

## Induction of *Plasmodium falciparum* strain 2300 dormant forms by artemisinin

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### ABSTRACT

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#### BACKGROUND

The presence of *Plasmodium falciparum* resistance and decreased efficacy of artemisinin and its derivatives has resulted in the issue of malaria becoming increasingly complex, because there have been no new drugs as artemisinin replacements. The aims of this research were to evaluate in vitro changes in ultrastructural morphology of *P. falciparum* 2300 strain after exposure to artemisinin.

#### METHODS

The research used an experimental design with post test only control group. Cultures of *P. falciparum* 2300 strain in one control and one mutant group were treated by exposure to artemisinin at  $IC_{50} 10^{-7}$  M for 48 hours. Ultrastructural phenotypic examination of ring, trophozoite and schizont morphology and developmental stage in the control and mutant group were done at 0, 12, 24, 36, 48 hours by making thin blood smears stained with 20% Giemsa for 20 minutes and examined using a microscope light at 1000x magnification.

#### RESULTS

Dormant forms occurred after 48 hours of incubation with  $IC_{50} 10^{-7}$  M artemisinin in the control group. In the mutant group, dormant forms, trophozoites with blue cytoplasm and normal schizont developmental stages were seen. Ultrastructural phenotypic morphology at 0, 12, 24, 36, 48 hours showed that in the control group dormant formation already occurred with exposure to  $IC_{50} 10^{-7}$  M, while in the mutant group dormant formation occurred only with exposure to  $IC_{50} 2.5 \times 10^{-5}$  M.

#### CONCLUSION

Exposure to artemisinin antimalarials in vitro can cause phenotypic morphological changes of dormancy in *P. falciparum* Papua 2300 strain.

**Keywords :** Artemisinin, *P. falciparum* 2300, phenotype, resistance

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## **Induksi bentuk dorman *Plasmodium falciparum* galur 2300 oleh artemisinin**

### **ABSTRAK**

#### **LATAR BELAKANG**

Resistensi parasit *P.falciparum* dan penurunan efek artemisinin dan derivatnya menyebabkan masalah malaria menjadi semakin kompleks, karena belum ada obat baru pengganti artemisinin. Penelitian ini bertujuan untuk menilai efek paparan artemisinin terhadap morfologi ultrastruktur *Plasmodium falciparum* galur 2300.

#### **METODE**

Penelitian menggunakan experimental design dengan post test only control group. Kultur *P. falciparum* galur 2300 kelompok kontrol dan kelompok mutan diberikan perlakuan paparan artemisinin dengan dosis  $IC_{50} 10^{-7}$  M selama 48 jam, kemudian dilakukan pengamatan gambaran ultrastruktur *P.falciparum* galur 2300. Pemeriksaan fenotipik ultrastruktur stadium perkembangan dan morfologi ring, trofosoit dan skizon pada kelompok kontrol dan kelompok mutan dilakukan setiap 12 jam setelah dipapar artemisinin. Selanjutnya pada jam ke 0, 12, 24, 36, 48 dibuat hapusan darah tipis yang diwarnai dengan Giemsa 20 % selama 20 menit dan dilakukan pemeriksaan menggunakan mikroskop cahaya perbesaran 1000x.

#### **HASIL**

Bentuk dorman terjadi setelah 48 jam diinkubasi artemisinin dosis  $IC_{50} 10^{-7}$  M pada *P. falciparum* 2300 kelompok kontrol sedangkan pada kelompok mutan *P. falciparum* 2300 gambaran fenotipik ultrastruktur terdapat stadium perkembangan bentuk dorman, trofozoit dengan sitoplasma berwarna biru dan skizon normal. Gambaran fenotipik ultrastruktur pada kelompok kontrol dan kelompok mutan pada jam ke 0, 12, 24, 36, 48 menunjukkan pada kelompok kontrol dengan paparan dosis  $IC_{50} 10^{-7}$  M sudah terjadi bentukan dorman sedangkan pada kelompok mutan dengan paparan dosis  $IC_{50} 2.5 \times 10^{-5}$  M baru terjadi bentuk dorman.

