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Antimalarial Effects of Menadione on *Plasmodium falciparum* FCR-3 Strains: In Vitro Study

Fiyandi Afianda,[1] Hesti Lina Wiraswati[1,2*], Bachti Alisjahbana[3,4], Nisa Fauziah[2,4]

[1]*Oncology and Stem Cell Working Group, Faculty of Medicine, Universitas Padjadjaran, Bandung*
[2]*Parasitology Division, Department of Basic Biomedical Science, Faculty of Medicine, Universitas Padjadjaran, Bandung*
[3]*Departement of Internal Medicine, Faculty of Medicine, Universitas Padjadjaran, Bandung*
[4]*Infection Study Center, Faculty of Medicine, Universitas Padjadjaran, Bandung*
**Corresponding Author: Hesti Lina Wiraswati*

Abstract:
Background:*Falciparum malaria is the most common type of malaria found in Southeast Asia, including Indonesia. Today, Artemisinin Combination Therapy (ATC) is the main therapy as well as a strategy to fight chloroquine resistance. This drug against parasite infection through oxidative stress mechanism. However, resistance to this drug has been found in several regions in Southeast Asia. Therefore, research to find new drugs for malaria is very important. On the other hand, menadione is a quinone group that is able to trigger oxidative stress. Thus this study aims to determine the activity of menadione in inhibiting the development of Plasmodium falciparum cells in finding new malaria drug candidates.*
Materials and Methods*: Menadione was tested on infected Red Blood Cells (RBC) with Plasmodium falciparum, in various concentrations. The analysis included microscopic observations to assess the reduction in the number of RBC using a hemocytometer, and the confluency of RBC as well as Plasmodium falciparum in culture.*
Results*: The results showed that menadione can cause a decrease in the number of red blood cells due to Plasmodium falciparum infection by 82% at a concentration of 8μM. This result is also in line with the decrease in the number of Plasmodium falciparum in the culture medium after menadione treatment.*
Conclusion:*Menadione showed inhibitory activity on the development of Plasmodium falciparum and maintain RBC from lysis. These results indicate that menadione has capacity as anti-malarial drug candidate.*
Key Word*: Artemisinin; Malaria; Menadione; Plasmodium falciparum; Oxidative stress.*

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I. Introduction

According to the data of World Health Organization (WHO), there were 219 million cases of malaria worldwide in 2017. Malaria falsifarum caused by Plasmodium falciparum is the most common malaria in Southeast Asia, including Indonesia. Annual Parasite Incidence (API) index-which shows the amount of malaria positive case per 1000 population-in the period of one year still has a high value especially in eastern Indonesia such as Papua, East Nusa Tenggara, and Maluku. Those regions have API of 50-100 in category of *High Cumulative Incidence II*.[1]

Since the initial discovery of the disease, some therapeutic drugs have been used. Some of them are, quinine, the combination of dihydrofolate-reductase inhibitor, such as proguanil, chlorproguanil, pyrimethamine, trimethoprim, and combination drugs of sulfa or sulfadoxine-pyrimethamine. Unfortunately, the resistance to these drugs has occured.[2–4] Several of those drugs have been no longer widely used for malaria therapy. The artemisinin compounds or the combination of artemisinin with other compounds, further referred as ACT or *Artemisinin Combination Therapy*is a malaria therapy that is recommended by WHO until today.[5] Also, ACT has become the recommended drug for malaria treatment by Republic of Indonesia Ministry since 2004.[6]

Artemisinin works against *Plasmodium falciparum*through induction mechanism of oxidative stress which will further inhibit the development and reduce the parasitemia of the parasite. The mechanism of oxidative stress makes artemisinin an antimalarial drug that can replace the previous one.[7,8] Unfortunately, the resistance proof towards artemisinin has been found in several regions such as Vietnam, Myanmar, and in the border regions of China and India with Myanmar as of July 2016.[2,5–7,9]Therefore, the research that proves the potency of antimalarial from other stress agents is indispensable. On the other hand, menadione is an oxidative stress agent that has been widely used in the discovery research of new drug candidate for all kinds of diseases.[8]Moreover, menadione has the ability to induce oxidative stress through different process with arteminisin. Arteminisin

induces stress through heme interaction on its endoperoxide part, meanwhile menadione induces stress through the process of direct electron reduction.[10–12]Thus, this research aims to find out medione activity in inhibiting the growth of *Plasmodium falciparum*cellin order to search the new malaria drug candidate.

