mg/kg artesu-
nate and the control batch. There was a significant (P = 0.03) increase in gametocytogenesis in the batch trea-
ted with 5 mg/kg artesunate two-fold higher compared with H0 before treatment and it was increased by half in the batches of mice treated with 10 mg/kg ferroquine and by less than a quarter in the batch of mice treated with 5 mg/kg ferroquine. The reduc-
tion in gametocytogenesis observed in the batch of mice treated with 10 mg/
kg artesunate and the control batch decreased almost 1.5-fold compared to H0, whereas gametocytogenesis increased in all the batches of treated mice five hours post-treatment. The increase was almost two-fold for the group treated with 5 mg/kg artesunate (significant, P = 0.006) and approxi-
ately 1.5-fold for the other groups treated with 10 mg/kg artesunate, 5 and 10 mg/kg ferroquine, compared with H0 before treatment for every group.

The gametocytogenesis was thus higher with 5 mg/kg artesunate both at H1h30 and at H5. At H48, gameto-
cytogenesis decreased for all treatments including the controls.

On the other hand, morphological changes were seen from the first hour of treatment until H24 on optical and electronic microscopy (data not shown) (figure 5).

### Table 2. Doses of drugs administered for experiments on gameto-
cyte infectivity in mosquitoes.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Batches</th>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Batch 1</td>
<td>Ferroquine</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Batch 2</td>
<td>Ferroquine</td>
<td>5</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>One batch</td>
<td>Artesunate</td>
<td>5</td>
</tr>
<tr>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>One batch</td>
<td>Ferroquine</td>
<td>10</td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>One batch</td>
<td>Artesunate</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Drugs administered orally.
<sup>b</sup> Drugs administered subcutaneously.

### Effects of ferroquine and artesunate on gametocyte infectivity

Mice for each experiment were exposed to mosquitoes (30 mosquitoes) at H0 before administration of drugs and after 30 minutes of feeding. The mice were treated with the two drugs (H1h30 and H5). The number of oocysts reduced in all mosquitoes, regardless of the drug used and the dose; the inhibitory effect was significant. With 5 mg/kg ferroquine, the reduction in oocyst numbers was about
Discussion

Many factors influence the transmission of malaria, such as impact point of the drug, dose, strain and time of action in vivo or in vitro, species of host infected etc., and the results reported by others may seem contradictory [2, 3]. The studies on production and infectivity of gametocytes of rodent *Plasmodium* are difficult to standardize and interpret. The parasitaemia levels (whether near or during a crisis) have an essential inhibitory role due to the release of toxic substances [15] and gametocytaminaemia may vary from one individual mouse to another. The experimental conditions also vary in different laboratories, thus generating contradictory results and thereby blurring transmission experiments.

Gametocytaminaemia, which was studied quantitatively by evaluating the number of gametocytes post-treatment, increased at H1h30 and H5 post-treatment (figure 4B). Buckling and Read [16] reported augmentation of gametocytogenesis with murine plasmodium *P. chabaudi* (chloroquine-sensitive lines) and in human plasmodium. This is in agreement with our in vivo results with *P. yoelii*, which showed that subtherapeutic treatment with 5 mg/kg and 10 mg/kg ferroquine and artesunate increased gametocytogenesis H1h30 and H5 following treatment. The same results were found in vivo by Beavogui et al. [17], Buckling et al. [18], Hogh et al. [4] and Puta and Manyando [19] with sulfadoxine-pyrimethamine, and in vitro with eight antimalarial drugs by Peatey et al. [20].

In contrast to our results, Gautret et al. [3] showed that subcurative doses of chloroquine had no effect on gametocytogenesis of *P. v. petteri* during the 3 days following the end of treatment. The difference in behaviour among murine *Plasmodium* species related to specific intrinsic characteristics was clearly demonstrated by Gautret et al. (1997), following administration of phenylhydrazine to mice, a drug that induces strong reticulocytaemia and haemolytic anaemia. There was an increase in gametocyte numbers in mice infected with *P. chabaudi* but not in those infected with *P. v. petteri*, demonstrating that the gametocytogenesis of two species of *Plasmodium* was triggered differently when the parasites developed in the reticulocytes.

Our results pertaining to the effects of artesunate on gametocytogenesis disagree with the results of Pukrittaya-kamke et al. [21] which indicated that artesunate is a potent inhibitor of gametocytogenesis of *P. falciparum* with several subcurative doses daily (3.3 mg/kg on the first day and then 1.65 mg/kg for a further 6 days), but we did not compare the number of gametocytes at the same times post-treatment in the same species, or with the same time periods. The heterogeneity of gametocytaemia (age, transmission power) complicates interpretation of gametocytaemia counts. The different gametocyte stages remain to be studied. It was necessary to evaluate qualitatively the role of gametocytes (infectivity) post-treatment.

