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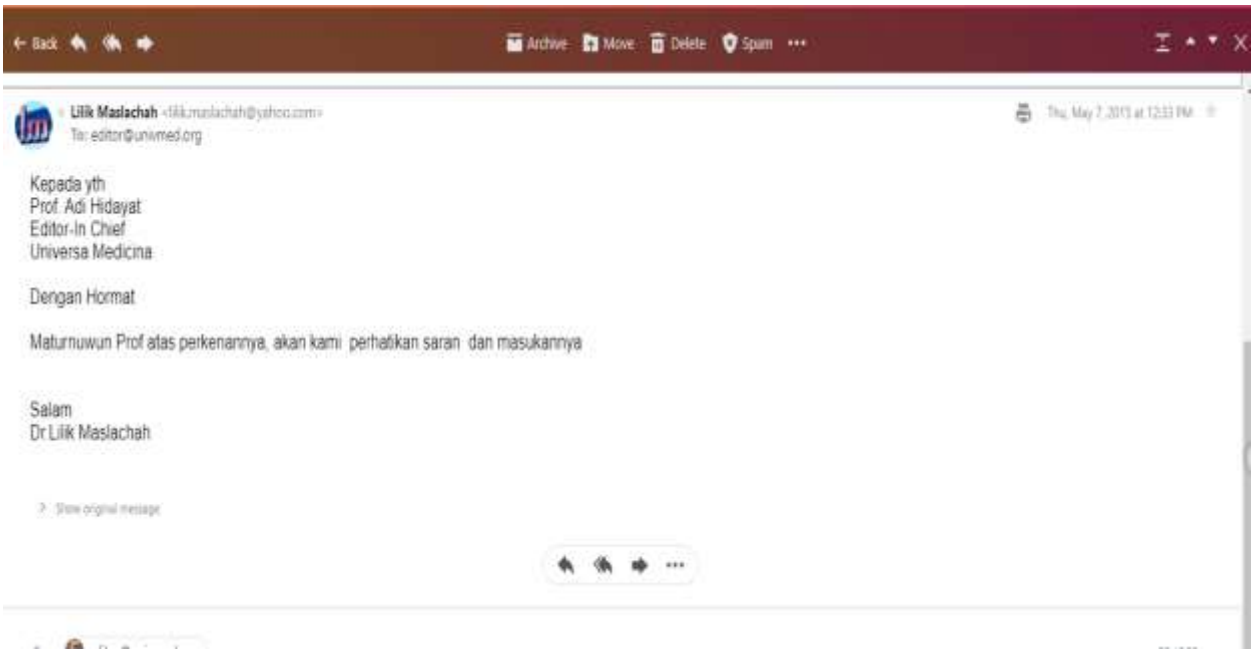

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Maturnuwun Prof atas perkenannya, akan kami perhatikan saran dan masukannya

Salam
Dr Lilik Maslachah

Ultrastructure Morphology of *Plasmodium falciparum* Papua 2300 Mutants Strain Because of In Vitro Artemisinin Exposure

15 kata, upayakan maksimal 12 kata dan sudah mengarah ke Kesimpulan

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Correspondence Dr.Lilik Maslachah Department of Basic Veterinary Medicine , Faculty of Veterinay Medicine, Airlanggga University Kampus C Mulyorejo Surabaya 60115. (031) 5992785 Fax (031) 5993015 Email: lilik.maslachah@yahoo.comAbstract

Background **220 kata, upayakan sekitar 250 kata**

The presence of the P. *falciparum* resistance and decrease of efficacy against artemisinin and its derivatives resulting in the issue of malaria becomes increasingly complex. Malaria becomes one of the world's health problems have not been resolved to date, because there hasn't been a new drug artemisinin replacement. This research aims are to give evidences that repeated exposure of artemisinin in vitro change Ultrastructure morphology of *Plasmodium falciparum* Papua 2300 Strain

Methods

The research used experimental design with post test only control group design.In-vitro culture of *P. falciparum* Papua 2300 strain. 2. treated regularly *Plasmodium falciparum* Papua 2300 strain by repeated exposure of artemisinin (IC_{50} concentration) alongside with the degree parasitemia calculation and detection Ultrastructure morphology of *P. falciparum* ~~The research had been worked during the March until October 2014 in Tropical Diseases Hospital Airlangga University and the Faculty of Veterinary Medicine Airlangga University.~~

Results

The result of ultrastructure morphology a dormant shape was occured after 48 hours incubation of IC50 artemisinin in control groups of *P. falciparum* 2300. In mutan groups ultrastructure morphology a dormant shape, trophozoit with blue cytoplasm image and normal schizont development stage. The result so showed dormant shape was occured in mutan groups but with increased artemisinin exposure dose.

Conclusion

Artemisinin exposure in vitro can cause phenotypic a dormant shape in *Plasmodium falciparum* Papua 2300.

Key word : Artemisinin, *P.falciparum* Papua 2300 , phenotype, resistance

Gambaran Ultrastruktur Mutan *Plasmodium falciparum* galur Papua 2300 yang Terpapar Artemisinin In Vitro

Abstrak

Latar Belakang 177 kata upayakan seklitar 250 kata

Resistensi parasit *P.falciparum* dan penurunan effikasi terhadap artemisinin dan derivatnya menyebabkan masalah malaria menjadi semakin komplek. Malaria masih menjadi salah satu masalah kesehatan di dunia yang belum dapat diselesaikan sampai saat ini, karena belum ada obat baru pengganti artemisinin. Penelitian ini bertujuan untuk membuktikan bahwa paparan artemisinin berulang in vitro dapat menyebabkan perubahan morfologi ultrastruktur *P. falciparum* galur Papua 2300.

Metode

Penelitian menggunakan *experimental design* dengan *post test only control group.* .Kultur *P .falciparum* galur Papua 2300 diberikan perlakuan Paparan artemisinin dengan dosis IC_{50} berapa lama paparan diberikan kemudian menghitung derajat parasitemia dan gambaran ultrasrtuktur *P.falciparum.* Uraikan cara perhitungan parasitemia dan pemeriksaan ultrastruktur ~~Penelitian dilakukan mulai bulan Maret sampai dengan bulan Oktober 2014 di Rumah Sakit Penyakit Tropis Universitas Airlangga dan Fakultas Kedokteran Hewan Universitas Airlangga.~~

Hasil

Hasil gambaran morfologi ultrastuktur bentuk dorman terjadi setelah 48 jam diinkubasi artemisinin dosis IC_{50} pada *P. falciparum* 2300 kelompok kontrol. Pada kelompok mutan *P. falciparum* 2300 gambaran morfologi ultrastruktur ada satadium perkembangan berupa bentuk dorman, trofozoit dengan sitoplama berwarna biru dan shizon normal.

Kseimpulan

Hasil ini menunjukkan bentuk dorman terjadi dikelompok mutan tetapi dengan peningkatan dosis paparan artemisinin.

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

BACKGROUND

Malaria is one of the infectious diseases that spread around the world ranging from the tropics, sub-tropical to cold climates. Malaria still be a public health problem in more than 90

countries, inhabited by 2.4 billion people or 40% of world population. In 2008, World Health Organization (WHO) estimates that there were approximately 243 million cases of malaria and 886.000 people die because of malaria, most of them occur in children under the age of five in sub-Saharan Africa due to falciparum malaria.[1]

Indonesia is a tropical country, 35% of the population live in areas which has a high risk of malaria, from 293 districts / cities in Indonesia, 167 districts / cities is a malaria endemic area. It is estimated that approximately 30 million cases of malaria each year, Although it has made the implementation and eradication program of malaria since 1959, but until right now the morbidity and mortality are still high. Most of the mortality by malaria infection in Indonesia caused by *Plasmodium falciparum* (*P. falciparum*).[2]

Antimalarial medicines such as chloroquine, sulfadoxine, pyrimethamine, sulfadoxine-pyrimethamine combined and artemisinin has been used in Indonesia and some parts of the world. Many use of first layer antimalarial medicines are resistant.

Prevention efforts against malaria have been carried out, but the morbidity and mortality of malaria in some countries are still high. Among the factors that cause difficulties prevention against malaria, *Plasmodium* resistance factor to antimalarial medicine is a factor which is most difficult to overcome because of a mutation in the genome of *Plasmodium* is difficult to control.[3]

The newest medicine for malaria therapy which is used until right now is artemisinin and its derivatives, but there has been an indication that the *Plasmodium* parasites resistant to this medicine.[4] Clinical results already shown that two patients in Cambodia who had been infected with *Plasmodium falciparum* resistant to artesunate.[5] Because of that, the eradication of malaria becomes more complex and dangerous. It becomes one of the health problems in the world that

can not be resolved until today because there isn't any medicine that can substitute artemisinin yet. The development of *Plasmodium* resistance to antimalarial medicine which is faster than new antimalarial medicine discovery becomes a consideration for seeking solutions to the accurate and efficient therapeutic management of malaria.

Resistance of *Plasmodium falciparum* to artemisin antimalarial medicine could occur due influenced by internal factors of *Plasmodium falciparum,* due to changes in the parasite itself because exposure of medicine to survive and adapt to environmental changes. The results shown that the deceleration development life cycle and the induction of the expression of genes that express proteins (protein overexpression) as one of the important mechanisms for the *Plasmodium* parasite to free themselves from the effects of antimalarial medicine and still be able to survive.[6] The other research that have been done by monitoring genes molecular that is involved in *Plasmodium falciparum* resistance to antimalarial medicine. [7,8] The research results found that the combination of artemisinin resistance is expected due to mutations in the pfatpase6 gene.[7]

Although the mechanism of artemisinin medicine resistance has not been known but it is suspected that the are changes on the antimalarial medicine resistance in the level of phenotypic, proteomics, and genotypic in *Plasmodium*. At the level of genotypic, one of the changes due to a mutation in the pfatpase6 gene and upregulation of expression of gene transcription. The relationship of repeated artemisinin antimalarial medicine exposure in phenotypic, proteomics and genotypic on chloroquine resistant *Plasmodium falciparum* has not been proved. Because of that, research is necessary to be done.

Apakah perbedaan studi sejawat dengan penelitian yang telah dilakukan?

Tambahkan objective of this study

METHODS

Research design

The research used am experimental design with post test only control group design. Cultures of *P. Falciparum* 2300 strain control group and mutant group exposured with artemisinin. Culture, parasitaemia and morphological examinations conducted at the Tropical Diseases Hospital of Airlangga University and Veterinary Medicine Faculty of Airlangga University. The research was conducted from March to October 2014.

Research sample

Sample that is used in this study is *P. falciparum* 2300 strain that had been resistant to chloroquine as a control and *P. falciparum* 2300 strain mutant as treatment group that had been resistant to chloroquine and artemisinin.