#### **KESIMPULAN**

Bentuk dorman terjadi di kelompok mutan hanya dengan peningkatan dosis paparan artemisinin.

**Kata kunci :** Artemisinin, fenotip, *P. falciparum* galur 2300, resistensi

## **INTRODUCTION**

Malaria is one of the infectious diseases with a global distribution, ranging from the tropics, subtropics to temperate climates. Malaria is still a public health problem in more than 90 countries that are inhabited by 2.4 billion people or 40% of the world population. In 2008, the World Health Organization (WHO) estimated that there were approximately 243 million cases of malaria and 886.000 deaths because of

malaria,<sup>(1)</sup> most of them in sub-Saharan Africa due to falciparum malaria in children under the age of five years. In addition to its impact on health, malaria imposes a heavy economic burden on individuals<sup>(2)</sup> and entire economies.<sup>(3)</sup>

Prevention efforts against malaria have been carried out, but the morbidity and mortality rates of malaria in some countries are still high. Among the factors that cause difficulties in malaria prevention, *Plasmodium* resistance to antimalarial medications is the factor which is

most difficult to overcome because of the occurrence of mutations in the genome of *Plasmodium* that are difficult to control.<sup>(1)</sup>

The most recent and currently used drugs for malaria therapy are artemisinin and its derivatives, but there have been indications that the *Plasmodium* parasites are now resistant to these drugs.<sup>(4)</sup> A clinical study found two patients in Cambodia who had been infected with *Plasmodium falciparum* (*P. falciparum*) to be resistant to artesunate.<sup>(5)</sup> Because of that, the eradication of malaria has become more complex and dangerous. It is one of the world health problems that have to date not yet been resolved because of the absence of artemisinin substitutes. The development of *Plasmodium* resistance to antimalarial drugs which occurs faster than the development of new antimalarials becomes a consideration for seeking solutions to the accurate and efficient therapeutic management of malaria.

Resistance of *P. falciparum* to artemisinin may be influenced by internal factors of *P. falciparum*, due to changes in the parasite itself to allow it to survive and adapt to environmental changes caused by drug exposure. The results of studies have pointed to the deceleration of the developmental life cycle and the induction of the expression of genes that code for proteins (protein overexpression) as one of the important mechanisms for the *Plasmodium* parasite to free themselves from the effects of antimalarial medication and still be able to survive.<sup>(6)</sup> Another research study was conducted by molecular monitoring of the genes that are involved in *P. falciparum* resistance to antimalarials.<sup>(7,8)</sup> The research results found that combined artemisinin resistance may be due to mutations in the *P. falciparum* adenine triphosphatase 6 (pfatpase6) gene.<sup>(7)</sup>

Although the mechanism of artemisinin resistance is unclear, it is suspected that there are changes in antimalarial resistance at phenotypic, proteomics, and genotypic levels in *Plasmodium*. At the genotypic level, one of the changes are due to a mutation in the pfatpase6 gene and upregulation of expression of gene

transcription.<sup>(9)</sup> The relationship of repeated artemisinin exposure with phenotypic, proteomics and genotypic changes in chloroquine resistant *P. falciparum* has not been proved, necessitating the present study.

Previous research on the *P. falciparum* F32-Tanzania strain that was exposed for 3 years to artemisinin at low concentrations ranging from 0.01  $\mu\text{M}$  up to 10  $\mu\text{M}$  for 100 exposure times. Drug-selected F32-ART parasites were the result of treatment with high doses of artemisinin (ART) for 48 h to 96 h, after which the parasites were returned to normal culture without the drug for 21 days until parasitemia reached 5%. The drug-selected parasite F32-ART could recover from ART treatment in a much shorter time than the F32-Tanzania did and survive.<sup>(10)</sup> Another research showed that *P. falciparum* strains GC06 and CH3-61 before and after selection with artemisinin at increasing concentrations from 0 to 20 nM and 0 to 100 nM, respectively, the  $\text{IC}_{50}$  value of the selected and viable GC06 parasites increased.<sup>(11)</sup> This study was conducted to evaluate the effect of in vitro artemisinin exposure at  $\text{IC}_{50}$  doses on the morphological changes in *P. falciparum* 2300 mutant strain.

