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Protein Profile and Hematological Parameters of Mice post Injected with Irradiated *Plasmodium berghei*

✉ Imam Rosadi¹, Mukh Syaifudin², Dewi Elfidasari¹

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¹Department of Biology, Faculty of Science and Technology, University of Al Azhar Indonesia, Indonesia

²Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency, Indonesia

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Abstract

Indonesia had a high number of malaria cases where the treatments are only perceived by 10% of patients in health facilities. The resistance of *Plasmodium* sp. to drugs causes the difficulty of controlling malaria. The use of irradiation to produce malaria vaccine material is being explored until nowadays. In previously study that dose rate of gamma irradiation delivered to parasite was at the low dose rate (380 Gy/hour) which produced several doses that not be able to attenuate the parasite. This research aimed to increase dose rate (740 Gy/hour) and to determine the dose irradiation effect of 150-250 Gy to the protein profile, erythrocyte and leukocyte numbers in mouse blood infected by *P. berghei* erythrocytic stage. Results showed that there was an influence of dose rate of 740 Gy/hour for doses of 150-250 Gy in treated mice and no difference in erythrocyte, leucocyte and body weight with negative control. Dose of 150 Gy was the optimal dose to attenuate parasites. The doses more than 150 Gy effectively attenuate the parasites. The changes of protein profile marked by the appearance of higher number of bands at molecular weight of 30-37 kDa that predicted as merozoite surface protein of *P. berghei* and/or antibodies elicited in mouse.

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✉ Correspondence Author:
Jl. Sisingamangaraja, Kebayoran Baru Jakarta Selatan, 12110
E-mail: imambiotek@gmail.com

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INTRODUCTION

Southeast Asia is one of the regions with high mortality rate of malaria. There were 28 million cases of malaria found in Southeast Asia from 216 million cases in the world in 2010 (Autino *et al.* 2012). Indonesia as the island nation that located in the South-East Asian region has quite high prevalence (number of events) of malaria. It was noted that Indonesia had a number of malaria paining of 1.85 per 1,000 inhabitants in 2009. Treatments of malaria are only perceived by 10% of the total number of patients in health facilities (KEMENKES 2011).

Malaria is a disease that can be found in animals and human which caused by protozoa of obligate intracellular of *Plasmodium* infection. There are four *Plasmodium* sp. that infecting human namely a *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium falciparum*. *Plasmodium falciparum* is the most dangerous parasite because it can cause of death in children and pregnant women (Zuzarte-Luis, Mota, and Vigário 2014; Lamikanra *et al.* 2007; Krishna *et al.* 2015).

Plasmodium sp. in human has similarities in morphology, genetic and life cycle with *Plasmodium* sp. that attacks animals. This is the basis of research in parasitological, immunology and the malaria vaccine development using an animal model of rodent such as mouse (Mlambo and Kumar 2008). There were two phases of *Plasmodium* development that divided into schizonts and sporogony phases. The main points of both phases are parasite pathogenicity infection from host to vector and vector to host (Antino *et al.* 2012; Delves *et al.* 2012). Based on their life cycle of infection into human/animals body that there were divided into three phases that are liver stage, blood stage, and vector stage (Delves *et al.* 2012).

In the blood stage, *Plasmodium* released merozoites into circulation to invade blood cells. The number of merozoites that released by *Plasmodium* depend into species of *Plasmodium* to perpetuate the asexual life cycle. *Plasmodium* are also released a large amount of toxins that cause the innate immunity activation. The most dangerous of *Plasmodium* blood stage also showed by manifestations (cerebral malaria or anemia) and cytoadherence ability. Cytoadherence is the ability of parasite to adhere with several organs vascular endothelium (lung, heart, brain, liver and kidney), placenta and subcutaneous adipose tissue (Antino *et al.* 2012).

In this study, we developed our research about *Plasmodium berghei* that has a level of vi-

olence similar to *P. falciparum* which causes death in the blood stage. *P. berghei* is a parasite that attacks the rodents. The parasite *P. berghei* has been widely used for malaria vaccine development research (Mlambo and Kumar 2008; Demicheli *et al.* 2006; Jobe *et al.* 2007). The resistance of *Plasmodium* sp. to drugs and malarial mosquitoes to the insecticides causes the difficulty of controlling malaria. The alternative to prevent of malaria disease is vaccine (Asih *et al.* 2012).