Experimental design

Plasmodium cultures were divided into two treatment groups, there are control group and mutant group with artemisinin exposure which the concentration is known as IC_{50}.

Uraikan cara pemberian artemisinin, dosisnya, berapa lama, had been exposed to repeated artemisinin and mutated.. Kontrolnya diberikan apa?

Culture and morphological examination

Plasmodium falciparum 2300 strains do thawing with Rowe method in liquid nitrogen. Every 1 ml solution of the erythrocyte suspension is taken and mixed with 9 ml of complete medium plus 15% human serum type O and put into the cultur flask and incubated in a CO_2 incubator at 37°C, 5% CO_2, 5% O_2 and 90% N_2. Medium replacement is done carefully every 48 hours using a sterile

Pasteur pipette. A little sediment is taken to make a smear to see parasitaemia, then added with 9 ml of medium for each culture bottle and incubated again.

Fenotiping observations of morphological developmental stages of *P. Falciparum* 2300 strain intra erythrocytic cycle is carried out from synchronized and not synchronized culture that had been incubated for 48 hours. Plasmodium cultures were divided into two treatment groups, there are control group and mutant group with artemisinin exposure which the concentration is known as IC_{50}. Developmental stage observation and morphological of ring, trophozoite and schizont in the control group and mutant group which the concentration is known as IC_{50} are done on the 0, 12, 24, 36, 48 hours by making a thin blood smear that is stained with 20% Giemsa for 20 minutes and examined using a light microscope with 1000x magnification. [6,9]

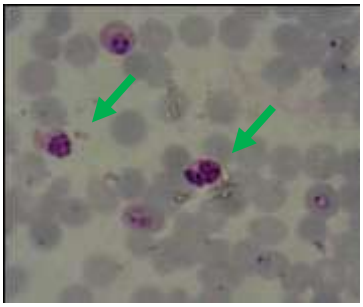
Uraikan cara perhitungan parasitemia dan pemeriksaan morfolgi

Data analysis

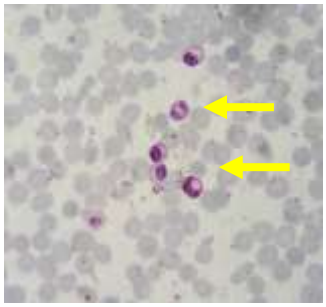
Data results of morphological (ultrastructure) studies of *Plasmodium falciparum* Papua strains control and mutant group were analyzed descriptively. Bukankah ada kontrolnya, mengapa tidak dibandingkan dengan control?

RESULTS

The examination's result of culture growth of *Plasmodium falciparum* Papua 2300 strains in the control group and the group of mutants is presented on the figure 1 below.



A



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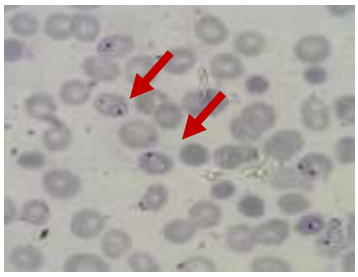
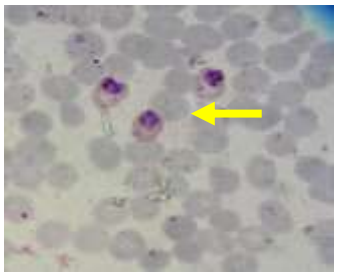


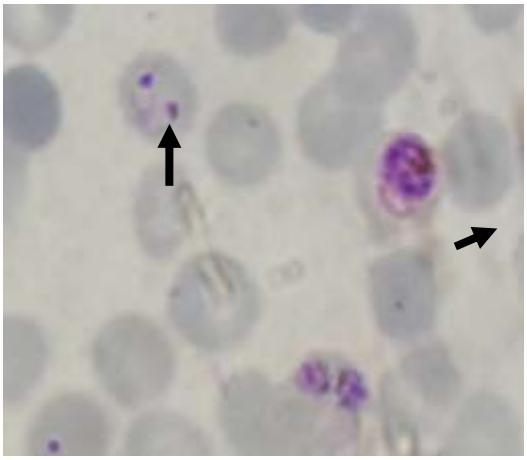


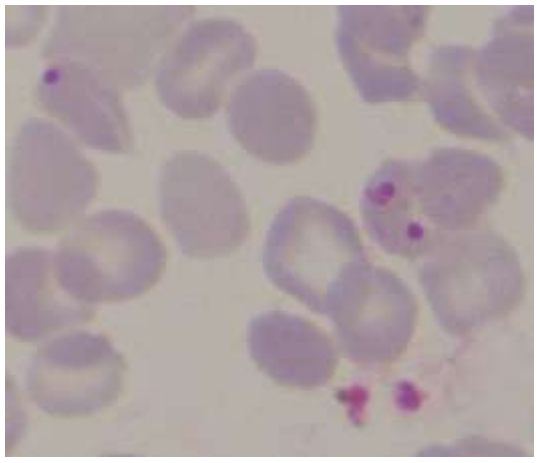
C

D

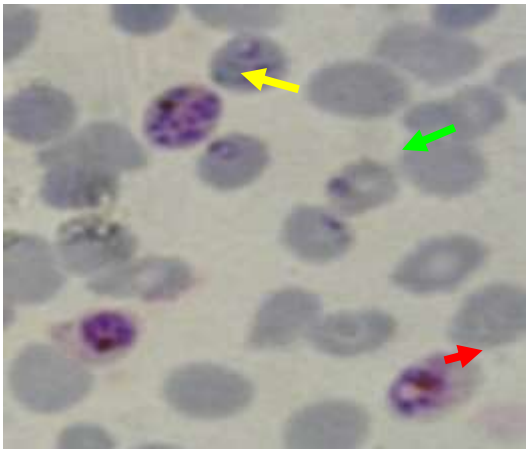
Figure1 A.B.C.D : The control group of morphological development and growth of *Plasmodium falciparum* Papua 2300 strains (A, B) and mutans (C, D) at a ring stage (red), trophozoites (yellow) and schizonts (green). Giemsa staining with 1000x magnification.

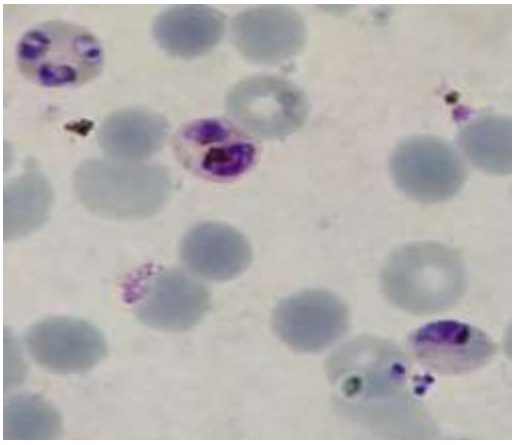
The results of morphological *Plasmodium falciparum* Papua 2300 strains for control and mutants group before and after 48 hours of being exposed to artemisinin anti-malarial medicine is presented in the figure 2 below.Figure 2 apakah juga A B C dan D





Parent of *P.falciparum* Papua 2300 strain **Parent of *P.falciparum* Papua 2300 strain after being exposed to artemisinin 10^{-7} M for 48 hours.**



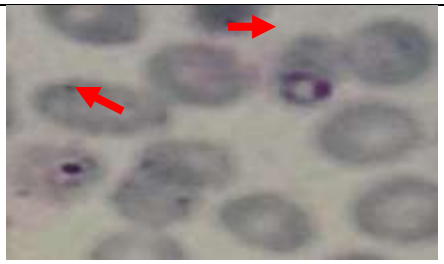


Mutants of *P. falciparum* Papua 2300 strain

Mutants after being exposed to artemisin 10^{-7} M for 48 hours

Figure 2 parent and mutant *P. falciparum* morphology after being exposed for 48 hours to artemisinin with IC50 dose. 1000x magnification. Giemsa staining. Black arrow: dormant, red: ring, yellow: trophozoites, green: schizonts, blue: merozoites. Giemsa staining.

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



	0 hour	
Control		Artemisinin with 10^{-7} M concentration

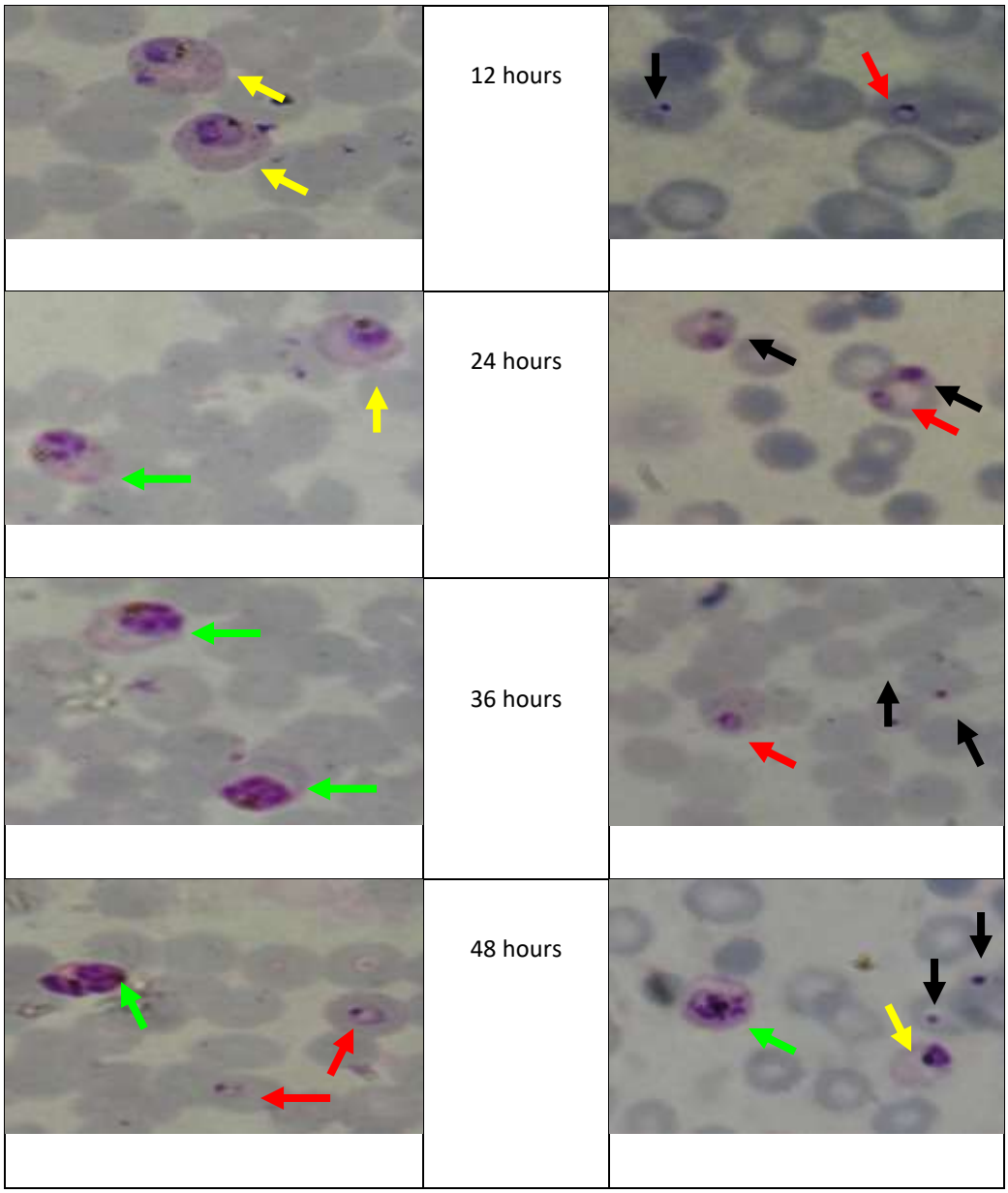
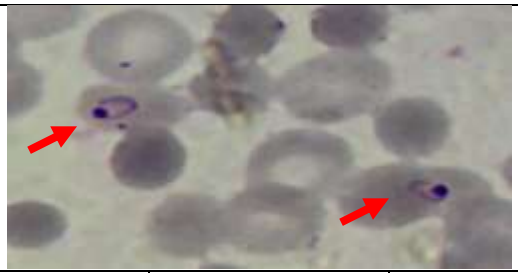