## METHODS

### Research design

The research used an experimental design with post test only with control group. Cultures of *P. falciparum* 2300 mutant strain were used in the control group and mutants. Parasitemia and morphological examinations were conducted at the Faculty of Veterinary Medicine, Airlangga University, from March to October 2013.

### Research sample

The samples that were used as controls in this study were cultures of *P. falciparum* 2300 mutant strain that had become resistant to chloroquine, whereas for the mutant cultures of *P. falciparum* 2300 mutant strain were used that had become resistant to both chloroquine and artemisinin.

### Experimental design

The Plasmodium cultures were divided into two treatment groups, i.e. the control group and the mutant. The control group was exposed to artemisinin in vitro at a concentration equivalent to  $IC_{50} 10^{-7}$  M, whereas the mutant was exposed to artemisinin at a dose of  $IC_{50} 2.5 \times 10^{-5}$  M, both groups being exposed for 48 hours. Ultrastructural phenotypic examination of ring, trophozoite and schizont morphology and developmental stages were observed in both groups at 0, 12, 24, 36, 48 hours.

### Culture and morphological examination

Cultures of *P. falciparum* 2300 mutant strain that had been stored in liquid nitrogen were thawed by the Rowe method. One millimeter of the erythrocyte suspension was taken and mixed with 9 ml of complete medium plus 15% human serum type O, then put into a culture flask and incubated in a  $CO_2$  incubator at  $37^\circ C$ , under 5%  $CO_2$ , 5%  $O_2$  and 90%  $N_2$ . Medium replacement was done carefully every 48 hours using a sterile Pasteur pipette. A sample of the sediment was taken to make a smear to determine parasitemia, then 9 mL of medium was added to each culture flask and the culture was incubated again.

Phenotypic observations of the morphology and developmental stages of the intra-erythrocytic cycle of *P. falciparum* 2300 strain were carried out on synchronized and non-synchronized cultures that had been incubated for 48 hours. Plasmodium cultures were divided into two treatment groups, i.e. the control group and mutant group with artemisinin exposure in vitro at  $IC_{50} 10^{-7}$  M for 48 hours. Observations on developmental stages and morphology of ring, trophozoite and schizont stages in the control group exposed to artemisinin in vitro at  $IC_{50} 10^{-7}$  M and in the mutant group exposed to artemisinin in vitro  $IC_{50}$  at  $2.5 \times 10^{-5}$  M were done at 0, 12, 24, 36, 48 hours on thin blood smears stained with 20% Giemsa for 20 minutes and examined using a light microscope at 1000x magnification.<sup>(6,12)</sup>

### Data analysis

Morphological (ultrastructural) data of *P. falciparum* 2300 strain control and mutant groups were compared and analyzed descriptively

## RESULTS

Figure 1 shows the differing percentages of growth inhibition of *P. falciparum* 2300 strain between the control and mutant groups, both of which had been exposed to artemisinin at  $10^{-7}$  M concentration and incubated for 48 hours.

The morphology of *P. falciparum* Papua 2300 strains in the control and mutant groups before and after 48-hour exposure to artemisinin is presented in Figure 2 below.

The results of morphological description of artemisinin exposure every 12 hours in the control and mutant groups are presented in Figures 3 and 4 below.

As seen in Figure 2, the morphology of *P. falciparum* 2300 strain were different in the control group as compared with the mutant group, before and after treatment with artemisinin at  $IC_{50} 10^{-7}$  M concentration and 48 hours of incubation. From Figures 3 and 4 it is also apparent that the developmental morphologies of *P. falciparum* of the synchronized control and mutant 2300 strains after exposure to artemisinin at  $IC_{50}$  concentrations and 12-hourly observation, were different from each other.

