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Vitamin A induction in reactive oxigen intermediate and nitric oxide intermediate production againts *Plasmodium berghei*

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Abstract

Vitamin A is believed to trigger the monocyte cell proliferation and increase ROI and NOI compounds production that has functions as cell signaling and body's defense mechanisms. This study aims to understand the most potent dose of vitamin A as stimulus of monocytes proliferation and ROI and NOI production in animal's immune response in mice infected with *Plasmodium berghei* which using cellular immunity as the parameters. This study has design with Post Test-Only Control Group Design used Swiss Strain male mice aged 8 weeks. A total of 24 mice were divided into four groups randomly. Giving vitamin A, administered orally at one week before and one hour after infection. All the mice infected with *Plasmodium berghei* were 10^7 intraperitoneally. ROI and NOI has examined and the results analyzed by ANOVA and Kruskal Wallis test with significance limit <0.05. Giving vitamin A has been increased the activity of macrophages and the production of ROI and NOI. In the treatment group it was also decreased the number of parasite. This study shows the 6.000 UL is the most potent vitamin A as a stimulus to increase monocyte activity against Plasmodium berghei in mice with malaria.

Keywords nitric oxide intermediate, *Plasmodium berghei*, reactive oxygen intermediate, vitamin A.

1. Introduction

Vitamin A is one of the most essential component that needed in the body's metabolism. Large consumption of this vitamin will led metabolism disorder and deficiency of it also has a negative effect. Deficiency of vitamin A has indicated health problem in human body. It is correlated with vitamin a function which is play roles in metabolism as coenzyme (Muller et al., 2007). It is should be consume in the proper amount. Based on research conducted by Shanker et al. (1999), Serghides & Kan (2002) showed vitamin A and β-carotene have abilities to help the body to against malaria. This is also supported by the fact that increasing of parasites the body comparable on deficiency of Vitamin A and β-carotene condition. Research by Hamzah et al., (2007) showed that retinol supplementation against *Plasmodium berghei* infection in vivo showed that retinol can reduce morbidity, or the serious condition possibility of malaria. The existence of sufficient vitamin A can help speed up the recovery from the acute phase of infection conditions (Varandas et al., 2001) and can lead to increased macrophages activity, by producing molecules ROI and NOI to weaken antigen (Muller et al., 2007).

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ROI and NOI generated from almost all metabolic reactions in cells throughout the body, but not all utilized. ROI and NOI is toxic metabolic waste, however, its needed as a signal course of cell reaction or as a part of defense mechanism. In the first path, ROS is a waste product resulting from the metabolism of cells originating in the mitochondria. During the process of oxidative phosphorylation to electron transport, mitochondria of cells through electrons releasing in large quantities and captured by the oxygen to react as ROS. ROS have an important role in all cell metabolism either directly or indirectly, as well as cell-damaging toxic at a certain threshold.

ROS are also produced from a variety of metabolic reactions in the immune cells, involves the types of enzymes with different purposes such as NADPH oxidase, which plays role to pathogen resistance. ROI is also a matter of the formation of Hypoclorad Acid (HOCl) with a catalyst and myeloperoxidase, and superoxide dismutase stored in the phagosome macrophage cells which are effective in microbial components digesting. Leakage that occurs during phagocytosis process takes place in the cytosol contaminating by ROS and lead oxidative stress (Quinn et al., 2006). It triggers the apoptosis program in case simultaneously.

Recent research, the scientist has been known that infections caused by parasites have triggered of iNOS / NOI production. These include Plasmodium are responsible for the emergence of malaria in many parts of the world. More than 198 billion report and 584 thousand dead case due to malaria, especially in rural area in Africa (WHO, 2015). Malaria itself due to *Plasmodium* pathogen genera, has been decreasing human quality life and productivities in social and economic development. Malaria is a disease which requirement's to be alarmed in Indonesia. In 2002, the number of malaria disease happen in the more than 0.47 per 1000 of Java-Bali populations and 22.3 per 1000 of outside Java-Bali population.