Figure 3 The morphology of *P. falciparum* 2300 strain that was synchronized in the control group and treatment with artemisinin IC50 concentration exposure was monitored every 12 hours with 1000x magnification. Giemsa staining. Black arrow: dormant, red: ring, yellow: trophozoites, green: schizonts, blue: merozoites.



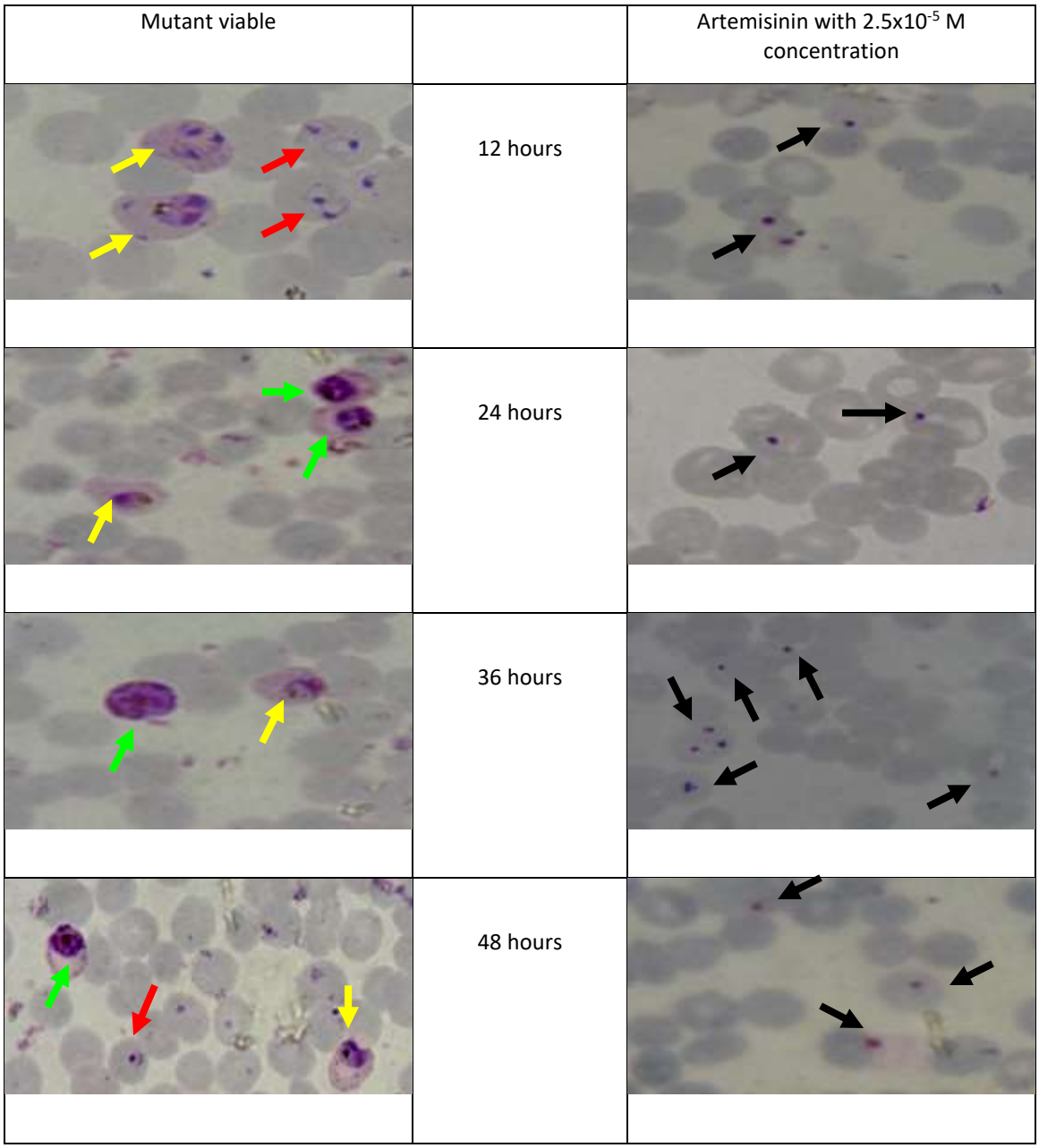


Figure 4 Morphology of *P. falciparum* 2300 strain which was synchronized on viable mutant group and treatment with artemisinin IC50 concentrations exposure was observed every 12 hours with 1000x magnification. Giemsa staining. Black arrow: dormant, red: ring, yellow: trophozoites, green: schizonts, blue: merozoites.

DISCUSSION

The results of the study in the control group that was exposed to artemisinin with 10^{-7} M concentration and incubated for 48 hours showed that the percentage of the growth is decreasing

for 35% and the growth inhibition is decreasing for 65%, morphological description showed that there is dormant formation. The mutant group that was exposed to artemisinin with 10-7 M concentration and incubated for 48 hours showed that the percentage of growth is 92% and the percentage of growth inhibition is 8%, morphological description showed that there is dormant formation, tropozoit with cytoplasm that still appears blue and the presence of normal schizont containing merozoites with brownish black pigment. The results of this study showed that the parent of *P. falciparum* 2300 strain requires medicine with smaller concentrations to inhibit *P. falciparum* growth compared to the mutant of *P. falciparum* 2300 strain that have been resistant to artemisinin, due to the greater concentration that is required to inhibit the growth of the parasites. It can already be seen from the observation of morphological description that the same artemisinin concentration (10^{-7} M), there are morphological changes compared to the control (K) group. In the mutant group, there are morphological changes in their developmental stages of the ring, schizonts and trofosoit are normal, so that the morphological description can describe that getting the same dormant period is required greater concentration of artemisinin.

The results of this study also showed that the dormant period can occur in a parasite strains that are resistant to artemisinin but it need a higher medicine concentration to be able to induce it. *Plasmodium* parasites that are resistant to artemisinin can not be into a dormant period if the drug concentration had been tolerant. It is possible that the dormant parasite uses innate mechanism to survive from the pressures (stressors), that is the concentration of medicine which can cause severe damage to the parasite, and the dormant parasites can also be triggered when a parasite growth is inhibited. [(10)]

Bagaimana hasil-hasil penelitian lainnya, apakah konsisten dengan hasil penelitian sejawat?

Stage of development and morphology of intra erythrocytic *P. Falciparum* 2300 strain that was synchronized in the control group (K) and treatment with artemisinin exposure with concentration from the IC_{50} values and were observed every 12 hours. In the control group, since 0 hours to 48 hours showed normal morphology development with faster growth compared with the group treated with artemisinin exposure concentration from the IC_{50} values. In the treatment group that was exposed to artemisinin showed dormant morphology developmental with core chromatin condenses and if it can survive with exposure to artemisinin, it only survives up to 24 hours after exposure, but shows the stages of development and morphological that is not perfect in the ring and trophozoites.

The results of this study showed that cell cycle development in the control group (C) run normally while the mutant group that was not exposed artemisin shows the life cycle of intra-erythrocytic development of *P. Falcifarum* is faster. The increased growth of *P. falciparum* that ever exposed to artemisinin is caused by upregulation of the genes transcription (multi-gene) that play a role in cell cycle regulation, transport substances from erythrocytes into the parasite, the enzymes that involved in the biosynthesis of purines in DNA synthesis and synthesis proteins that play a role in the adaptation of parasites in the environment from the early stage of parasite growth (16-20 hours) during the development of the ring becomes trophozoites and at the end of development (36-40 hours) when trophozoites develops into schizonts. [(11)]

In the group that was treated with artemisinin exposure concentration from the IC_{50} values shows the decreasing intra-erythrocytic development of *P. Falcifarum*'s life cycle. The results of this study is suitable with research that is conducted by Veiga et al. (2010) who did mefloquine exposure on three strains of Plasmodium (W2, 3D7 and FCB) compared with the control treatment, 40% of cell morphology showed slowdown development, Exposure to anti-malarial medicine that

can induce *pfmdr1, pfcrt, pfmrp1* and *pfmrp2* gene expression with increased 1.5 times. The same thing also happens after quinine exposure for 12 hours, there is a slowdown in the development of cell morphology. The results of this research can clarify a slowdown in the development of *P. falciparum* is very important mechanism for the parasite to be able to escape from the influence of anti-malarial medicine. [6]

Morphological dormancy in *P. falciparum* that were exposed to artemisinin is a defense mechanism for the parasite to be able to survive from the exposure of artemisinin anti-malarial medicine. Parasites will be able to grow normally after medicine's pressure 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 medicine, because in this situation, there is no DNA synthesis. [11]

A parasite that still survive in the trophozoite stage of exposure to artemisinin anti-malarial medicine with abnormal morphology, which forms a thickened cytoplasm. Morphological changes start at the trophozoite stage until the final phase of the life cycle of *P. falciparum* that had been exposed to artemisinin for 48 hours. Artemisinin exposure causes metabolic disorder of parasites that affect its growth and development. Those can be observed from the changes and the morphologic abnormalities. A change in this trophozoite stage because at this stage, the parasite began a process of active metabolism. Parasites began to grow bigger from the initial trophozoites into mature trophozoites. The extraction of nutrients from the erythrocyte's cytosol will be consumed quickly in the digestive vacuole that began to form. Hemoglobin degradation process into oligopeptides and heme by proteolysis in the digestive vacuole is began to occur in order to fullfil the nutritional needs of the parasite. [12,13]

Abnormal morphology and growth inhibition after artemisinin exposure is also caused by the inhibition of parasite protease enzyme (plasmepsin, falcipain and falcilisin) which is essential for parasite growth. Research that is conducted by Bonilla et al. [14] showed that plasmodium knockout mutants (triple and quadruple PM PM KO KO) in the protease enzyme causes a deficiency in the endosomal vesicles process that enters into the digestive vacuole and produce multilamellar bodies, causing a deficiency in hemoglobin digestion and obstacle hemozoin formation in the digestive vacuole so that causes slowing growth.