## DISCUSSION

The results of the study showed that in the control group that was exposed to artemisinin at  $10^{-7}$  M concentration and incubated for 48 hours, the growth percentage decreased to 35% and growth inhibition decreased to 65%. Morphological description showed that there were dormant forms. The mutant group that was exposed to artemisinin at  $10^{-7}$  M concentration and incubated for 48 hours had a growth

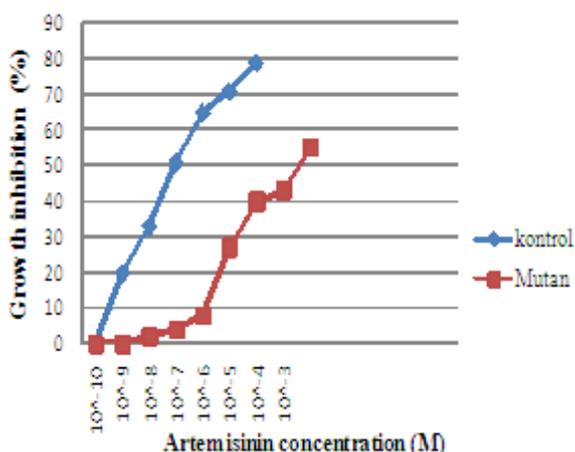


Figure 1. Percentage growth inhibition of *P. falciparum* 2300 strains in control and mutants exposed to artemisinin

percentage of 92% and growth inhibition of 8%. Morphological description also showed dormant forms, trophozoites with cytoplasm that still appear blue and the presence of normal schizonts containing merozoites with brownish black pigment (Figures 1 and 2). The results of this study showed that the parent *P. falciparum* 2300 strain requires smaller artemisinin concentrations

to show growth inhibition compared to the mutant *P. falciparum* 2300 strain that had become resistant to artemisinin, which required greater artemisinin concentrations to show growth inhibition of the parasites. It can already be seen from the morphological description that at the same artemisinin concentration ( $10^{-7}$  M), there are morphological changes in the mutant group when compared to the control group. In the mutant group, there are morphological changes in their ring, schizont and trophozoite developmental stages, which are normal. Therefore, from the morphological description it can be concluded that to obtain the same dormant period a greater concentration of artemisinin is required.

The results of this study also showed that the dormant period can occur in parasite strains that are resistant to artemisinin, but it needs a higher drug concentration for its induction. *Plasmodium* parasites that are resistant to artemisinin cannot be induced into a dormant period if there had been tolerance to the drug concentration. It is possible that the dormant