Research on vaccine with irradiation has started since 1967 by Nussenzweig that immunization of mice with irradiated sporozoite of *P. berghei* capable of protecting challenges with infective sporozoite (Hill 2011; Crompton, Pierce, and Miller 2010). Infective sporozoite that exposed to irradiation has suffered damage to the structure of the protein. Changes in the structure of this protein can be caused by protein denaturation, protein degradation or alteration of the structure of DNA (deoxyribonucleic acid). Changes in DNA structure can cause a particular protein synthesis or it generates new proteins (Tetrian *et al.* 2008).

Dose and dose rate of irradiation affecting the attenuation of infective parasites. The research that has been done previously in our laboratory was only at the low dose rate (380 Gy/h), but there is no information after irradiation at high doses rate (more than 700 Gy/h) on protein expression/profile alteration (Tetrian *et al.* 2008; Darlina and Tetrian 2006; Syaifudin, Nurhayati, and Tetrian 2008; Syaifudin *et al.* 2013). Protein profile test was needed to be conducted to determine the security levels of the irradiated samples and its purity.

It still needs to test protein profiles of *P. berghei* of erythrocytes stage through the analysis with SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) at certain doses of irradiation. Based on previous studies, doses of irradiation of 75 - 125 Gy were not effective to weaken the *P. berghei* in red blood cells (Darlina and Tetrian 2006). The optimum dose of irradiation was 150 to 175 Gy at dose rate of 380 Gy/hour. The optimal dose is the dose that can lower the infectiousness of *Plasmodium* in red blood cells of mice (Darlina and Tetrian 2006; Hoffman *et al.* 2002). Up to now there is no available data on protein profiles of *P. berghei* irradiated with doses of 0 Gy (positive control), 150, 175, 200 and 250 Gy at dose rate of 740 Gy/h. On this basis, it is necessary to do research aimed of analysis SDS-PAGE, erythrocyte and leucocyte number at high dose rate with doses of radiation are 150, 175, 200 and 250 Gy to get dose optimal of malaria

vaccine. The dose optimal can be reviewed that vaccine candidate also has characteristic as active or inactive vaccine. In the future, the blood stage of malaria can be prevented by vaccine with utilizing gamma irradiation at the high dose rate which was quickly than the lower dose rate.

METHODS

Parasites and Mice

Plasmodium berghei (strain Antwerpen-Kasapa, ANKA) infected mouse bloods were received from Eijkman Institute for Molecular Biology in Jakarta. Male Swiss-Webster mice (6-8 weeks old at the beginning of research) were purchased from SEAMEO TROPMED Regional Center for Community Nutrition University of Indonesia in Jakarta and were housed at the Animal Laboratory of The Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia (BATAN) and handled according to institutional guidelines. All procedures were reviewed and approved by the Animal Care and Use Committee National Institute of Health Research and Development, the Indonesian Ministry of Health.

Mice Infection

Two mice were intraperitoneally (IP) injected with about 1×10^6 *P. berghei*/ml parasitized blood and control mice were sham injected with uninfected blood (negative control) (Figure 1). The number of parasites in the blood (parasitemia) was monitored by Giemsa stained blood smears using light microscopy. Parasitemia, physical condition and body weight and survival of mice were noted.

Parasitaemia Monitoring

Parasitemia in mice from each infected group were monitored daily by conventional Giemsa staining starting on day 3 after infection. Thin blood films were prepared by tail bleeding, air dried, and methanol fixed before staining. Staining was done with a 10% Giemsa solution for 10 minutes at room temperature. Slides were evaluated at a 1000x magnification (oil-immersion) using a Nikon E200 Eclipse microscope by reading 20 fields per slide.

Irradiation of Blood Stage Parasites

Infected bloods of mouse with 20-40% parasitemia were irradiated *in vitro* within a gamma irradiator (Cobalt-60 of IRPASENA) of the Center for Application of Isotope and Radiation, BATAN to doses of 0 (positive control), 150, 175, 200 and 250 Gy at a high dose rate (740.0 Gy/hour). Each control and treatment groups consist of 3 swiss webster mice.

