ARTESUNATE INCREASES BACTERICIDAL EFFECT OF ISONIAZID-RIFAMPICIN TO CLINICAL MDR-TB ISOLATES

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Abstract— Tuberculosis is one of the infectious disease that rely on antibotics to eradicated. The emergence of first line anti tuberculosis drugs resistance aggravate this situation. Artesunate possesses the abilities to: (1) the production of free radicals to damage the bacterial membrane, (2) lower the antibiotic efflux pump proteins with lower expression mRNA Acra, AcrB, and TolC, (3) lower NO produced by bacteria, and (4) increase the hydroxyl radical in bacteria resulting in toxic effects on bacteria through modification of DNA bases and chain termination.

Objective : to determine whether artesunate has an antimicrobial activity that increases bactericidal effect of Isoniazid-Rifampicin (INH-RIF) against Isoniazid-Rifampicin resistant Mycobacterium tuberculosis.

Method : the method of laboratory experiment are the dilution method to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The concentration of MBC of Artesunate was determined by way of 3 levels dilution thus obtained Artesunate 1 (ART 1) 120 mg / ml, Artesunate 2 (ART 2) 60 mg / ml, and 3 Artesunate (ART 3) 30 mg / ml. This experiment was repeated three times.

Result : at the concentration INH-RIF-ART 1, INH-RIF-ART 2, and INH-RIF-ART 3 were found bactericidal effect to Isoniazid-Rifampicin resistant Mycobacterium tuberculosis.

Conclusion: this study showed that there was increasing bactericidal effect of comibined Isoniazid-Rifampicin-Artesunate against Isoniazid-Rifampicin resistant Mycobacterium tuberculosis.

Index Terms— Isoniazid, Rifampicin, Mycobacterium tuberculosis, Artesunate.

I. INTRODUCTION

Tuberculosis (TB) is one of the infectious disease that remains to be a global health problem. According to a data by the WHO, the case of TB has reached 8.6 million in occurrence and resulting to 1.3 million deaths in 2012[1]. In 2013, the number increased to 9 million cases of TB and 1.5 million deaths[2]. The fact that there was 9.6 million cases of TB and 1.5 million deaths in 2014 shows rapid escalation[3].

In 2004, it was estimated that each year there are 539,000 new cases and 101,000 deaths due to TB in Indonesia. Whereas in 2010 the estimated prevalence of TB cases was approximately 660,000 and the estimated incidence amounted to 430,000 new cases per year in 2010[4] [5]. In 2014, the incidence of TB in Indonesia reached 324 539 cases and 1,812 confirmed to have multidrug-resistant TB (MDR-TB)[6].

Treatment of patients with TB has become the world's attention, with the "End TB Strategy" programmed by WHO. Indicators of achievement of this program is the 95% reduction in the number of TB deaths in 2035 compared with 2015, a decrease TB incidence rate of 90% in 2035 compared to 2015, and zero cost to patients affected by TB in 2035[7]. This can be achieved by four principles[7], namely: Service and accountability of government, with monitoring and evaluation; The strong coalition with civil society organizations and the public; Protection and promotion of human rights, ethics and equity; Adaptation of the strategy and targets at the country level, with global collaboration. It also can be achueved by 3 Pillar[7], namely: integration, patient-centered care and prevention; Bold policies and supportive systems; Intensive research and innovation.

WHO (2014) reported a prevalence of MDR-TB in the world in 2013 approximately 480,000 people had MDR-TB and 210,000 deaths. In Indonesia, there were 680,000 cases of TB and 383 of them are known MDR-TB in 2011[2]. Regionally, it was confirmed that the provinces with the lowest number of cases, namely Yogyakarta, Bali and Central Java. While the provinces with the highest number of cases are North Sulawesi, Southeast Sulawesi and Gorontalo[8].