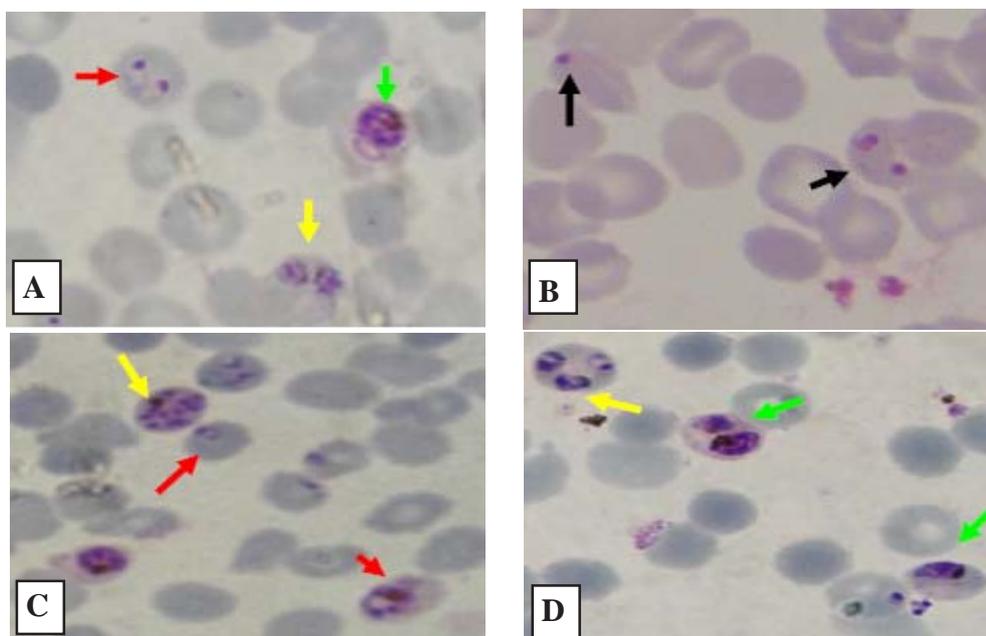


Figure 2. Parent and mutant *P. falciparum* morphology after being exposed for 48 hours to artemisinin at  $IC_{50}$  dose (1000x magnification). Giemsa staining. Arrow color codes: black = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite. (A) Parent *P. falciparum* 2300 strain, (B) Parent *P. falciparum* 2300 strain after being exposed to artemisinin  $10^{-7}$  M for 48 hours, (C) Mutant *P. falciparum* 2300 strain, (D) Mutant *P. falciparum* 2300 strain after being exposed to artemisinin  $10^{-7}$  M for 48 hours