Erythrocytes and Leukocytes Calculation

8 µL of blood was taken from the tail end of mice and then 3 µL of this blood was dissolved in 493 µL of Hayem for the examination of erythrocytes number, and other 5 µL was dissolved in 95 µL of Turk solution for leukocytes examination. The numbers of erythrocytes and leukocytes count were observed under microscope at 400x and 100x magnifications. The number of erythrocytes and leukocytes was calculated with the following formulas [a] and [b].

$$\text{Erythrocyte (cell/mm}^3\text{)} = (\Sigma \text{ cell} \times \text{DF}^*) / (0.0025 \text{ mm}^2 \times 0.1 \text{ mm}) \dots\dots\dots(a)$$

$$\text{Leucocyte (cell/mm}^3\text{)} = (\Sigma \text{ cell} \times \text{DF}^*) / (0.01 \text{ mm}^2 \times 0.1 \text{ mm}) \dots\dots\dots(b)$$

DF* = Dilution Factor

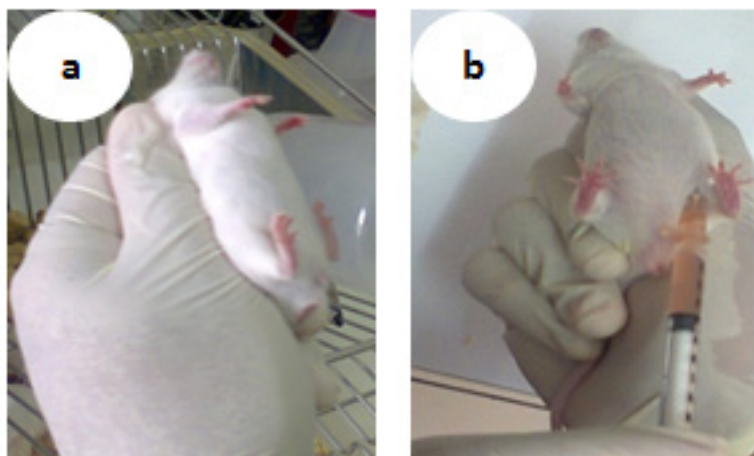


Figure 1. *P. berghei* breeding process, (a) mice holding tehcnique; (b) intraperitonelly injection of *P. berghei*

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis.SDS-PAGE was performed according to the methods of Laemmli, 1970 (Hoffman *et al.* 2002) and Theisen to the following group of samples: doses and dose rates of gamma ray for irradiation of *P. berghei* and days post infection and percentage of parasite density for non irradiated parasites. It was carried out on Bio-Rad gels composed of stacking gel (5% w/v) using 1.0 M Tris-glycine buffer containing 0.4% SDS at pH 6.8 and resolving gel (12%, w/v) using 1.5 M Tris-glycine buffer containing 0.4% SDS at pH 8.8. Protein sample was dissolved in phosphate buffer (5 mg/ml) and mixed with 6.22 µl of solubilization Tris-HCl buffer (pH 6.8) which contains 2% (w/v) SDS, 50% glycerol, a pinch of bromophenol blue and reduced with 0.9 µl of 2-mercaptoethanol in boiling water for 5 min. Protein sample was loaded onto each well of the acrylamide gel and electrophoresis (Bio-Rad Laboratories, Hercules, CA) was conducted at constant current of 100 volts for 90 minutes by a Bio-Rad electrophoresis power supply unit. A standard broad range of protein marker was also run into every gel. After electrophoresis, gels were stained with Commassie-250 blue solution for one night and then was fixed with fixative solution (methanol: acetic acid = 3 : 1) for 1 hour and in the water for another 1 hour and photographed with Gel-doc imaging system (Bio-rad).

To determine molecular weights of protein, a standard curve was constructed from the distances migrated by each marker protein. Then the distance migrated by the sample proteins can be plotted and their molecular weights calculated by interpolation with Excel Program.

RESULTS AND DISCUSSION

***Plasmodium berghei* Breeding**

P. berghei harvested after reaching more than 30% parasitaemia, with an average of 34.03% on day 14 (Table 1). Blood containing 34.03% parasite then prepared for the positive control and irradiation. Treatment of blood samples are irradiated with high dose rate 740 Gy/hour respectively divided in a dose of 150

Gy, 175 Gy, 200 Gy and 250 Gy.

Mice Physical Observation

The experiment showed that appearance of physical symptoms in mice was occurred only in positive control (non-irradiated) group during the first two weeks following inoculation of parasites. These symptoms included mucous membrane echidna, paler toes and tail of mice infected with non irradiated *P. berghei*. Whereas mice injected with irradiated parasites at doses of 150-250 Gy did not show any symptoms of clinical effects that may caused by the weakness of parasites after irradiation to infect red blood cells. Clinical symptoms were not seen in this treated mice group that might be caused by the exposure to high dose rate (740 Gy/h) and result in dead of parasites. Symptoms seen on negative control mice were the decreasing of appetite and drink and their body weight (Lamikanra *et al.* 2007, Darlina and Tetriana 2006; Basir *et al.* 2015) (Figure 2).