Previous studies mentioned that the severity of malaria in human correlated with increased production and activity of NOI (Anstei et al., 1999). Increased levels of NOI has a positive impact because it can help kill parasites in the body. Another discovery shows that increased levels of NO also affects decreasing parasite adhesion to the endothelium of blood vessels, reduce interference micro vascular cytokine production and inhibit establishment of malaria. (Wink et al., 2011)

Based on previous studies, vitamin A is able to increase the macrophages activity. Macrophages will transform oxygen into reactive oxygen intermediates (ROI which is a reactive oxidizing agent that destroys microbes including plasmodium. Based on that, this research seeking to determine the optimal dose that can enhance macrophage phagocytosis ability shown by the index of phagocytosis and ROI production and NOI.

2. Materials and methods

This study is an laboratory experimental research, The Post Test-Only Control Group Design using experimental animals as objects of the research (Sudigdo et al., 2002). The number of samples is determined by the formula Federer for experimental research, as many as 24 mice were divided into 4 groups. This research was conducted in the Laboratory of Biochemistry, Biology Department of Universitas Negeri Semarang, Health Laboratory board of Yogyakarta and GAKI Faculty of Medicine, Universitas Diponegoro, Semarang

Plasmodium berghei obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, was cultured in mice Swiss Animal Laboratory Physiology Semarang State University. The experimental mice obtained from the Laboratory of Parasitology Faculty of Medicine, Universitas Gajah Mada, maintained in an iron cage measuring 50x30x20 cm, each cage contains 6 mice, fed with pellets BR2 and given to drink

enough water. Vitamin A is tablets contain Vitamin A acetate will be used to, each tablet contains 6000 IU. It is soluble in the olive oil to get proper concentration.

Samples were taken from male mice Swiss strain from peritoneal exudate cells (PEC). Mice feed obtained from the Food and Nutrition PAU UGM. Reagents needed are: Roswell Park Memorial Institute (RPMI), solution Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS) 10%, 70% alcohol, penicillin and streptomycin, 3% acetic acid, Latex beads, absolute methanol, Phosphate Buffered Saline (PBS), physiological saline, and NH4Cl

Plasmodium uses and mice sampling

This research has been used *Plasmodium berghei* as infectious agent and mice as host, because it is easier to do the manipulation on the host so that it can be studied the immunological changes that occur during malaria infection. *Plasmodium berghei* is hemoprotozoa that causes malaria in rodents.

Purposive samples taken from male Swiss mice strains that are genetically the same nature, healthy, activity and normal behavior, age 8 weeks weighing 28.80 to 31.20 grams. Grouping is done randomly and weighed before and after treatment to avoid bias due to age and weight. Strains are selected is because the Swiss have been reported to induce cellular immune response when inoculated mice with *Plasmodium berghei*.

Treatment phase

Experiments were carried out with completely randomized design. Mice were divided into 4 groups adapted for 7 days and treated in the laboratory with adequately housed, fed standard and drink ad libitum until the 14^{th} day. Each group was treated as follows. Each group was infected with *Plasmodium berghei* in the inoculum injection through intraperitoneally. Inoculum was prepared by diluting a number of blood donors with parasitaemia 30-40% in RPMI 1640. Blood was taken a week before and 1 hour after the infected. As such treatment groups Group 1: Control (10^7 *Plasmodium berghei*); Group 2: 10^7 Plasmodium berghei + olive oil 1 cc; Group 3: 10^7 Plasmodium berghei + vitamin A dose of 3000 IU; Group 4: 107 Plasmodium berghei + vitamin A dose of 6000 IU.

ROI and NOI production macrophages measurement

ROI production of macrophages checked using Nitroblue Tetrazolium (NBT) Reduction Assay. With the superoxide anion / O_{2^-} in cultured macrophages induced by PMA, will lead to reduced NBT to form formazan precipitates. The result is read under a light microscope to measure the percentage and degree per 50 macrophages then were averaged and expressed in degrees 1-4.

Macrophages activated by microorganisms will produce several compounds as a mechanism to kill the invader. One such compound is NO. NO measurements macrophages done by Griess reagent reacting with macrophage culture supernatant of each experimental group.