Changes in morphology and growth obstacle because of protease enzyme had been reported by Salmon et al. [15]. In the final stages of the parasite that is incubated with E64 protease inhibitor for 6-12 hours, parasite growth is stopped in the schizont stage and inhibit the breakdown of mature schizonts to infect erythrocytes. This results is suitable with the E64 protease inhibitor effects on schizont maturation so that the parasite failed to enter the next cycle. It shows that the protease is required to produce a viable merozoites. Inability hemoglobin digestion because of protease inhibition will cause osmotic imbalance that causes death in schizont stage. [15]

Tambahkan keterbatasan penelitian, implication of this study and future directions of this study

CONCLUSION

Exposure artemisinin antimalarial medicine in vitro can cause phenotypic changes of dormancy morphological in *Plasmodium falciparum* Papua 2300 strains.

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15%. Terlalu banyak 5/15 (33%) Harap baca Petunjuk bagi penulis

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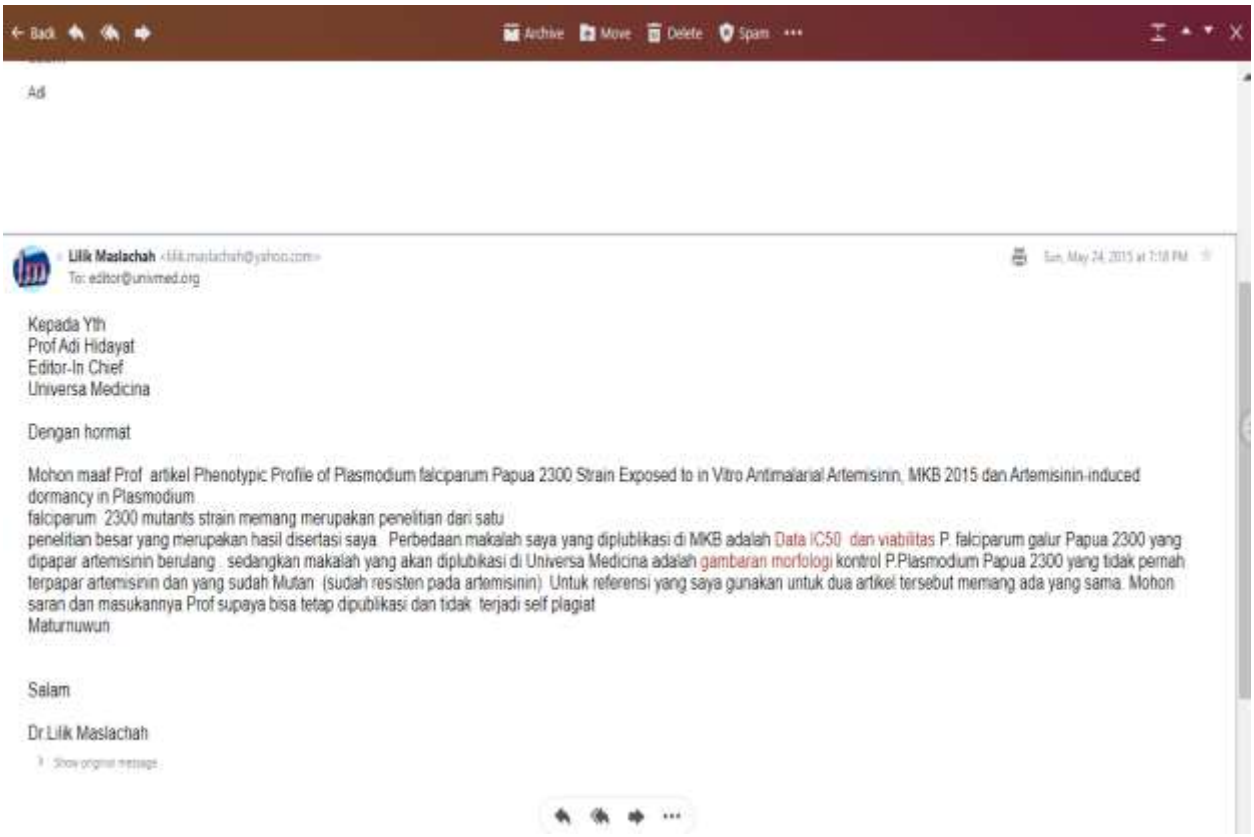

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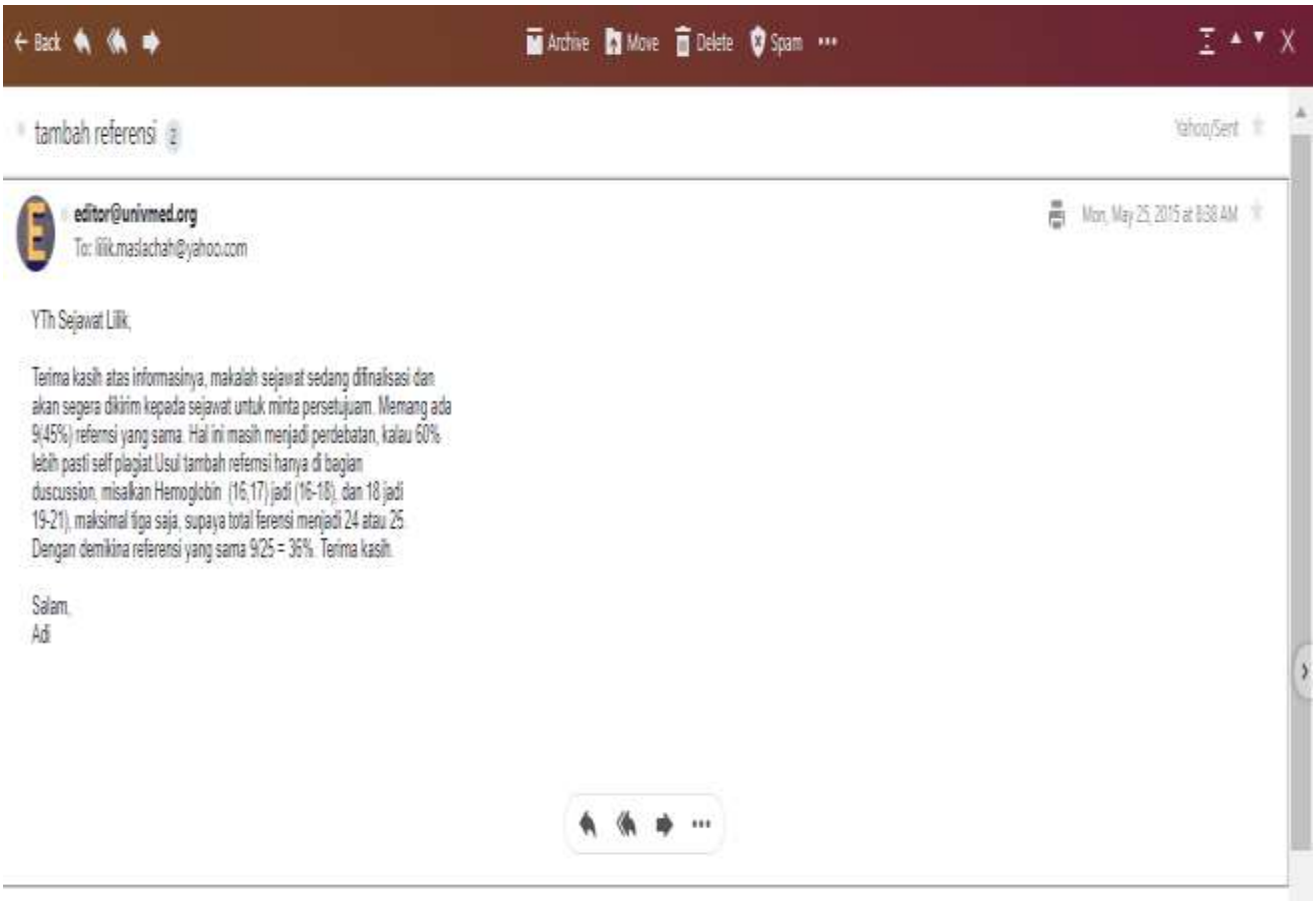

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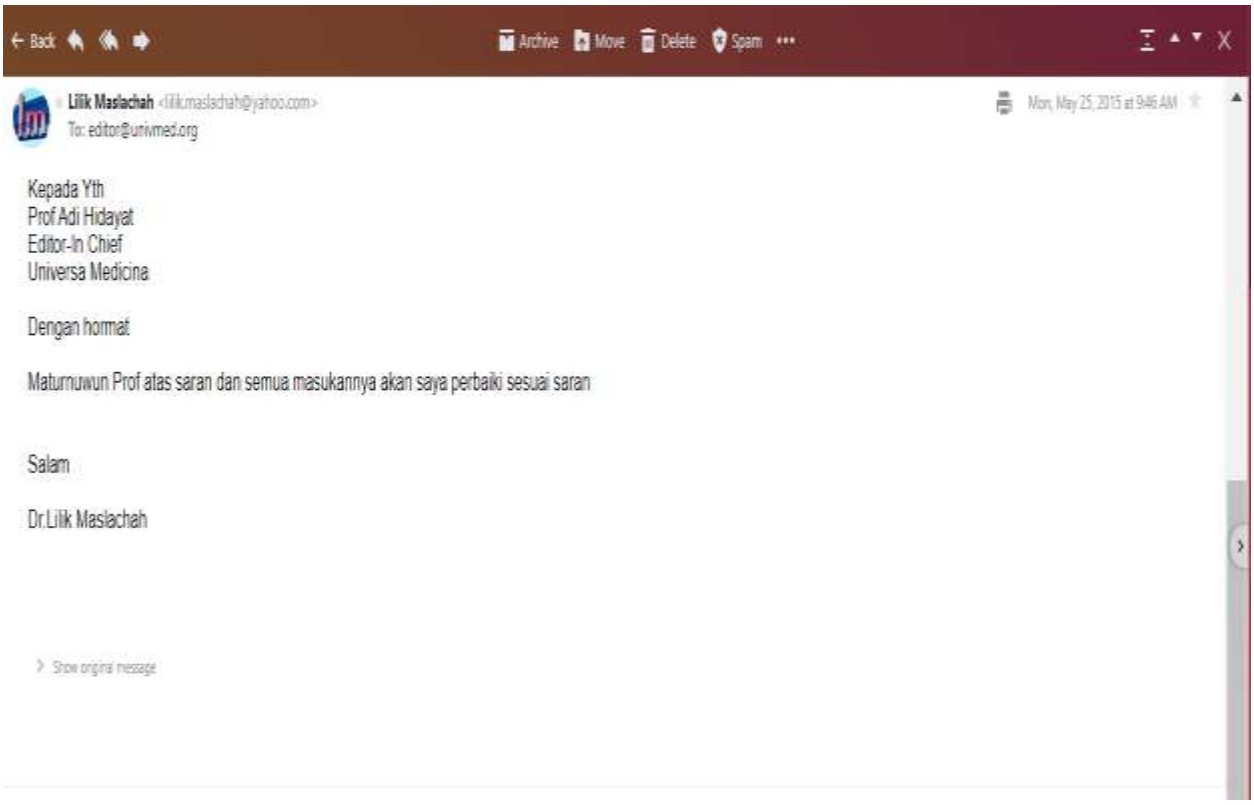