Erythrocytes and Leukocytes Calculation

Results of calculation of erythrocytes and leukocytes indicated that the negative control mice had the highest number of erythrocytes, while the positive control group had the lowest number of erythrocytes (Figure 3). Erythrocytes number in blood of mice that infected with irradiated *P. berghei* (150, 175, 200 and 250 Gy) was higher than the positive control but lower than that of the negative control. As seen in Figure 3 that the number erythrocytes in blood of positive control mice was decreased started on day 8 and get steady number until day 28 post infection.

Leukocytes are the cells of the immune system that are involved in defending the body against both infectious disease and foreign invaders such as parasites (Spiering 2015). The number of leukocytes in each treatment group did not show any difference among them, except for positive control. Positive control mice had an increased number of leukocytes from day 3 and relatively stable on the day of the 8th to 12th. The very high number of leukocytes was observed on days 14 and 16 (Figure 4). Increased number of leukocytes is due to the response of immune reac-

Table 1 Parasitemia before irradiation on day 14

Red Blood Cell (RBC)/ Erythrocytes	Parasite	(Parasite/RBC) x 100	Percentage of Parasite
2198	824	(824/2198) x 100	37.49%
2207	675	(675/2207) x 100	30.58%
Average	749.5	(749.5/22022) x 100	34.03%

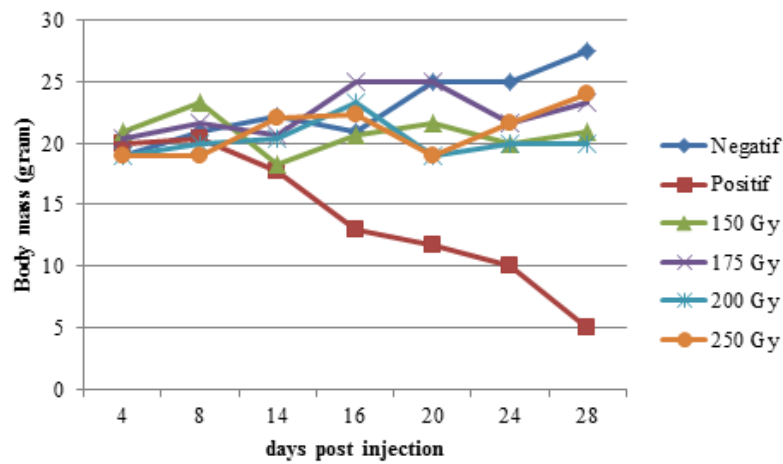


Figure 2 Body weight of mouse at days post injection with gamma irradiated *P. berghei*

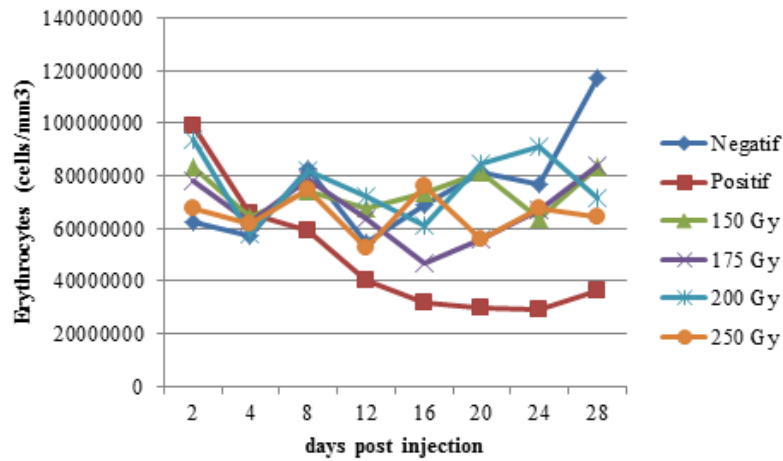


Figure 3. Erythrocytes number of negative control treatment, positive, 150, 175, 200 and 250 Gy