II. Material And Methods

Plasmodium falciparum cell used was strain FCR3 (chloroquin resistant) with ATCC registration number 30932. The basic medium used was RPMI 1640 (Sigma Aldrich, R8578), *Red Blood Cell* (RBC) and blood serum obtained from human donors with the blood type of O, *giemsa*coloring, *Phosphate Buffered Saline*(PBS, Sigma Aldrich), and menadione(Sigma Aldrich, M5625). The count of red blood cell was conducted using hemocytometer. The cell culture was carried out in the *flask*of 25 cm^2. Medione testing was done in the *plate* of six wells.

The making of Complete Medium. The medium used for *Plasmodium falciparum* culture was complete medium added with RBC. Complete medium was made by mixing RPMI 1640 with blood serum at a ratio of 9:1. Serum was obtained by using centrifugation on the *whole blood* with speed of 1600 rpm for 10 minutes. Serum found on the supernatant was separated and inactivated by conducting heating on temperature at 56^{o}C for 30 minutes.

The preparation of RBC. RBC was obtained by using centrifugation on the *whole blood* with speed of 1600 rpm for 10 minutes. The pellet produced was separated from its supernatant, then washed with RPMI 1640 twice. The pellet was then resuspended with RPMI 1640 at a volume ration of 1:1. The supply of RBC was then placed at temperature of 4^{o}C.

The Culture of *Plasmodium falciparum Cell.* The supply of *P. falciparum* in the freezer -20^{o}C was diluted using *waterbath*on temperature at 37^{o}C for 10 minutes. The supply was then centrifuged with speed of 1600rpm for 10 minutes. The pellet produced was washed using RPMI 1640 twice, then resuspended using the same medium. The cell was cultured using complete medium with RBC added. The cell was preserved in the 5% CO_2 incubator on temperature at 37^{o}C. The medium was regularly replaced twice a week. After 1 week of perseverance, the cell was ready to use for the next treatment.

The making of Menadione Solution. Menadione powder preparation of 0.05 gram was dissolved in absolute ethanol of 2.9 ml that produced initial menadione supply with concentration of 0.1 M. The supply was then diluted with RPMI 1640 to attain concentration t 2µM, 4 µM, dan 8 µM. The fresh menadione solution was used for cell treatment.

The Menadione Treatment on *Plasmodium falciparu Culture.* *Plasmodium falciparum* cell was cultured in 1 flask for 24 hours, with the amount of RBC set (to obtain 1 million of RBC per well in plate of 6 wells). On day 1, the cell was cultivated for the next treatment with menadione. Parallel to it, the existence of the parasite in RBC was detected using *Giemsa* coloring technique. The cultivation of the cell was carried out by transferring culture medium into the falcon tube. The cell was then washed twice with PBS and the solution was transferred to the same falcon. Afterwards, trypsin was added into the flask to release the glued cells in the flask surface, by using incubation for 5-10 minutes in CO_2 incubator. The cell suspension in the falcon used for cell treatment with menadione. The treatment was conducted by six well plates. The control used is the well contained medium, the well added with menadione with concentration of 8µM, and the well contained medium and *Plasmodium falciparum*. The treatment well consisted of medium added with menadione with respective concentration of 2µM, 4 µM, dan 8µM. Respective treatment well was made with twice repetitions. Those cells were incubated for 48 hours inside CO_2, then to be analyzed.

The coloring with *Giemsa.* The 10% of *Giemsa*10% dissolved in PBS was used in this research. The *Giemsa* coloring was started with inserting suspension of 10µl above the object glass. The formed preparat was fixed with methanol then given *Giemsa* coloring for 10 minutes. The result of *Giemsa* was observed on the 100x magnification to detect the presence of *Plasmodium falciparum* inside RBC.

Analysis. The observation of cell culture result was conducted twice, at hour of 0 and at hour of 48. The observation result was the amount of RBC counted by using counting chamber or hemocytometer. The density of RBC and *Plasmodium falciparum* cell outside RBC set by conducting under microscope observation.