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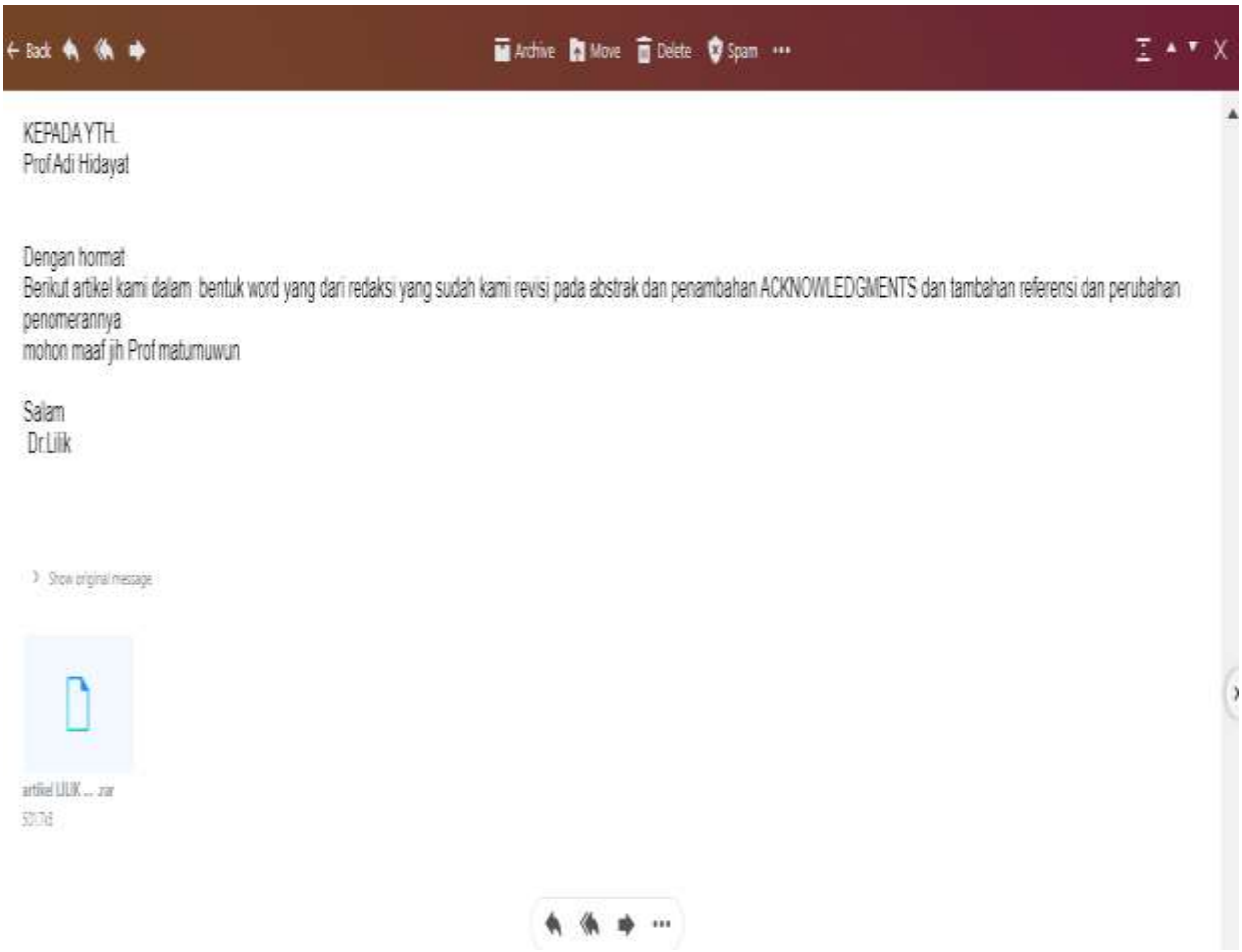
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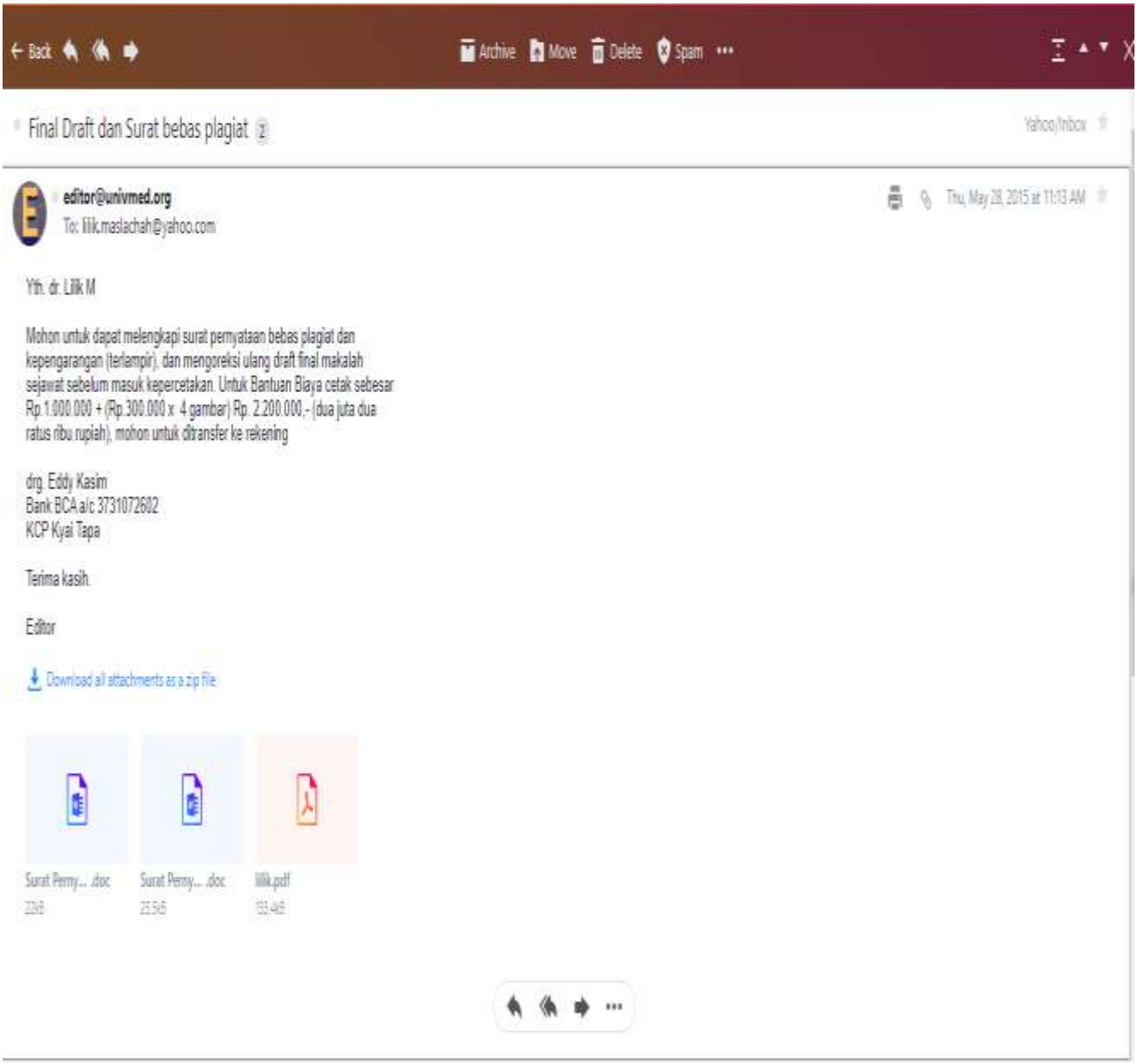
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Induction of *Plasmodium falciparum* strain 2300 dormant forms by artemisinin

Lilik Maslachah*, Yoes Prijatna Dachlan, Chairul A.Nidom*, and Loeki Enggar Fitri*****

ABSTRACT

BACKGROUND

The presence of the P. *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^{-5}??? (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} $2.5x10^{-5}$ M.

CONCLUSION

Dormant forms occur in *P. falciparum* 2300 strain mutants only after exposure to high artemisinin concentrations.

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 komplek, karena belum ada obat baru pengganti artemisinin. Penelitian ini bertujuan untuk menilai efek paparan artemisinin terhadap morfologi ultrastruktur P. 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^{-5} ??? (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 dimulai dari jam ke 0, 12, 24, 36, 48 dengan membuat 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 sitoplama 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} $2.5x10^{-5}$ M baru terjadi bentuk dorman.

KESIMPULAN

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

***Kata kunci** : Artemisinin, fenotip, Plasmodium 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,[1] 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 [2] and entire economies.[3]

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.[1]

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.[4] A clinical study found two patients in Cambodia who had been infected with *Plasmodium falciparum* to be resistant to artesunate.[5] 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 *Plasmodium falciparum* to artemisin may be influenced by internal factors of *Plasmodium 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.[6] Another research study was conducted by molecular monitoring of the genes that are involved in *Plasmodium falciparum* resistance to antimalarials.[7,8] The research results found that combined artemisinin resistance may be due to mutations in the *P. falciparum* adenine triphosphatase 6 (pfatpase6) gene.[7]

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.[9] The relationship of repeated artemisinin exposure with phenotypic, proteomics and genotypic changes in chloroquine resistant *Plasmodium falciparum* has not been proved, necessitating the present study.