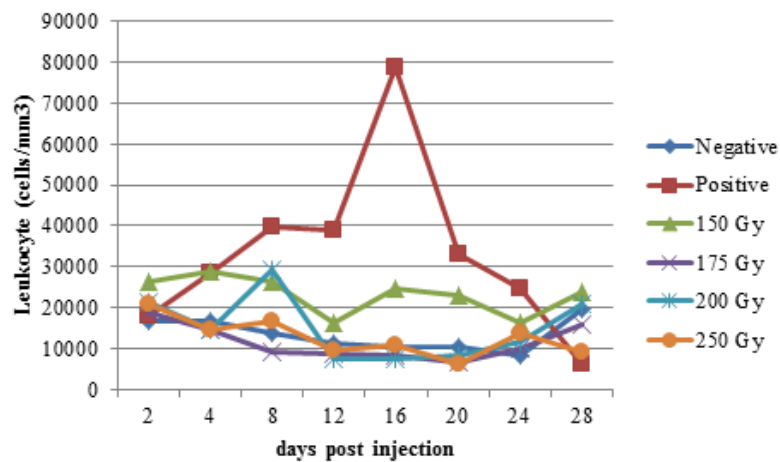


Figure 4. Mice leukocytes number of negative control treatment, positive, 150, 175, 200 and 250 Gy

tions caused by *P. berghei* infection.

As seen in Figure 4 that among irradiated *P. berghei* treatment mouse, dose of 150 Gy showed a highest number of leukocytes that started

on day 4. This result indicated that treatment of 150 Gy contains a parasite that has already weakened due to radiation dose rate of 740 Gy/h, thus potentially as an active vaccine (Syaifudin,

Nurhayati, and Tetriana 2008). Based on the results of this research it was known that dose of 150 Gy at a dose rate of 740 Gy/h is the optimum dose to weaken the parasites. *P. berghei* treated with 175-250 Gy supposedly died due to its high dose rate of irradiation.

Analysis of Protein Profile

Studies of protein profiles after irradiation in vaccine development are important aspect to know the purity of product as well as its safety as vaccine materials. When two proteins are covalently crosslinked due to irradiation or other causes, the gel migration patterns of both proteins shift in relation to the uncrosslinked proteins. Therefore, if antibodies, for example, that detect each target protein are available, the most straight forward method to detect the shift of the interacting proteins is by SDS-PAGE and Western blot analysis. Protein concentrations may change as a result of gamma-ray irradiation of either their structures or bonds. Changes can be caused by structure denaturation and protein degradation, as well as changes of deoxyribonucleic acid (DNA). This DNA change may lead to increase protein synthesis or formation of new proteins (Syaifudin 2014). Protein profiling is an emerging independent subspecialty of proteomics that is rapidly expanding and providing unprecedented insight into biological events. Quantitative evaluation of protein levels can be accomplished with protein profiling, which shows unique expression patterns such as between experimental and con-

trol.

On gel SDS/PAGE after high-dose rate (740 Gy/h) was delivered into red blood cells of mice of negative control group (without parasitic infections) there were 10 protein bands. These bands were corresponded to molecular weights of 132, 125, 112, 97, 88, 74, 59, 45, 37, 33, 21, 18 and 15 kDa, respectively (Figure 5). In positive control (with parasitic infections) there were 13 protein bands. Protein with molecular weights of 45, 37 and 33 kDa were not found in the negative control group. Therefore, these three bands are thought to be parasitic proteins or proteins related to immune system in mouse body as result of irradiation. Thicker bands were also observed that corresponded to the higher dose of irradiation. This is due to formation of new bands of protein induced by irradiation.

At the dose rate of 380 Gy/h there were 13 bands seen on gel that were equal to positive control while only 10 bands in negative control. These proteins had the molecular weights of 133, 104, 91, 80, 72, 58, 46, 39, 34, 30, 18, 14 and 10 kDa, consecutively. Whereas in control negative there were no protein bands with molecular weight of 39, 34 and 30 kDa (Figure 6).

Our previously research found that protein profile in mouse blood infected with *P. berghei* was different between irradiated and non irradiated sample. Infected samples had higher number of bands compared to uninfected (control) sample indicating the presence of new proteins (imported) into host cell of malaria. Irradiation may