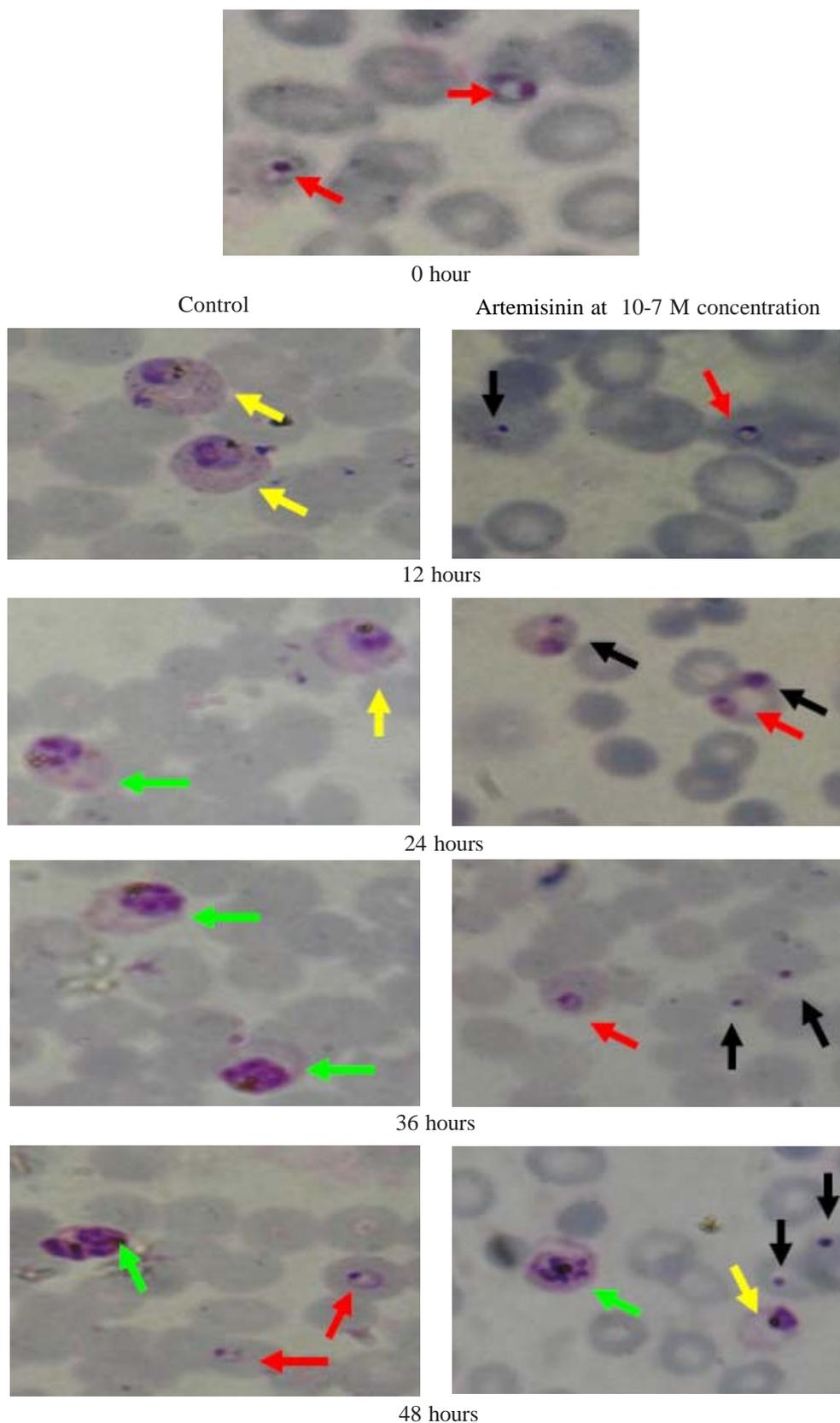


Figure 3. Morphology of *P. falciparum* 2300 strain synchronized in the control group, treated with artemisinin at  $IC_{50}$   $10^{-7}$  M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