This study was conducted to evaluate the effect of in vitro artemisinin exposure at IC_{50} doses on the morphological changes in *Plasmodium falciparum* 2300 mutant strain that had become resistant to artemisinin and already had mutations in the pfatpas6 genes.[9] Previous research on the *P. falciparum* F32-Tanzania strain that was exposed for 3 years to artemisinin at low concentrations ranging from 0.01 µM up to 10 µM 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.[10] 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 IC_{50} value of the selected and viable GC06 parasites increased.[11]

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} $2.5x10^{-5}$ 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 *Plasmodium 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°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 $2.5x10^{-5}$ 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.[6,12]

Data analysis

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

RESULTS

Figure 1 shows the differing percentages of growth inhibition of *Plasmodium 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 *Plasmodium 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.

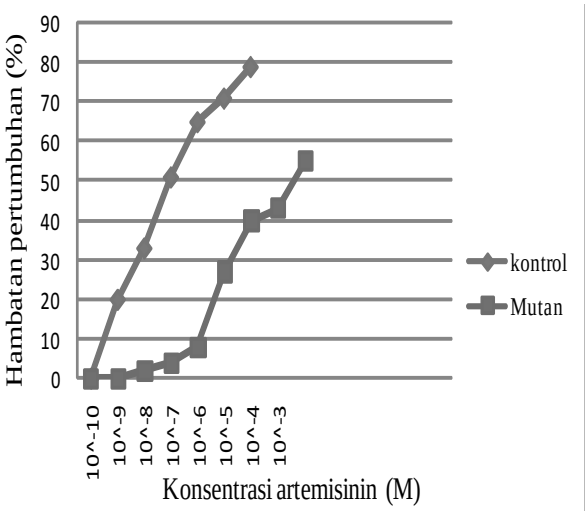

Figure1. Percentage growth inhibition of *Plasmodium falciparum* 2300 strains in control and mutants exposed to artemisinin

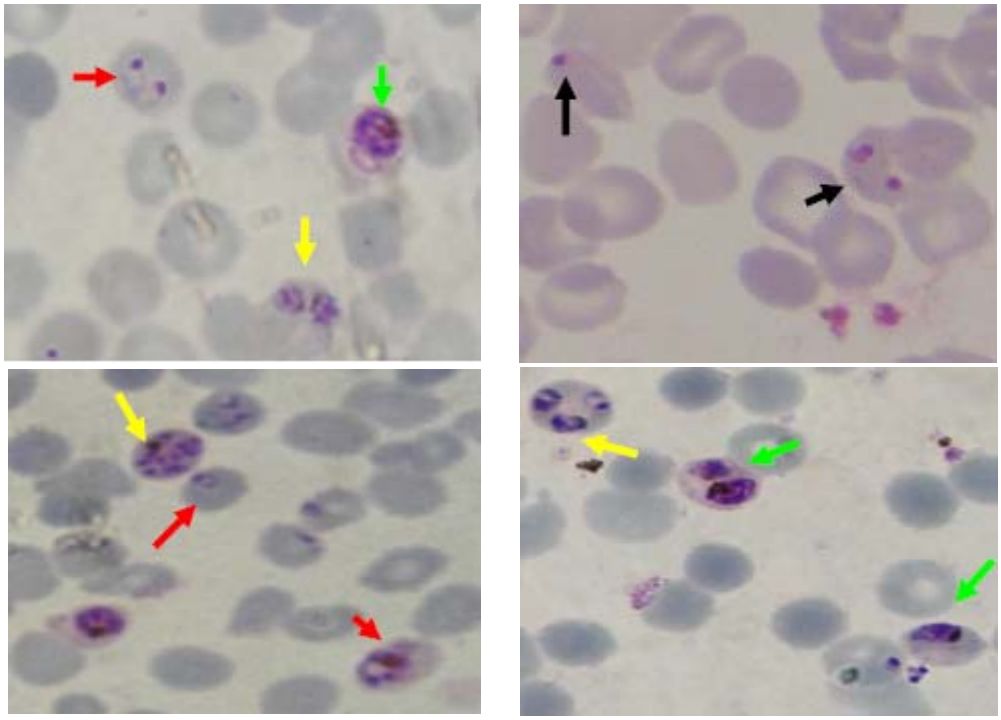

Figure 2. Parent and mutant *P. falciparum* morphology after being exposed for 48 hours to artemisinin at IC_{50} dose (1000x magnification). Giemsa staining. Arrow color codes: black = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite. (A) Parent *P.falciparum* 2300 strain, (B) Parent *P.falciparum 2*300 strain after being exposed to artemisinin 10^{-7} M for 48 hours, (C) Mutant *P. falciparum* 2300 strain, (D) Mutant *P.falciparum 2*300 strain after being exposed to artemisin10^{-7} M for 48 hours

As seen in Figure 2, the morphology of *Plasmodium 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 *Plasmodium 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 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.

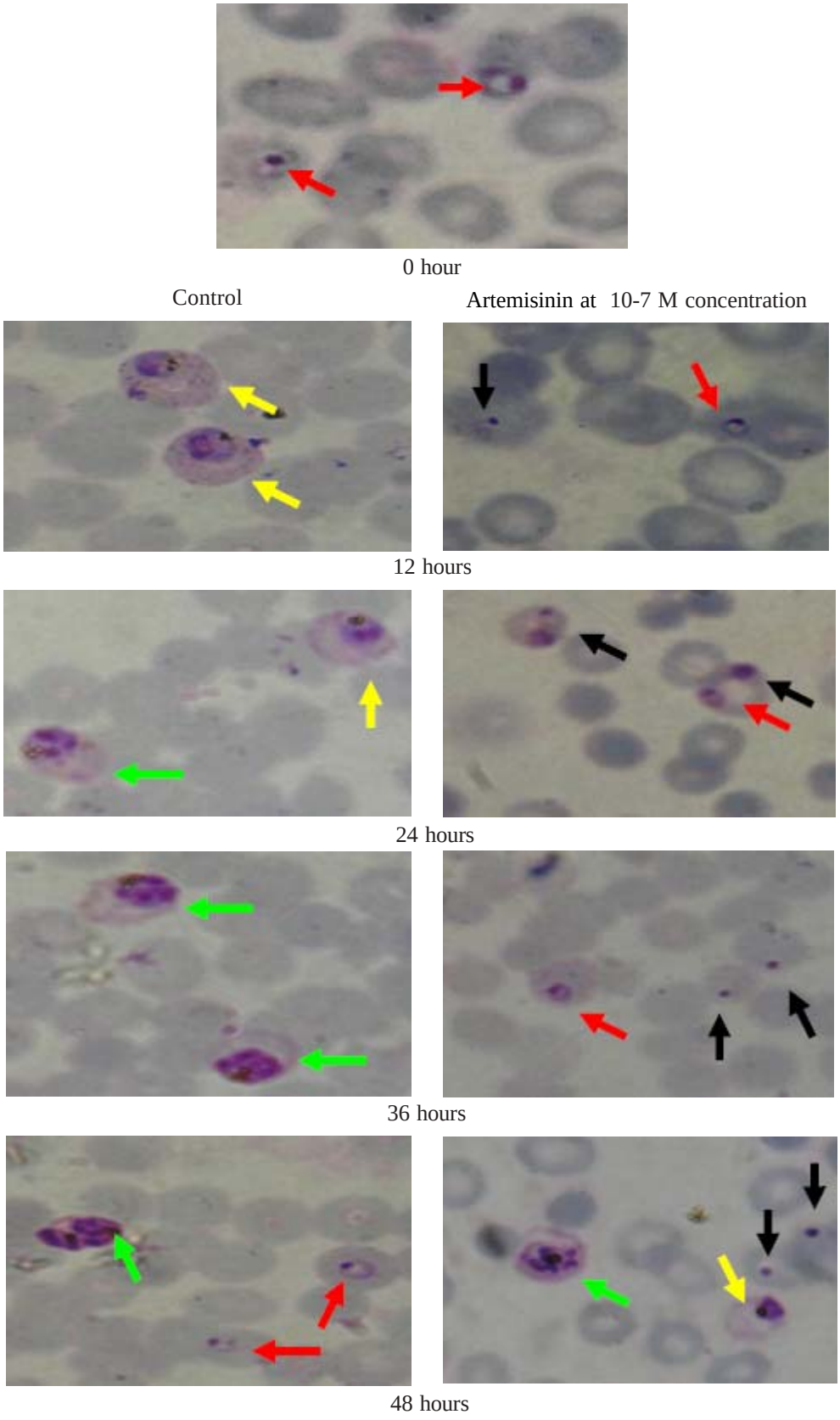

Figure 3. Morphology of *P. falciparum* 2300 strain synchronized in the control group, treated with artemisinin at IC_{50} 10^{-7} M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