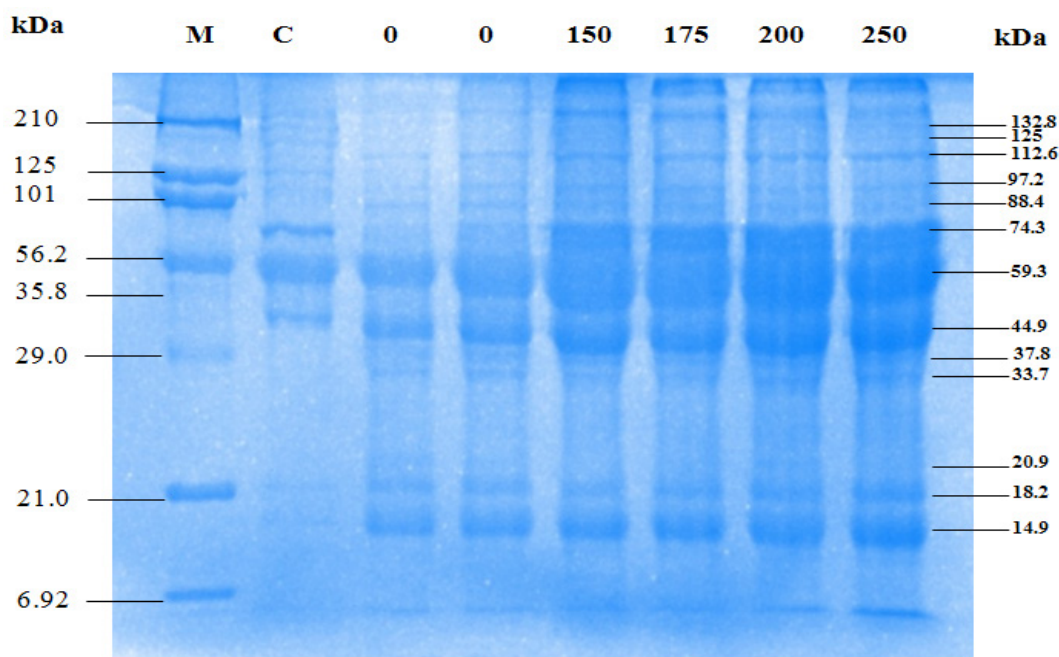


Figure 5. Profile protein in blood after irradiation at the high dose doses (740 Gy/hour) before injected into mouse; M = marker; C = negative control; 0 = positive control; 150-250 = dose of 150-250 Gy

also cause the appearance of new proteins. Profile protein was also affected by the percentage of parasite in the blood, the stages of the life cycle of parasit, species of parasit that infected blood, and certainly dose of irradiation (Syaifudin *et al.* 2013).

According to Cortes, Winograd, and Wisser (2003) that a protein of *P. berghei* has molecular weight of 45 kDa. That protein influences on the parasit infection and the mediation of parasite to penetrate the membranes of red blood cells. In 2004, specifically protein of *P. berghei* MSP7 (merozoites surface protein 7) was isolated by recombinant (Tewari *et al.* 2004). Research by Tewari *et al.* (2004) showed the MSP7 that found in *P. berghei* has a 42 kDa molecular weight. MSP7 is a protein found in *P. yoelii*, *P. berghei* and *P. falciparum*. MSP7 is not protein that causes the death of the parasite but affecting its growth where growth is slower than the wild type. Subsequent studies that have been done by Bagai, Pawar and Kumar (2010) showed that a protein antigen of *P. berghei* has molecular weights of 43 and 48 kDa. Protein of 48 kDa is an immunogenic response and has higher molecular weight than the 43 kDa. Based on the results obtained in this research, protein with molecular weights of 45 kDa and 39 kDa were thought can be protein of *P. berghei*.

Activation key to the emergence of serine protease is MSP. This protein as pure enzyme migrated as bands with SDS-PAGE analysis with molecular weight of about 30 kDa or 37 kDa (Akuba and Amlabu 2013; Wickham,

Culvenor, Cowman 2003). Research by Yahya & Kadir (2013) on protein profile analysis with SDS-PAGE of *P. berghei* of erythrocytes stage found proteins with the sizes of 120, 60, 30 and 14 kDa. Protein with 30 kDa of size is protein that control (inhibit) the growth of *P. berghei* of intraerythrocytes phase (Yahya and Kadir 2013). Based on the results of our study, protein with sizes of 37, 34, 33 and 30 kDa were suspected as protease protein that inhibit the growth of malaria parasit. Proteins with a molecular weight of 43, 35 and 30 kDa were not seen in the negative control. Therefore these proteins were thought to be proteins of *P. berghei* or antibodies. Research stated that protein with a molecular weight of 43 is the protein of *P. berghei* (Tewari *et al.* 2004; Bagai, Pawar and Kumar 2010). Protein with molecular weight of 35 kDa is serine protease and 30 kDa of size is a protein that control (inhibit) the growth of *P. berghei* in intraerythrocytes stage (Akuba and Amlabu 2013; Wickham, Culvenor, Cowman 2003)

Protein profiling at days post irradiation found that there was no alteration in bands on SDS-PAGE for negative control group. However in the positive control there was an alteration in bands with the disappearance of some bands that may be due to the destruction of red blood cells of mice (Figure 7). *P. berghei* at erythrocytic stage that attenuated by irradiation would be a non-recombinant inactive vaccine (Syaifudin, Nurhayati, and Tetriana 2008). This type of vaccines needs to be challenged by injecting wild type of *P. berghei* (non irradiated parasit). New bands ap-