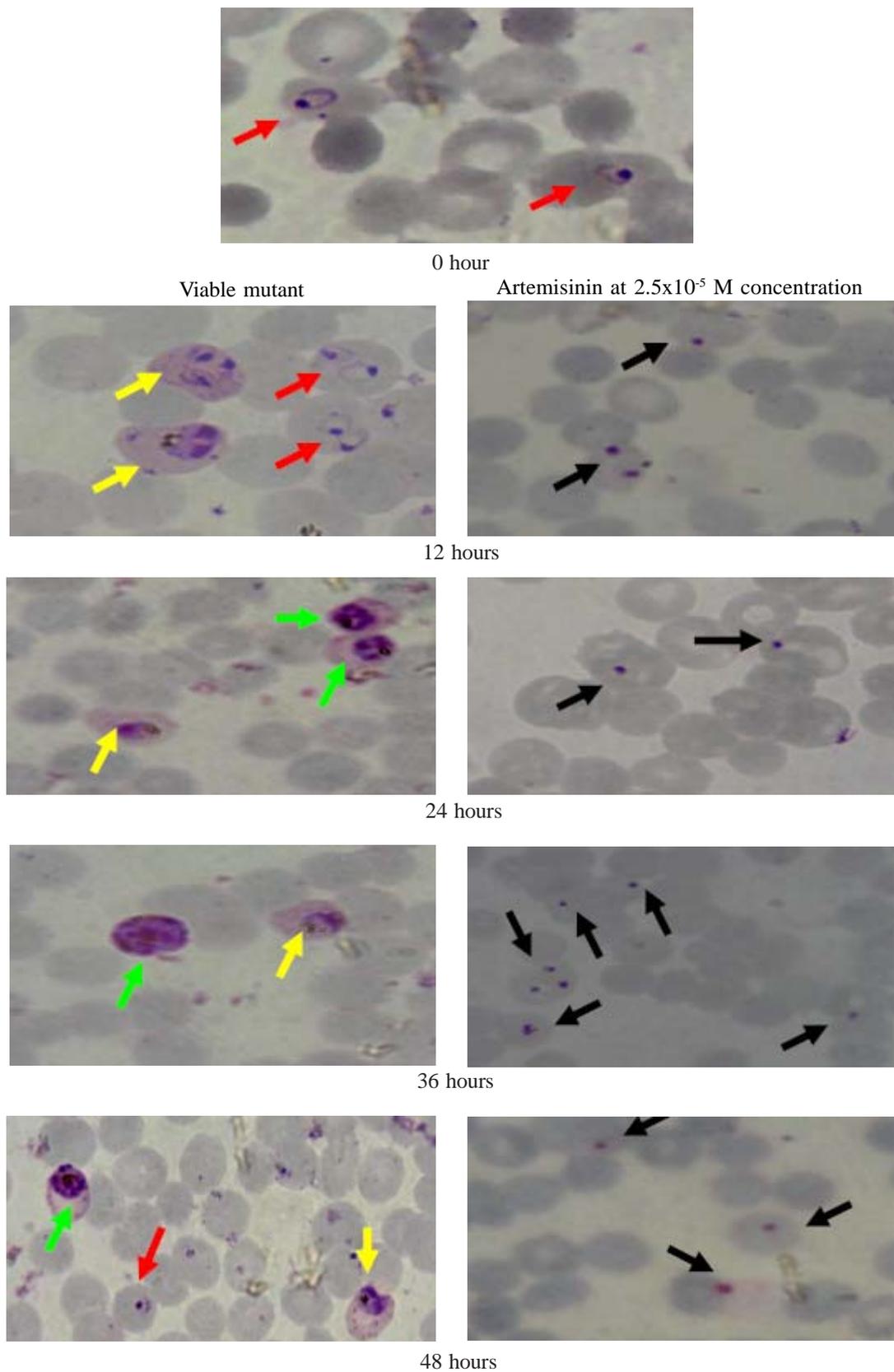


Figure 4. Morphology of *P. falciparum* 2300 strain synchronized in the viable mutant group, treated with artemisinin at  $IC_{50}$   $2.5 \times 10^{-5}$  M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

parasites use innate mechanisms to survive the stressor, i.e. the drug concentration that can cause severe damage to the parasite, and that the dormant parasites can also be triggered when parasite growth is inhibited.<sup>(13,14)</sup>

The results of this research are in agreement with those of research conducted on *P.falciparum* GC06 and CH3-61 strains before and after selection with artemisinin at increasing concentrations of 0 to 20 nM and 0 to 100 nM, respectively, where viable parasites showed an increase in IC<sub>50</sub> strain values after selection with artemisinin. IC<sub>50</sub> strain values increased in the first GC06 strain from 3.1 ± 0.1 nM to 12.5 ± 1.6 nM and in the first CH3-61 strains that from 28.8 ± 1.3 nM to 58.3 ± 4.5nM.<sup>(11)</sup> The results of research on *P. falciparum* Tanzania F32 exposed to artemisinin for 3 years at low concentrations ranging from 0.01 µm up to 10 µm for 100 exposure times, resulting in the selection of F32-ART strains, showed that at higher concentrations of artemisinin exposure (35 µm and 70 µm) for 96 hours, only the F32-ART strain was able to survive.<sup>(10)</sup>

Stage of development and morphology of the intra-erythrocytic cycle of *P.falciparum* 2300 strain that was synchronized in the control group, treated by artemisinin exposure at IC<sub>50</sub> concentration and observed every 12 hours, from 0 to 48 hours showed normal morphology development with faster growth compared with the group treated with artemisinin exposure at IC<sub>50</sub> concentration. The treatment group that was exposed to artemisinin showed dormant morphology development with nuclear chromatin condensation and if able to survive exposure to artemisinin, it only survives up to 24 hours after exposure, with imperfect ring and trophozoite stages.