Our results demonstrated the reduction in infectivity for both drugs and doses. Reductions in oocyst count were slightly different according to the drug and dose at H1h30 and at H5: oocyst numbers reduced at H1h30 and H5 post-treatment with 5 mg/kg ferroquine were reduced by 60% and 80% ± 1, and 80% ± 3 and 90% ± 3 with artesunate compared to H0. With the 10 mg/kg dose at H1h30 and H5, oocyst numbers were reduced by 70% ± 5 and 90% ± 2 post-treatment with ferroquine and by 80% ± 3 and 98% ± 2 with artesunate compared to H0 (table 4). Comparable results were observed in in vitro studies with *P. falciparum* by Chotivanich et al. [22] who reported that artesunate had a potent effect on gametocyte infectivity in *A. dirus*. At a low concentration (1.5 ng/mL), 90% inhibition of infectivity was observed with both quinine and primaquine, but at relatively high concentrations the inhibition of infectivity in vitro by quinine was 67% and by primaquine it was 44%. According to the results of [23], artesunate at a total dose of 100 mg over 6 days inhibited the infectivity of *P. falciparum* gametocytes. Chen et al. [24] and Price et al. [25] stated that artemisinin derivatives can influence the infectivity of gametocytes of *P.
Figure 4. Development of parasitaemia (A: ---) and gametocytaemia (B: ...) within 48 hours after treatment with ferroquine (FQ) and artesunate (AS) compared to controls. The X-axis represents time and the Y-axis the % of parasites (A) and the % of gametocytes (B) exposed.

Figure 4. Développement de la parasitémie (A: ---) et de la gamétocytémie (B: ...) 48 heures après le traitement à la ferroquine (FQ) et l’artésunate (AS) comparé aux contrôles. L’axe des X représente le temps et l’axe des Y le % de parasites (A) et le % de gamétocytes (B).
falciparum by more effective blocking of the transmission of P. falciparum malaria than mefloquine. Puri and Dutta [7] found a drastic reduction in mosquito infectivity and oocyst development post-treatment of P. cynomolgi with elubaquine at subcuratives doses of 0.63, 1.25, 1.87 and 2.5 mg/kg in rhesus monkeys, thus demonstrating a dose-dependent effect. Complete inhibition was obtained at H5 post-treatment with doses of 3.75 and 5 mg/kg, the mature gametocytes subsequently becoming non-infective to mosquitoes. The percentage of inhibition of the infectivity of gametocytes of P. v. petteri in mosquitoes at H1 and H12 post-treatment with chloroquine (5 mg/kg) was about 40% and 60%, respectively, while there was no significant effect on transmission H12 post-treatment with 1 mg/kg chloroquine [3]. Comparable results were reported by Klein et al. [26], who observed a temporary inhibition of transmission of gametocytes of human plasmodial parasite P. vivax by Anopheles darlingi at H4 post-treatment with 600 mg/kg chloroquine. Ichimori et al. [27] with P. yoelii nigeriensis and Gautret et al. [9] with P. chabaudi showed an increase in mosquito infectivity after treatment with chloroquine. As mentioned above, our study revealed the presence of damaged gametocytes (morphological changes and pigment clumping) after treatment (data not shown), and Kombila et al. [28] previously observed that the gametocytes of P. falciparum were deformed 3 hours after treatment with artemether.

Our results show that subcurative doses of ferroquine and artesunate have an effect on gametocytogenesis of P. y. yoelii following treatment, especially 5 mg/kg artesunate at
H1h30 and H5. In our experiments with *P. y. yoelii*, a general reduction in infectivity of about 60-80% at H1h30 and 75% (73%-100% (98%)-100% at H5 post-treatment was observed (table 4), as reported by others [7, 22].

**Conclusion**

We performed an *in vivo* study of the effects of artesunate and ferroquine on the gametocytogenesis of the rodent malaria parasite *P. y. yoelii* and its vector *A. stephani*. We also studied the effects of the same drugs on the infectivity of gametocytes. Subcurative doses (5 and 10 mg/kg) of both ferroquine and artesunate increased gametocytogenesis at H1h30 and H5 post-treatment, the most effective being 5 mg/kg artesunate. We also observed a decrease in the infectivity of gametocytes of *P. y. yoelii* in mosquitoes both at H1h30 and H5 post-treatment. These results should be confirmed with a study on a revised distribution of the different gamocyte stages and the transmission stages from gamocyte to oocyst development in the mosquito. The number of gametocytes post-treatment was not directly correlated with their infectivity in mosquitoes.

Studies comparing the effects of ferroquine and artesunate on gametocytogenesis and on the infectivity of gametocytes have to be performed with several dosages and strains (resistant and sensitive) over longer periods.
(H24, H48) post-treatment and to explore the relationships between gametocyte development stages and infectivity.

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