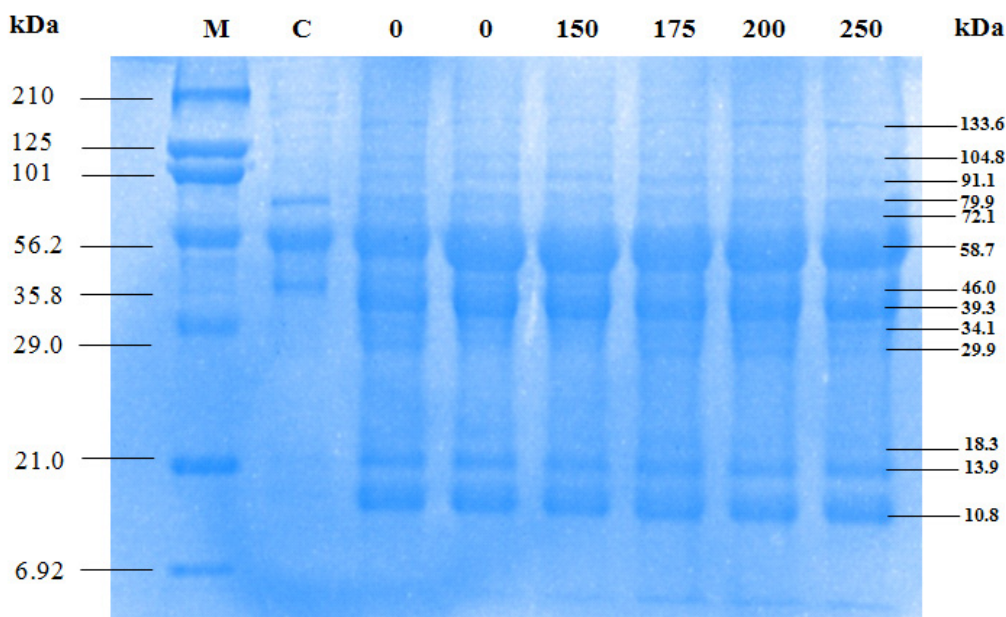


Figure 6. Profile protein after irradiation at the low dose doses (380 Gy/hour) before injected into mouse; M = marker; C = negative control; 0 = positive control; 150-250 = dose of 150-250 Gy

peared after irradiation at doses of 150-250 Gy beside due to *P. berghei* protein, it also as an antibody protein. Antibodies formed as immune response of mouse may result in memory antibody of *P. berghei*. Our results indicated that new protein was memory protein that may destroy *P. berghei* as an antigen.

For positive control, we analyzed the profile of protein of *P. berghei* from day 2 to 16. At day 2 to 10 the molecular weights of protein were

decreased. Molecular weight of proteins of negative control were 59, 44, 37, 12, 9 and 8 kDa. At day 2 there was a same in the weight of molecular between positive and negative controls. At day 12 to 16 the molcular weight only consisted of 36, 30, 12, 9 and 8 kDa (Figure 8). Bands that disappeared was though due to the destroyed erythrocytes. Protein with sizes of 36 and 30 kDa were both did not seen in negative control and still appeared till day 16.