Statistical analysis

Before the test the hypothesis, data was input in the computer files and cleaning, after it carried out a descriptive statistical analysis. In the descriptive analysis, calculated values of central tendency (mean and median) and distribution (Standard Deviation). Results of the

study tested the distribution of data is the Kolmogorov-Smirnov and showed normal distribution. Obtained measurement results indicate a normal distribution so that hypothesis testing the levels of macrophage phagocytosis index using One-way ANOVA test followed by Least Significant Difference (LSD). ROI and NOI production w used nonparametric test considering ROI and NOI calculation data in ordinal scale, ie the nonparametric Kruskal–Wallis test followed by Mann Whitney U test statistical analysis assisted with SPSS 13 for windows. Significant value in this research when variables analyzed had $p < 0.05$.

3. Results and discussion

This study used vitamin A doses that equivalent to the recommended dose for children who are patients with chickenpox. During the trial period. Observations made include the general state of the mice, weight gain at the end of the study, and spleen weighing is done after mice were switched off. During the experiment the mice circumstances well, showing no signs of hipervitaminosis A, such as hair loss. This means that doses of vitamin A given to the mice in the amount of 3000 IU and 6000 IU does not cause poisoning symptoms.

Mice in the Control group and P1 appear to be inactive on day 6-8 post-infection. Groups of mice were given vitamin A does not appear amendment during treatment until mice were killed on day 10 post-infected. Parasitaemia in group 1 and group 2, began to be detected on day 2 post infection, followed by a rapid increase in parasitaemia and lead to death. The deaths occurred in its group on days 8-10 post infection with parasitaemia between 70% - 90%, while group 3 and group 4, a new parasite was detected on day 4 post-infection. In the third group there was an increase of 9% parasitaemia on day 6 to 15% at day 8 and to 31% on the 10th day whereas the second group of 11% on day 6 to 24% on day 8 and to 15% at day 10 (see Figure 1).

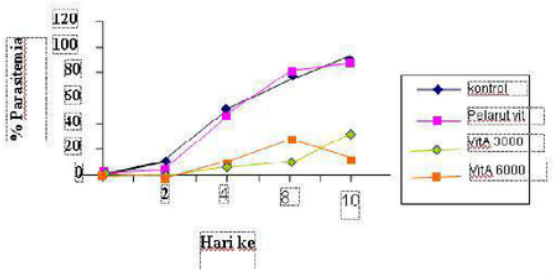

Figure 1. Parasitemia index in the experimental mice infected by 10^7 *Plasmodium berghei.*

Macrophages phagocytosis ability

Macrophage phagocytosis index is highest in the P3, reached (3.91683 ± 0.227190), while the lowest average was found in control, namely 4 14433 ± 0.336419) (see Table 1).

The test results Least Significant Difference (LSD) showed tha 4 here was no significant difference between Control group to P1. The test results showed no significant difference between control with P2 and P3. The test results also showed a significant difference between P2 with P3.

ROI production in group 4 was higher than Control group, group P1 and P2, which reached 3.5333 ± 0.16476, while the lowest average was found in group P1 (2.3800 ±

0.39985). Mann Whitney U test indicate that there is no real significant differences between control to P1 (p = 0.336) and group 3 (p = 0.107). Mann Whitney U test results also showed that there was a real each difference between Control group to P3 (p = 0.004) and P2 with P3 (0.004)

Table 1. Amount of ROI and NOI products by macrophages induced vitamin A in mice infected by *Plasmodium berghei.*

No.	Groups	Phagocytosis index	ROI macrophages production	NOI macrophages production
1.	control	(1,144 ± 0,336)a	(2.526 ± 0.108)[a]	(0.303 ± 0.589)[a]
2.	P1	(1,154 ± 0,274)a	(2,380 ± 0.400)[a]	(0,293 ± 0.013)[a]
3.	P2	(1,523 ± 0,241)b	(2,723 ± 0.214)[a]	(0.366 ± 0.024)[b]
4.	P3	(3,917 ± 0,227)c	(3,533 ± 0,165)[b]	(4,011 ± 0.641)[c]