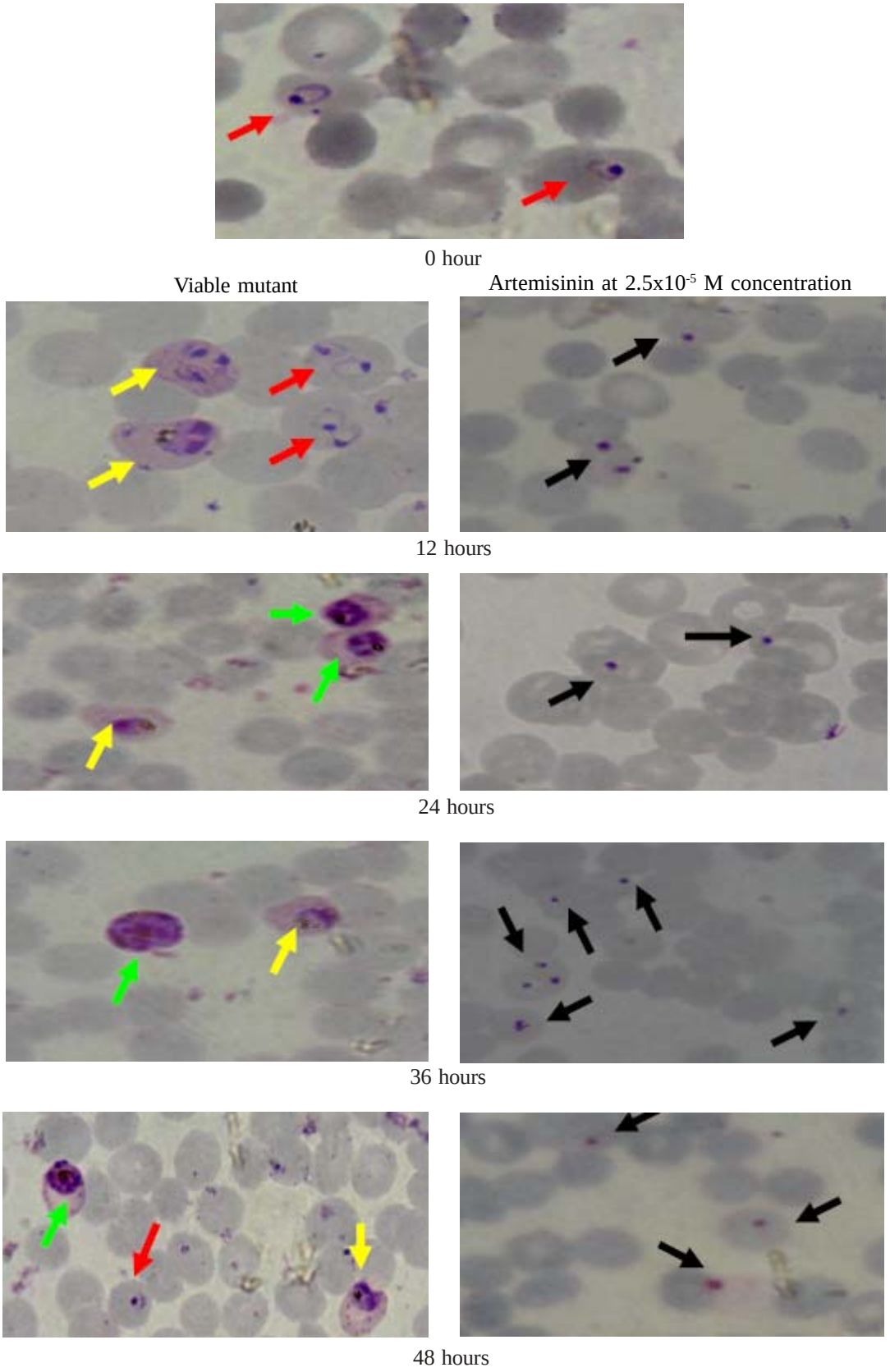

Figure 4. Morphology of *P. falciparum* 2300 strain synchronized in the viable mutant group, treated with artemisinin at IC_{50} $2.5x10^{-5}$ M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

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 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.[13]

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_{50} strain values after selection with artemisinin. IC_{50} 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.[11] 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.[10]

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_{50} 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_{50} 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. falcifarum* in the mutant group that was not exposed to artemisin 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.[10]

In the group that was treated with artemisinin exposure at IC_{50} concentration, there was decreased intra-erythrocytic development of *P. falcifarum*. The results of this study are comparable to those of research by Veiga et al.[6] 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, pfcrt, 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.

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.[10,14,15]

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 fullfil the nutritional needs of the parasite.[16,17]

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.[18] 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.[19,20]

One limitation of this research is that it was conducted only with the *Plasmodium 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 *Plasmodium 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 *Plasmodium 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 *Plasmodium falciparum* Papua 2300 strain.

ACKNOWLEDGMENTS
Tambahkan

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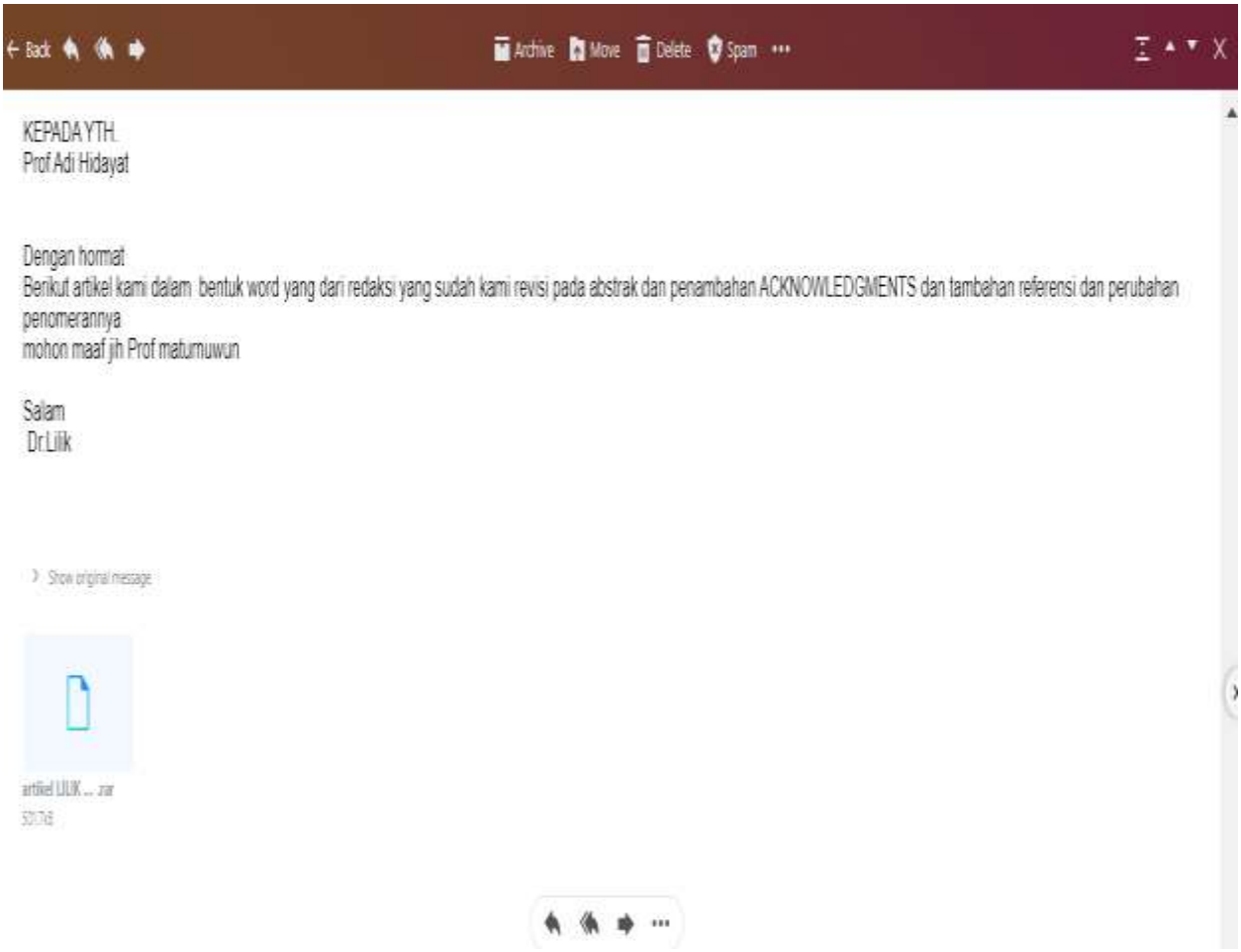
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Induction of *Plasmodium falciparum* strain 2300 dormant forms by artemisinin

Lilik Maslachah*, Yoes Prijatna Dachlan, Chairul A.Nidom*, and Loeki Enggar Fitri*****

ABSTRACT

BACKGROUND

The presence of the P. *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} $2.5x10^{-5}$ M.

CONCLUSION

Dormant forms occur in *P. falciparum* 2300 strain mutants only after exposure to high artemisinin concentrations.

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 komplek, karena belum ada obat baru pengganti artemisinin. Penelitian ini bertujuan untuk menilai efek paparan artemisinin terhadap morfologi ultrastruktur P. 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^{-5} 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 dimulai dari jam ke 0, 12, 24, 36, 48 dengan membuat 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 sitoplama 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} $2.5x10^{-5}$ M baru terjadi bentuk dorman.

KESIMPULAN

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

***Kata kunci** : Artemisinin, fenotip, Plasmodium 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,[1] 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 [2] and entire economies.[3]

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.[1]

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.[4] A clinical study found two patients in Cambodia who had been infected with *Plasmodium falciparum* to be resistant to artesunate.[5] 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 *Plasmodium falciparum* to artemisin may be influenced by internal factors of *Plasmodium 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.[6] Another research study was conducted by molecular monitoring of the genes that are involved in *Plasmodium falciparum* resistance to antimalarials.[7,8] The research results found that combined artemisinin resistance may be due to mutations in the *P. falciparum* adenine triphosphatase 6 (pfatpase6) gene.[7]

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.[9] The relationship of repeated artemisinin exposure with phenotypic, proteomics and genotypic changes in chloroquine resistant *Plasmodium falciparum* has not been proved, necessitating the present study.

This study was conducted to evaluate the effect of in vitro artemisinin exposure at IC_{50} doses on the morphological changes in *Plasmodium falciparum* 2300 mutant strain that had become resistant to artemisinin and already had mutations in the pfatpas6 genes.[9] Previous research on the *P. falciparum* F32-Tanzania strain that was exposed for 3 years to artemisinin at low concentrations ranging from 0.01 µM up to 10 µM 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.[10] 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 IC_{50} value of the selected and viable GC06 parasites increased.[11]

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} $2.5x10^{-5}$ 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 *Plasmodium 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°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 $2.5x10^{-5}$ 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.[6,12]

Data analysis

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

RESULTS

Figure 1 shows the differing percentages of growth inhibition of *Plasmodium 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 *Plasmodium 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.

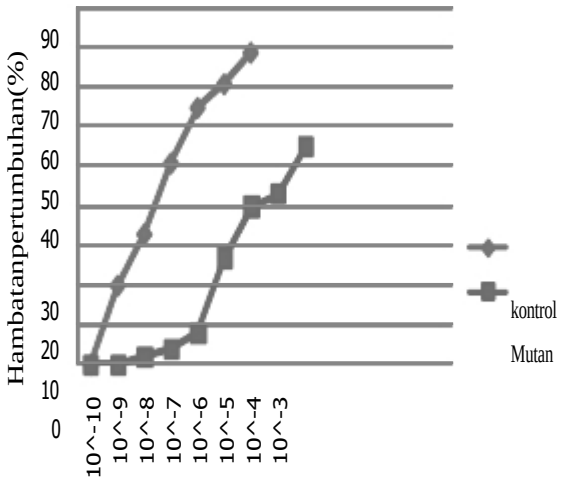

Figure1. Percentage growth inhibition of *Plasmodium falciparum* 2300 strains in control and mutants exposed to artemisinin