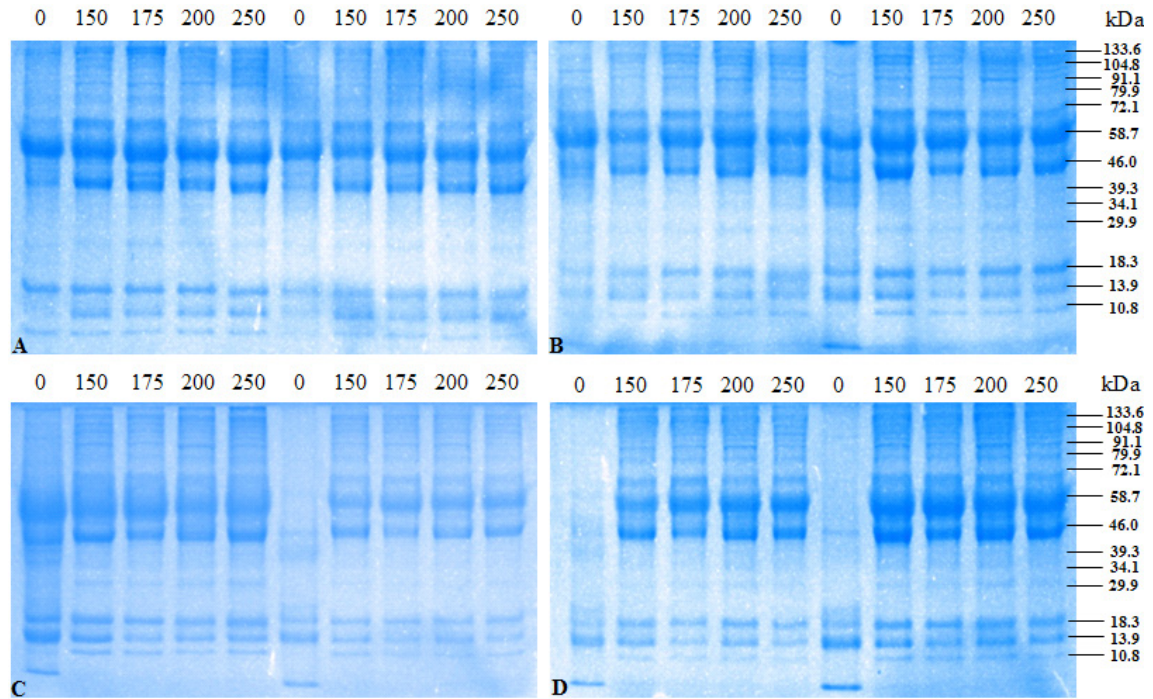


Figure 7 Protein profile in blood of the positive control mouse blood post infections with 150, 175, 200, 250 Gy irradiated *P. berghei* at days of (A) 3 and 4; (B) 6 and 8; (C) 10 and 12; (D) 14 and 16 post infections

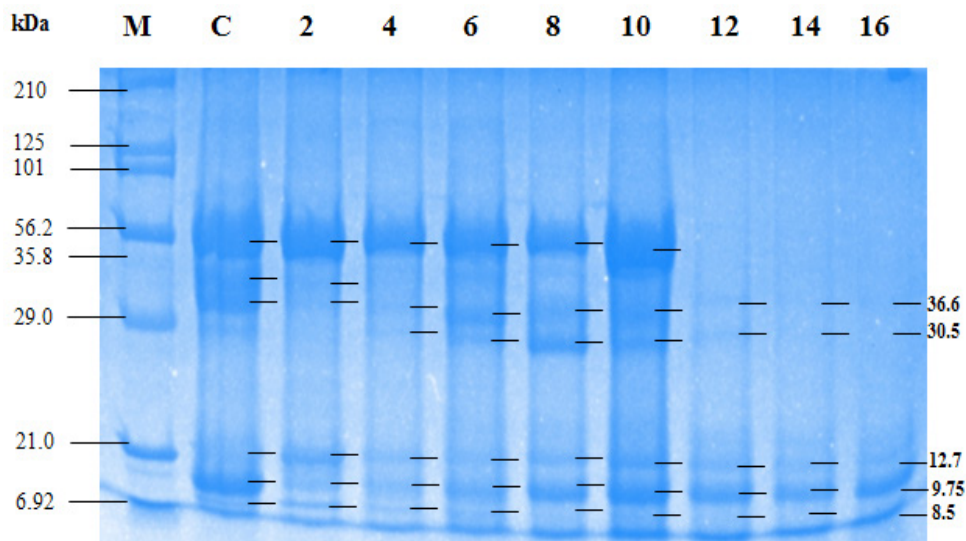


Figure 8. Alteration of protein profiles of *P. berghei* at day 2 to day 16 post injection; M = marker; C = negative control

Protein with size of ± 43 kDa was predicted as erythrocytic cell protein (antibody) of mouse because it disappeared during days of observations. These proteins are not surely as specific protein because there are about 400 putative proteins in *P. berghei*. These 400 proteins were corresponding to about 8% of genes, of which 225 proteins were related to the virulence of parasit and 160 proteins were included in host erythrocytes and parasit interaction (Marti *et al.* 2004). This suggested that futher research is needed to make sure the type of protein such as with western blotting method.

CONCLUSION

The research showed that there was no significantly differences result of 740Gy/h dose rate at doses of 150, 175, 200 and 250 Gy to erythrocytes, leukocytes and body weight of negative control mouse group. Dose of 150 Gy was optimal dose of irradiation to attenuate/inactivate parasites. Alteration of profile protein was characterized by the additional amounts of bands with molecular weight of 37-30 kDa allegedly as MSP of *P. berghei* and/or antibodies in mouse body.

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