Artesunate is water soluble semisynthetic derivatives of artemisinin which is more tolerable and less toxic compared to artemisinin which is a sesquiterpene lactone extracted from the plant *Artemisia annua*[9][10][20]. Artesunate minimizes oxidative stress formation of nitric oxide (NO) in *Bacillus*

licheniformis. This mechanisms can reinforce the activity of antibiotics because bacteria can detoxify NO antibiotics. So when combined with rifampin, artesunate may enhance the bactericidal effects of rifampicin against *Bacillus licheniformis*[11][12] [13]. In addition, artesunate can be bound to the heme groups that interfere with the conversion of H_2O_2 catalase. Hydroxyl radicals produced from H_2O_2 cause toxic effects on the bacterial DNA through base modification and termination of the chain. The study by Li *et al* (2011) showed that artesunate can degrade mRNA expression *Acra, AcrB,* and *TolC*, which is important protein pump efflux of antibiotics in gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*[13][15][16][17]. Artesunate was well tolerated by patients. Nevertheless, dihidroartemisinin can accumulate in cerebrospinal fluid during repeated administration of artesunate. Fever, itching, abnormal bleeding, hematuria, mild and reversible electrocardiographic abnormalities and haematological, such as first-degree heart block and neutropenia, sometimes found as side effects. However in general clinical use of artesunate is safe[18] [19].

Based on this background, this study aims to determine the potential effects of an ingredient that can be combined with antibiotics so that the effect of antibiotics becomes stronger. One of the ingredients that have been proven to strengthen the effect of antibiotics is artesunate, an antimalarial drug. The cell wall structure of *Mycobacterium tuberculosis* contains complex thick lipid layer. Some lipophilic drugs are difficult to penetrate the cell wall. Artesunat has easy solubility in the solution of lipids[21] [22] [23] [24] [25][26][27]. The problem is whether artesunate can also be used in combination with isoniazid and rifampicin and also whether artesunate has an antimicrobial activity that increases bactericidal effect of Isoniazid-Rifampicin (INH-RIF) against Isoniazid-Rifampicin resistant Mycobacterium tuberculosis.

II. MATERIAL AND METHODS

Making the artesunate solution, Isoniazid and Rifampicin

Each drug is dissolved until it reaches a specified concentration. Artesunate 8000 µg/ml dan isoniazid 0,2 µg/ml dissolved in physiological saline solution due to its hydrophilic characteristic. While rifampin 1 µg/ml dissolved with methanol, because it is slightly soluble in water. Then, All solutions are sterilized by *Millipore* filtration technique[14] [28].

Making the bacteria suspension

Isolates were subcultured on liquid medium Middlebrook 7H9 for two weeks at a temperature of 37°C, isolated by centrifugation (2000 g, 10 minutes), then suspended in saline, clumps of bacteria were separated by a bath-type sonicator. The inoculum was diluted to a concentration of $3x10^6$ cfu / ml until it reaches the standard turbidity of McFarland number 1[29].

The effect of bactericidal or sensitivity to Mycobacterium tuberculosis

Bacterial suspension was applied to the Middlebrook 7H9 liquid medium that has been mixed with a solution of isoniazid 0,2 µg/ml and rifampicin 1 µg/ml, with additional artesunate contained 8000 µg / ml and medium without artesunate. After that, stored in an incubator at 37 ° C for 14 days. Then, the bacterial suspension was observed with the standard conventional proportional method, on Middlebrook 7H10 medium for bacterial sensitivity test. 100 µl of bacterial suspension was dropped on the surface of the medium agar. Then, the medium was kept in an incubator at 37°C for 14 days. Susceptibility testing performed on Mycobacterium tuberculosis strains of MDR-TB with 3 replication and bacteria control Mycobacterium tuberculosis H37Rv ATCC 27 294[14] [29] [30] [31].

III. RESULT

This research was an experimental research on the effect of artesunate to bacteria growth of clinical isolates of MDR-*Mycobacterium tuberculosis*. The method used is the dilution method to determine the MIC and MBC. In this research, artesunate with a concentration of 8.000 µg/ml derived from the solid artesunate powder and dissolved in physiological saline solution. Based on the journal, researchers determine the MBC by the dilution with 3 levels. Thus, were obtained Artesunate 1 (ART 1) 120 mg / ml, Artesunate 2 (ART 2) 60 mg / ml, and Artesunate 3 (ART 3) 30 mg / ml.

In this research, it needs 28 solid medium of 7H10 as treatment and 4 solid medium of 7H10 as control. On H37Rv sample with artesunate addition can be observed the growth of the bacteria at week 2 after planting to solid medium 7H10, and when observations punctuated by using a loop, there can be seen the colonies characteristic, dry-coarse, white-black, and granular. In patient 1 with addition ART 3, there were colonies of *Mycobacterium tuberculosis* in the media. Similarly, control media H37Rv, patient 1, patient 2, and patient 3. There were colonies of Mycobacterium tuberculosis (see Table 1). Colonies of bacteria appear on solid media 7H10 with treatment as follows, H37Rv treated with ART 2, ART 3, INH + RIF, patient 1 treated with ART 3, Patient 2 treated with ART 3, and in patient 3 does not appear colonies (see Table 1).