[a,b] Different letters indicate significant differences at the level of 5%

The mean production NOI macrophages in P3 higher than control group, P1 and P2, which reached 4,011 ± 3641, while the lowest average was found in the treatment control group (0.303 ± 0.589). Mann Whitney U test results showed that there was no significant difference between the control, P1 (p = 0.873) and Control group to P2 (p = 0.078). There is a noticeable difference in the results between Control group with P3 (p = 0.004) and P2 with P3 (0.004)

Immunity against *Plasmodium berghei* involve phagocytosis by macrophages activated, in which activation occurs through one of the cytokines produced by T cell. Cytokines produced by these T cells is IFN-γ that stimulates and activates macrophages to producing cytokines, arachidonic acid metabolites and various substance as killers of pathogens, including ROI, NOI and lysozyme enzyme secreted into the phagosome (Abbas, 2003).

Macrophages which is activated will transform oxygen molecules into Reactive Oxygen Intermediates (ROI) which is a reactive oxidizing agent that destroys microbes. In this study, vitamin A supplementation can improve ROI and NOI production of macrophages and giving vitamin A with the highest dose of 6000 IU ROI and NOI increased production of macrophages. Basically Vitamin A in the form of provitamin and carotenoids are easier to be absorbed. Provitamin A is absorbed converted into retinoldehyde in the digestive system and more easily absorbed by the cytoplasm (Lobo et al., 2010). Retinoldehyde in the cytoplasm changed into retinoic acid that will bind to the site Retinoids A Receptor (RAR) and retinoid X receptor (RXR). The binding of Retinoic Acid Complex-RAR / RXR make a form of heterodimer DNA in cell and trigger the transcription of acute phase response proteins such as IL-12 and TNF α also increase phagocytic activation (Zappa-Gonzales et al., 2007).

Increased production of ROI will lead to increased production of H_2O_2, O_2^- and OH^- (Rhee et al. 2005). Production O_2^{--} states macrophage phagocytic capacity of the existing power. Kim et al. (2001) states that interference with the production of O_2 will cause bacteria imperfect deletion and may increase the risk of infection in a patient.

After phagocytosis, macrophages will killing the parasites by forming toxic NOI to bacteria. Increased NOI production associated with macrophage activity as phagocytic cells in accordance with the results of this research. With the resulting significant increase in NOI, will improve the effectiveness of macrophages to perform phagocytic activity.

ROI and NOI are mediators that have an important role in the mechanism of protozoa. Synergies between ROI and NO to form peroxynitrite which will increase the killing power to

against *Plasmodium berghei*. Retinoic acid binds to the complex Retinoic Acid Receptor and or retinoid X receptors which will trigger the proliferation of monocytes into Dendritic Cells (Hengesbach et al., 2004).

ROS molecules such as superoxide anion from the group O_2, H_2O_2, OH^{--} other molecules such as ON (OOK) resulting in dysfunction and cells destruction. It is because of the high level ROI and NOI affinity and activity. The damage may occur due to a disturbance in 1) pathways or metabolic activity (Newsholme et al. 2007, 2009). 2) resulting in damage to both the cell membrane structure and DNA also proteins structure (Chandra et al., 2000; Limon-Pacheco & Gonsebatt, 2009)

There is additional evidence of the role of free oxygen or molecule ROI and NOI in helping the body's defense system, both natural and artificial, as for the role, among others modulation lymphocyte cells to secrete cytokines and trigger apoptosis regulation of the immune cells. Some components of the transduction pathways have been identified as a target intracellular reactive toward NOI and ROI (Bogdan, 2000)

4. Conclusion and remarks

This study shown vitamin A was able to improve ROI and NOI production as well as lower index of parasitaemia in significantly. The most optimum dose in this study was 6,000 IU. Vitamin A has potential to stimulate monocyte activity against *Plasmodium berghei* in mice with malaria. Furthermore, it is needed further research to see potential damage in the body's organ due to high doses enough.

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