The results of this study showed that cell cycle development in the control group proceeds normally while *P.falciparum* in the mutant group that was not exposed to artemisinin had a faster intra-erythrocytic life cycle. The increased growth of *P.falciparum* that had been exposed to artemisinin is caused by upregulation of gene

transcription (multi-gene) that plays a role in cell cycle regulation, transport of substances from erythrocytes into the parasite, the enzymes involved in the biosynthesis of purines in DNA synthesis and synthesis of proteins that play a role in the adaptation of parasites to the environment in the early stage of parasite growth (16-20 hours) during the development of the ring forms into trophozoites and at the end of development (36-40 hours) when trophozoites develop into schizonts.<sup>(10,15)</sup>

In the group that was treated with artemisinin exposure at IC<sub>50</sub> concentration, there was decreased intra-erythrocytic development of *P. falciparum*. The results of this study are comparable to those of research by Veiga et al.<sup>(6)</sup> who performed mefloquine exposure on three strains of Plasmodium (W2, 3D7 and FCB). Compared with the control treatment, 40% of cell morphology showed retarded development. Exposure to anti-malarial medications caused a 1.5-fold increase in *pfmdr1*, *pfprt*, *pfmrp1* and *pfmrp2* gene expression. Similarly, upon quinine exposure for 12 hours, there was a slowdown in the development of cell morphology. The results of this research demonstrate that a slowdown in the development of *P. falciparum* is a very important mechanism for the parasite to be able to escape from the influence of anti-malarial medications.<sup>(6,16)</sup>

Morphological dormancy in *P.falciparum* that have been exposed to artemisinin is a defense mechanism for the parasites to be able to survive from the exposure of artemisinin anti-malarial medication. The parasites will be able to grow normally after the pressure from the drug is removed. In this dormant period, parasites can survive in a few days by slowing down the process of metabolism to limit the effects of the medication, because in this dormant period there is no DNA synthesis.<sup>(10,17-19)</sup>

Parasites that survive in the trophozoite stage from exposure to artemisinin have abnormal morphology, with formation of condensed cytoplasm. In *P.falciparum* that have been exposed to artemisinin for 48 hours,

morphological changes occur from the trophozoite stage until the final phase of the life cycle. Artemisinin exposure causes metabolic changes in the parasites that affect its growth and development, as can be observed from the morphological abnormalities. The changes start to occur at the trophozoite stage because at this stage, the parasites begin a process of active metabolism, growing larger in size from initial trophozoites into mature trophozoites. The nutrients extracted from the erythrocyte cytosol will be consumed quickly in the digestive vacuole that has started to form. The hemoglobin degradation process into oligopeptides and heme by proteolysis in the digestive vacuole is initiated to fulfill the nutritional needs of the parasite.<sup>(20,21)</sup>

Abnormal morphology and growth inhibition after artemisinin exposure is also caused by the inhibition of parasite proteases (plasmepsin, falcipain and falcilysin) which are essential for parasite growth. Research by Bonilla et al.<sup>(22)</sup> showed that *Plasmodium* knockout mutants (triple and quadruple Plasmepsins Knockout Mutants, PMKO) in the protease enzyme have a deficiency in the endosomal vesicles that enter the digestive vacuole and produce multilamellar bodies, causing a deficiency in hemoglobin digestion and inhibition of hemozoin formation in the digestive vacuole, thus slowing growth.

Barriers artemisinin in endocytosis causes an inhibition of transport vesicle fusion into the digestive vacuole, so that the digestion of hemoglobin does not occur or is blocked. The relationship between endocytic transport and cellular signal transduction pathways that lead to inhibition of endocytosis will slow the growth of the parasites and result in reduction of parasitemia and parasite mortality.<sup>(23,24)</sup>

One limitation of this research is that it was conducted only with the *P. falciparum* Papua 2300 strain. A better way would have been to use more than one strain, so that the results could be compared. The implication of this research is that it may explain the mechanism of the development of resistance to artemisinin through

phenotypic dormancy of *P. falciparum* which can cause a decrease in artemisinin efficacy and give rise to recrudescence and artemisinin treatment failure. Further research needs to be done to visualize the ultrastructural changes of digestive vacuoles and mitochondria of *P. falciparum* by transmission electron microscopy (TEM) and to determine changes at proteomic and genomic level, then performing an in vivo research in experimental animals as models of artemisinin resistance in humans.

## CONCLUSION

Exposure to artemisinin antimalarials in vitro can cause phenotypic morphological changes of dormancy in *P. falciparum* Papua 2300 strain.

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