III. Result

The *Giemsa* coloring conducted to see the presence of *Plasmodium falciparum* cell inside RBC. The result of *Giemsa* showed that there was a ring structure inside RBC cell. This showed that *Plasmodium falciparum* cell successfully infected RBC. (Figure no 1)

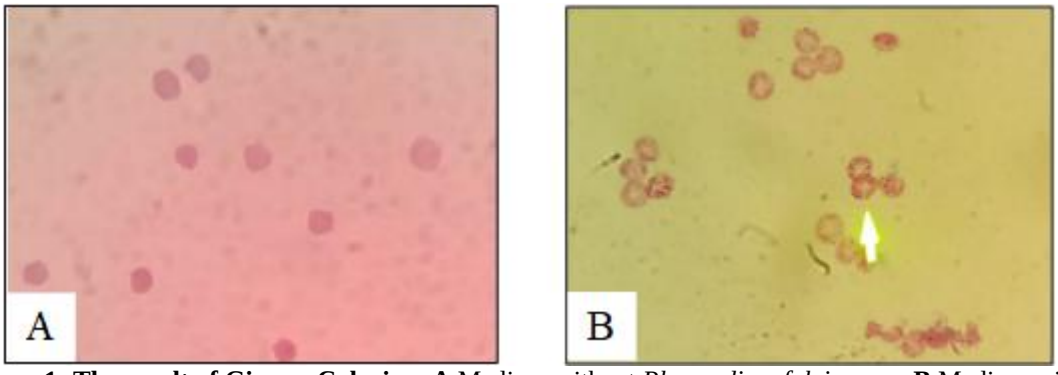


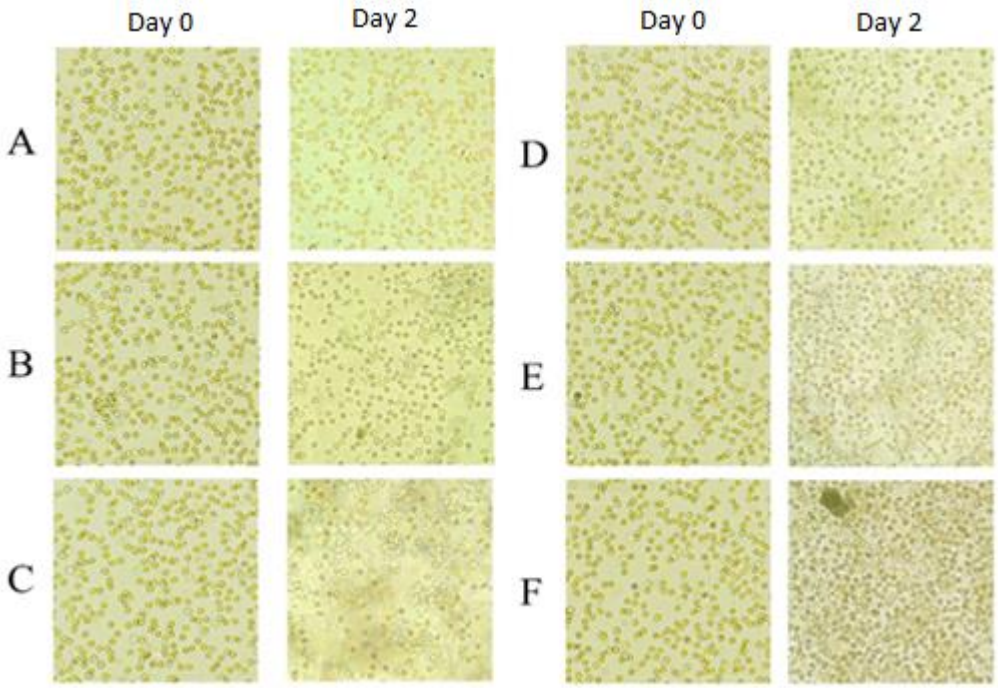
Figure no 1: The result of Giemsa Coloring. A. Medium without *Plasmodium falciparum*, **B.**Medium with *Plasmodium falciparum*
Description: The arrow showed the ring structure of *Plasmodium falciparum* inside the cell

The treatment with menadione was started by ensuring that menadione didn't cause the RBC to get lysis. This can be carried out by comparing the decreased amount of RBC on the control well that consisted of medium without menadione addition with control well that consisted of medium with menadione addition with concentration at 8µM. The count result of cell on both wells showed the decrease of 66% on well without menadione treatment and 65% on well given menadione treatment. (Figure no 2, Table no 1). This indicates that menadione doesn't cause RBC to get lysis.