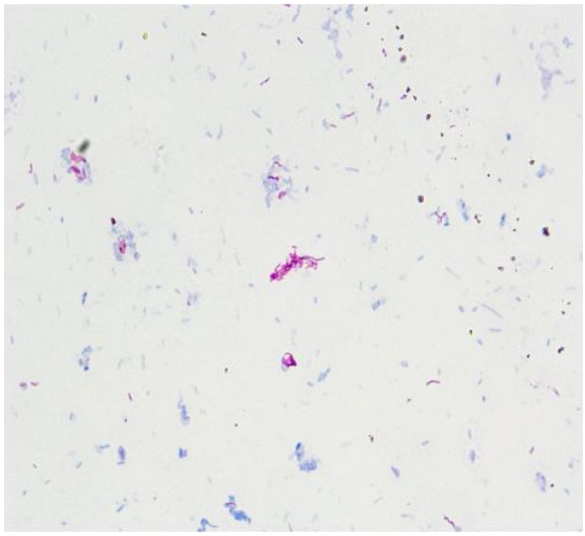

Fig.I: Ziehl neelsen staining on colony of Clinical MDR-TB Isolates

Table I: Artesunate inhibition test results in various concentrations on clinical MDR-TB isolates

Sample / Treatment	H37RV	Px 1	Px 2	Px 3	x̄
ART 1	-	-	-	-	-
ART 2	1	-	-	-	0.25
ART 3	2	7	1	-	2.5
INH + RIF	4	-	-	-	1
INH + RIF + ART 1	-	-	-	-	-
INH + RIF + ART 2	-	-	-	-	-
INH + RIF + ART 3	-	-	-	-	-
Control	40	6	30	4	20

IV. DISCUSSION

Artesunate Effects on Bacterial Growth of Clinical MDR-TB Isolates

Based on the results of the research, concentrations of artesunate with the good bactericidal effect on Mycobacterium tuberculosis occurs in ART 1 (120 mg / ml) and ART 2 (60 mg / ml). In this research, were tested the inhibitory anti bacterial drugs of artesunate against clinical MDR-TB isolates in liquid media 7H9 and solid media 7H10 were tested in this research. Based on the research by Li, *et al*, (2011), the concentration of bactericidal artesunate against Mycobacterium tuberculosis can be determined from the Minimal Inhibitory Concentration (MIC) artesunate against Mycobacterium tuberculosis, which is 8.000 µg/ml. Lethal dose of artesunate on research conducted by Natalang, O., *et al*, (2008), is determined by increasing the concentration of artesunate (30-100-300-500-3000 ng / mL) for 3 hours, washed twice with Roswell Park Memorial Institute (RPMI) and re-incubated at 37°C for 24-30 hours, until the next re-invasion. Re-invasion of parasites was assessed by Giemsa staining. Lethal dose artesunate is the minimal concentration needed to prevent 100% re-invasion. Lethal dose of artesunate on the parasite *P. falciparum* was 780 nM[33]. In this research, artesunate is diluted, in order to obtain artesunate 1, 2, and 3 with the following doses of 120 mg / ml, 60 mg / ml, 30 mg / ml. In accordance with the Federer formula, the research was conducted three times replication.

Artesunate produces free radicals and may cause damage to the plasmodium parasite membrane, through peroxidation of phospholipids[33]. In addition, artesunate binds heme groups catalase and interferes with the conversion of H_2O_2. Hydroxyl radicals causes toxic effects on the bacterial DNA through base modification and termination of the chain. The study by Li, *et al* (2011) showed that artesunate can degrade mRNA expression *Acra*, *AcrB*, and *TolC*, which is important protein pump efflux of antibiotics in gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*[13] [15] [16] [17].

Artesunate creates impacts on the parasite in several ways, namely through metabolic pathways, affect encoding chaperones, tranporters, kinases, Zn-finger proteins, transcription, proteasome degradation, in oxidative stress and in cell cycle regulation. In general pathways that can lead to death parasite is a disorder in the metabolism of purine / pyrimidine, mitochondrial electron transport chain, proteasome-dependent protein degradation or the robustness of the food vacuole[32] [33].

Artesunate is considered prodrugs that are activated to generate carbon-centered free radicals or reactive oxygen species (ROS). Artesunate modulates oxidative stress on the parasite and reduces the levels of antioxidants and glutathione (GSH) in the parasite. Artesunate-derived free radicals cause damage to cellular targets via alkylation[32] [33].