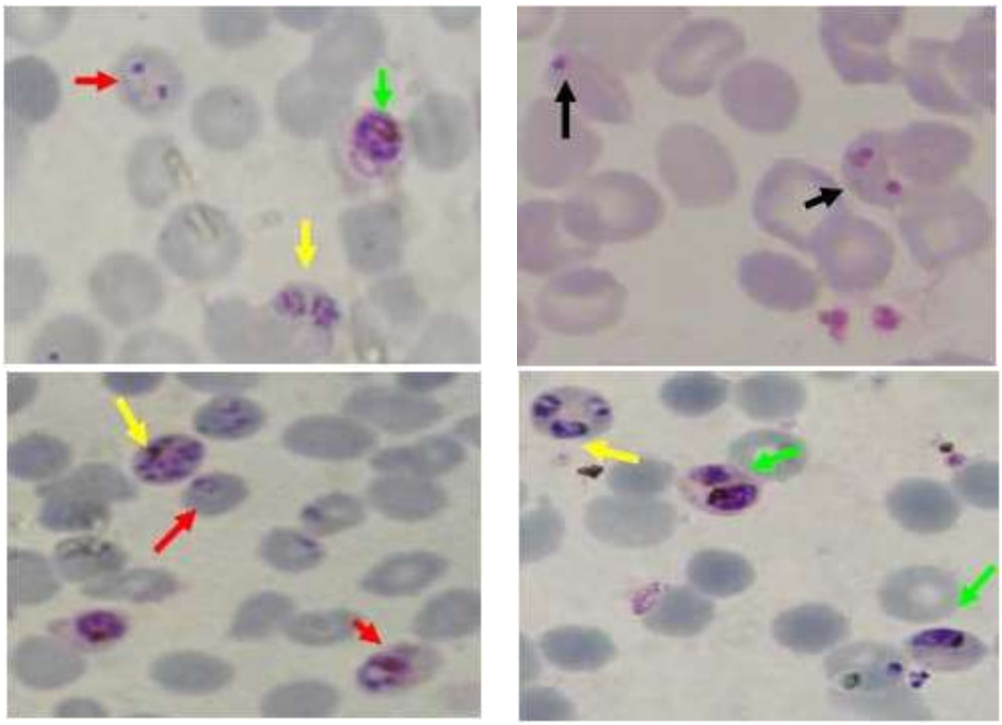

Figure 2. Parent and mutant *P. falciparum* morphology after being exposed for 48 hours to artemisinin at IC_{50} dose (1000x magnification). Giemsa staining. Arrow color codes: black = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite. (A) Parent *P.falciparum* 2300 strain, (B) Parent *P.falciparum 2*300 strain after being exposed to artemisinin 10^{-7} M for 48 hours, (C) Mutant *P. falciparum* 2300 strain, (D) Mutant *P.falciparum 2*300 strain after being exposed to artemisin10^{-7} M for 48 hours

As seen in Figure 2, the morphology of *Plasmodium 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 *Plasmodium 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 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.

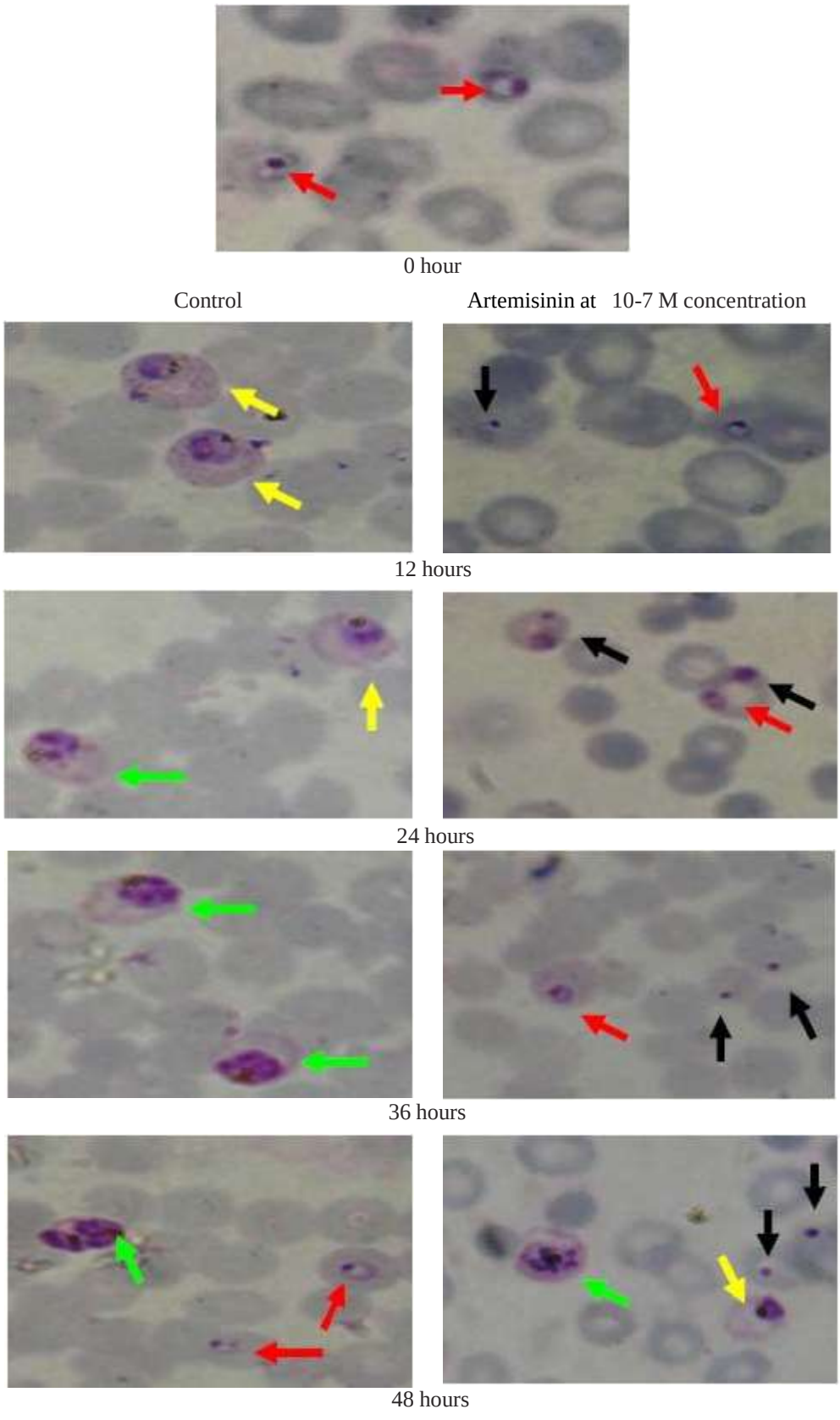

Figure 3. Morphology of *P. falciparum* 2300 strain synchronized in the control group, treated with artemisinin at IC_{50} 10^{-7} M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

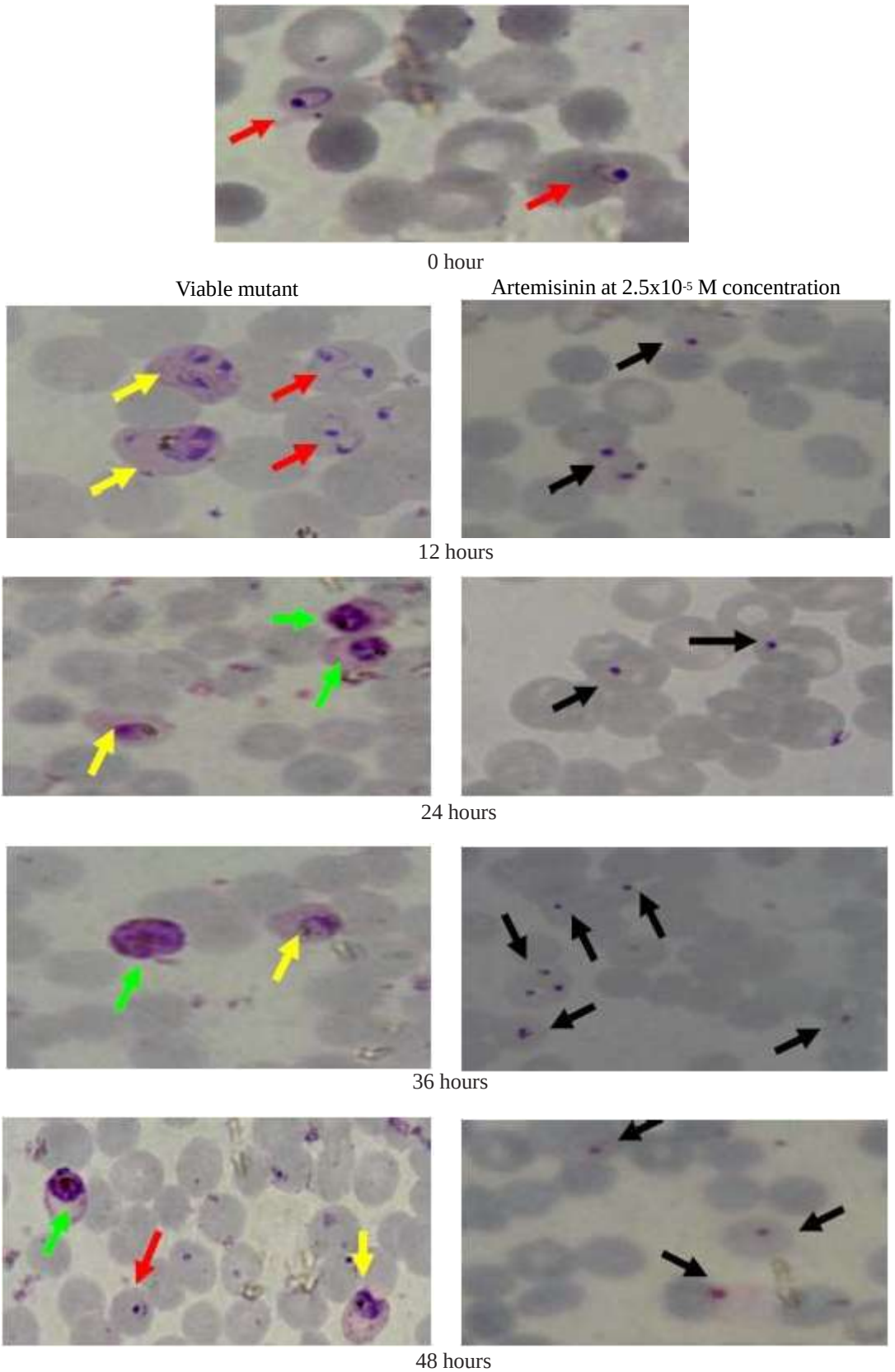

Figure 4. Morphology of *P. falciparum* 2300 strain synchronized in the viable mutant group, treated with artemisinin at IC_{50} $2.5x10^{-5}$ M concentration, and monitored every 12 hours (1000x magnification). Giemsa staining. Black arrow = dormant, red = ring, yellow = trophozoite, green = schizont, blue = merozoite

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 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.[13,14]

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_{50} strain values after selection with artemisinin. IC_{50} 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.[11] 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.[10]

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_{50} 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_{50} 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. falcifarum* in the mutant group that was not exposed to artemisin 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.[10,15]

In the group that was treated with artemisinin exposure at IC_{50} concentration, there was decreased intra-erythrocytic development of *P. falcifarum*. The results of this study are comparable to those of research by Veiga et al.[6] 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, pfcrt, 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.[6,16]

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.[10,17,18,19]

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 fullfil the nutritional needs of the parasite.[20,21]

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.[22] 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.[23,24]

One limitation of this research is that it was conducted only with the *Plasmodium 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 *Plasmodium 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 *Plasmodium 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 *Plasmodium falciparum* Papua 2300 strain.

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