Table no 1: Average Observation

	RBC						The amount of *Plasmodium falciparum* outside RBC
	At hour of-0			At hour of-48			
	Cell Amount	Cell density estimation (per mm^2)	Confluency (%)	Cell Amount	Cell density estimation (per mm^2)	Confluency (%)	
Control							
Medium	160000	318987	80	55000	299836	70	++++
Medium + Menadione 8 µM	160000	314767	80	54000	288887	70	-
Medium + *Plasmodium falciparum*	160000	305485	80	6800	93488	20	-
Treatment							
Menadione 2 µM + medium + *Plasmodium falciparum*	160000	303797	80	9500	223193	60	+++
Menadione 4 µM + medium+*Plasmodium falciparum*	160000	302953	80	24000	262778	70	++
Menadione 8 µM + medium + *Plasmodium falciparum*	160000	305485	80	29000	268674	70	+

(-) none, (+) small, (++) tolerably, (+++) many, (++++) so many



Figureno 2: Microscopic observation result of Menadione treatment on *Plasmodiumfalciparum culture.***A**. Medium, **B**.Medium + Menadione 8µM, **C.**Medium + *Plasmodium falciparum*,**D.** Menadione 2µM + Complete Medium + *Plasmodium falciparum*,**E.** Menadione 4µM + Complete Medium + *Plasmodium falciparum*,**F.** Menadione 8 µM + Complete Medium + *Plasmodium falciparum*

The inhibitory activity of *Plasmodium falciparum* growth was measured by comparing the amount of RBC lysis on the treatment well with control well that consisted of medium and *Plasmodium falciparum*. The calculation result showed there was decreased percentage of RBC lysis on treatment well with Menadione if compared to control well. The percentage of RBC lysis on treatment well with concentration at 2µM, 4µM, and 8µM was as of 94%, 85%, and 81% respectively, had a smaller value compared to the percentage of RBC lysed on control well was as of 95%. (Figure 2, Table 1) This showed that Menadione has the inhibitory effect of *Plasmodium falciparum* growth indicated by the lessening of RBC that was lysis.

The smallest percentage of RBC lysis was found on the well with Menadione concentration at 8µM, was as of 82%. The treatment of Menadione concentration at 2µM and 4µM showed the bigger percentage of RBC lysis, which was of 85% on concentration at 4µM, and 94% on concentration at 2µM. (Figure 2, Table 1). The comparison result showed that the biggest inhibitory activity of *Plasmodium falciparum* growth occurred on Menadione treatment with concentration at 8µM.

The reduction of RBC lysis amount after having Menadione treatment confirmed by comparing confluence of RBC and the cell density calculation between treatment well and control well. The result showed that the density and confluence of RBC on treatment well with Menadione was higher than on control well containing medium and *Plasmodium falciparum.* The highest density and confluence was observed on the well with Menadione treatment on concentration at 8µM. The observation result of l *Plasmodium falciparum* cell confluence also showed the lowest percentage on the well with Menadione treatment with concentration at 8µM. Based on the calculation result of the RBC amount, as well as *Plasmodium falciparum* cell confluence outside RBC, concluded that Menadione had the effect of preserving the RBC lysis and inhibiting *Plasmodium falciparum* growth with the biggest effect observed on treatment with Menadione in concentration at 8µM.

IV. Discussion

The result of Menadione treatment on *Plasmodium falciparum* culture supported the activity of inhibiting Plasmodium cells growth by this drug. Based on the obtained data, Menadione was proved to reduce the RBC lysis due to *Plasmodium falciparum* infections at the small lysis percentage of 82% on Menadione concentration at 8µM.

Menadione is a member of 1,4-naphthoquinone class that can induce the oxidative stress in cells as proved in the research conducted by Loog G, et. al. Based on that study, the mechanism of menadione in inducing oxidative stress was started with reduction process that formed unstable compounds of semiquinone which then can subsequently change into Menadione and release the stress agent.[12] The effect of malaria from artemisinin

which is the most potent drug for malaria has also action mechanism through oxidative stress induction though in different metabolic pathway from Menadione that is through the reaction of electron transfer which involves heme and endoperoxide rings.[13]The oxidative stress ability might become the basic of the antimalarial effect that the menadione has to be observed, though the more in-depth research about the definite mechanism of the antimalarial effects from Menadioneis is still indispensable.

V. Conclusion

The testing of *Plasmodium falciparum* strain FCR-3 culture with menadione showed that menadione was able to cause the decrease of the RBC lysis amount due to *Plasmodium falciparum* infection, that are of 82%, 85%,and 94% respectively on the concentration at 2µM, 4µM, and 8µM. This is in line with the density of *Plasmodium falciparum* cells in the medium which decrease successively from small to big, on the menadione concentration at 8µM, 4µM, and 2µM. Those results showed the potency of menadione as the new antimalarial drug candidates. Further research needs to be conducted to find out the mechanism of menadione action in inhibiting the growth of *Plasmodium falciparum*.

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