Heme is generated from the digestion of hemoglobin in the parasite food vacuole. Heme is toxic to the parasite and must be detoxified through polymerization to form 'hemozoin' (malaria pigment). Heme-artemisinin able to interact with *P. falciparum* histidine-rich protein II (PfHRP II), heme polymerase, and to replace the heme from PfHRP II, thereby inhibiting heme polymerization and formation of hemozoin. Artemisinin also increase the breakdown hemozoin[32] [33].

Artesunate also has a target protein that contributes to specific cysteine protease inhibition, which results in a decrease in hemoglobin decreasing and disrupt the function of the protein. P-type ATPase has been proposed as a specific target of artemisinin[32] [33].

Artesunate has also been shown to inhibit mitochondrial respiratory chain. Artesunate enables the electron transport chain, which produces ROS, which in turn damages the mitochondria[32] [33].

Effect of combination of Artesunate + Isoniazid + Rifampicin In Bacterial Growth of Clinical MDR-TB Isolates

Isoniazid is an antibiotic that is tuberculostatic. Its mechanism of action is by inhibiting the synthesis of mikolat acid which is an essential component of the bacterial cell wall.

Rifampicin / Rifampin is a semisynthetic antibiotic produced by *Streptomyces mediterranei* which is bactericidal. Its mechanism of action is to bind to the β subunit of RNA polymerase, thus inhibiting synthesis of RNA.

Artesunate could strengthen antibiotic activity. The production of free radicals be one of the mechanisms that support its activity against bacteria. Free radicals cause damage due to Plasmodium parasite membrane through phospholipid peroxidation[33].

In addition, research conducted by Zeng *et al* (2011) found that artesunate minimize oxidative stress formation of nitric oxide (NO) in *Bacillus licheniformis*. These mechanisms can reinforce the activity of antibiotics because bacteria can detoxify NO antibiotics. So when combined with rifampicin, artesunate may enhance the bacteridal effects of rifampicin against *Bacillus licheniformis*[11] [12] [13].

In addition, artesunate can be bound to the heme groups that interfere with the conversion of H_2O_2 catalase. Hydroxyl radicals produced from H_2O_2 causes toxic effects on the bacterial DNA through base modification and termination of the chain. The study by Li *et al* (2011) showed that artesunate can degrade mRNA expression *Acra*, *AcrB*, and *TolC*, which is important protein pump efflux of antibiotics in gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*[13] [15] [16] [17].

It was concluded that concentrations of artesunate with the good bactericidal effect to *Mycobacterium tuberculosis* which are, ART 1 (120 mg / ml) and ART 2 (60 mg / ml). Drug combinations with good bacteridal to INH + RIF + ART 1, INH + RIF + ART 2, and INH + RIF + ART 3; can be concluded combination of Isoniazid and Rifampicin with Artesunate can be used with lower concentrations, namely ART 3 (30 mg / ml).

Synergistic drug effects are the effects of two or more drugs that have the same therapeutic effect so they can strengthen the therapeutic effect, either with the same or different mechanism of action. Combination Anti Tuberculosis, Isoniazid and Rifampicin with Artesunate can improve the synergism of drugs through several ways, which increases the penetration of the drug into the cell or the target tissue, the target specific drug action, and molecules simultaneously work together. Thus, the drug dose of anti tuberculosis drug for patients with tuberculosis can be lowered. Thus, the OAT drug side effects can be minimized.

Based on the study by Li *et al* and Zeng *et al* (2011) artesunate and its derivatives can enhance the bactericidal of first-line anti-tuberculosis drugs, namely isoniazid and rifampicin in Clinical Mycobacterium tuberculosis strains of MDR-TB Isolates. Increased the bactericidal of isonoiazid-rifampin-artesunate through several ways, namely the production of free radicals, lowering the efflux pump protein, lowering the NO produced by the bacteria, and increasing the hydroxyl radical in bacteria.

The new findings on the research, concluded the effect of the increase bactericidal drug-combination of Isoniazid Rifampicin-Artesunate against *Mycobacterium tuberculosis* strains of MDR-TB, compared with only Isoniazid-rifampicin combination.

CONCLUSION

This research showed that there was increasing bactericidal effect of comibined Isoniazid-Rifampicin-Artesunate against Isoniazid-Rifampicin resistant *Mycobacterium tuberculosis.*

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