GSC Biological and Pharmaceutical Sciences

eISSN: 2581-3250 CODEN (USA): GBPSC2

Cross Ref DOI: 10.30574/gscbps

Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Probing the effects of increased protein intake on *Plasmodium berghei* parasitemia level in Swiss Webster mice

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GSC Biological and Pharmaceutical Sciences, 2023, 24(02), 067–074

Publication history: Received on 24 June 2023; revised on 31 July 2023; accepted on 03 August 2023

Article DOI: https://doi.org/10.30574/gscbps.2023.24.2.0309

Abstract

Malaria remains a significant global health challenge, with Indonesia being no exception. Upon infection, the host's immune system plays a pivotal role in combating the malaria parasite. Proteins, being essential macronutrients for supporting the immune system, hold particular significance. Therefore, this research aims to investigate the impact of supplementary protein intake on the parasitemia levels of *Plasmodium berghei* in Swiss Webster mice. This research followed an experimental with a Posttest-only Control Design. Swiss Webster mice were selected as the study subjects. As an adjuvant therapy, casein was administered as an additional protein intake to the subjects. The severity of the infection was assessed by measuring the parasite count per µL of blood on the final day of the research, following the Peter 4-days suppressive test protocol. The findings of this study unveiled a significant correlation between the dose of casein and the severity of the infection. Interestingly, the correlation exhibited a positive trend, which deviates from the anticipated results based on the research's theoretical framework. The presence of other variables that may have influenced these outcomes will be thoroughly explored and discussed in this article. From this result we hope it may spark some new question to bridge a new research idea in the future.

Keywords: Malaria; *Plasmodium berghei*; Protein; Parasitemia; Immunity; Plasmodium

1. Introduction

Based on the 2017 World Malaria Report, which covered 91 countries, there were 216 million malaria cases and 445,000 related deaths in 2015. This data emphasizes the ongoing significance of malaria as a global health issue, necessitating urgent research and interventions [1]. Malaria also become a health problem in Indonesia with cases spreaded through the country. The coarse and growth of malaria case in Indonesia can be seen in Annual Parasite Incidence (API) that've been recorded in each region in Indonesia, where the highest API were recoreded in the eastern side of indonesia [2].

Malaria is caused by the Plasmodium parasite, transmitted to humans via female Anopheles mosquitoes. *Plasmodium falciparum*, Plasmodium vivax, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* are the primary species capable of infecting humans. The clinical manifestation of malaria includes periodic fever, chills, and sweats, commonly referred to as the malaria triad symptom. This symptom's duration and severity are influenced by the distinct reproductive cycles of the Plasmodium species, resulting in variations in fever patterns. Furthermore, the Plasmodium's

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reproductive cycle induces haemolytic anemia, affecting human red blood cells [3]. Data from Indonesian Basic Health Research in 2018 revealed a predominance of *Plasmodium falciparum*-induced cases (86.4%), followed by *Plasmodium vivax* (6.9%), and a combined incidence of *Plasmodium ovale* and *Plasmodium malariae* (2%) [2]. *Plasmodium falciparum* emerges as the main antagonist in severe malaria due to its unique ability to infect red blood cells of all ages, and producing a significant output of 18-24 merozoites from each infected cell. The short reproductive cycle of this parasite (24-48 hours) also plays a pivotal role in exacerbating the severity of the disease. Consequently, the host's parasitemia level escalates rapidly, leading to a more severe outcome of malaria [4,5].

The blood smear method is utilized to assess the parasitemia level and determine the severity of the illness. The procedure involves examining a thick smear under a 1000x magnifying power microscope, counting the number of parasites encountered until 200 leukocytes are observed. By dividing this sum by 200, we obtain the ratio of parasites to leukocytes. To convert this ratio to parasites/μL of blood, we multiply it by 8000, which represents the average number of leukocytes in a microliter of human blood. Additionally, the thin blood smear can be used to assess parasitemia level, expressed as a percentage. This is achieved by calculating the number of infected red blood cells divided by the total number of red blood cells seen and then multiplied by 100. The count of red blood cells is determined by estimating the parasitemia level when examining the blood smear. If the level is high (>10%), 500 red blood cells are counted, while for very low levels (<1%), a count of 2000 red blood cells is recommended [5].

An effective method for researching malaria is through in vivo experiments. Among the subjects used for such research, the Swiss Webster mouse strain is particularly notable. Originally developed in 1926 by Dr. Leslie Webster, the Swiss Webster mouse is a selectively inbred model of the Swiss mouse colony, exhibiting favorable traits for conducting malaria research. This mouse strain serves as an excellent model for studying the functional characteristics of the human body system. With a lifespan of 1-2 years and body weights ranging from approximately 24-36 grams for females and 33-46 grams for males [6]. It is essential to note that different species of Plasmodium infect different host organisms. Therefore, in the context of malaria research, it is crucial to identify a Plasmodium strain that can effectively mimic the infection observed in humans.

Plasmodium berghei is one of four species known to affect rodents in West Africa. It has emerged as a highly suitable model for studying both malaria parasites and the corresponding human malaria infection. The parasite's attack on reticulocytes, similar to *Plasmodium falciparum*, involves 12-18 merozoites per cycle, leading to a severe infection accompanied by pronounced pathological symptoms. Unlike *Plasmodium vivax*, which infects humans and exhibits a dormant hypnozoite phase in the liver, there is no evidence yet of *Plasmodium berghei* undergoing a similar phase. As a result, a mice that has been infected with *Plasmodium berghei* often establishes a great murine model that effectively simulates *Plasmodium falciparum* infection in humans [7,8].

The immune system is a crucial for defending against pathogens, including Plasmodium. Two main types of immunity exist: natural and adaptive immunity (9). Notably, adaptive immunity stands out due to its specificity against particular pathogens and its ability to retain pathogen-related memories. A key element of adaptive immunity is humoral immunity, involving the secretion of immunoglobulins or antibodies by B cells. These antibodies play a vital role in neutralizing specific pathogens. Humoral immunity can be employed to combat protozoan pathogens like Plasmodium [10].

The immune system relies on various essential components, with proteins being a one of the crucial element. Proteins are vital macromolecules that serve multiple functions in the human body. They play both functional and structural roles, as seen in the cytoskeleton, which provides cellular framework, and enzymes, facilitating metabolic processes [11]. In the diet, proteins undergo a process of breakdown into monomers, specifically amino acids, which are absorbed through the intestines to form and replenish the body's proteins [12]. Determining the appropriate protein intake has been a subject of debate, with factors like activity level influencing the recommendations. According to the U.S. Recommended Dietary Allowance (RDA), adult humans require at least 0.8 g/kgBW/day of protein to prevent muscle wasting [13].

The immune system relies on proteins for various essential functions. These include their involvement in the formation of inflammatory mediator compounds and serving as building blocks for white blood cells. Notably, proteins play a significant role in the synthesis of immunoglobulins, critical components of immunity. Among the amino acids, glutamine stands serves as a precursor for nucleic acid synthesis and also plays a role in immune system components. Michael Gleeson, in his article review, emphasized that deficiencies in protein and micro-nutrients can have adverse effects on the body's immune system [14,15].

According to research conducted by Amaral J, it was observed that mice with a low protein diet exhibited a decline in the production of immunoglobulins or antibodies. However, when these mice were provided with a protein supplement in the form of casein, the levels of antibodies returned to normal. Casein, as a supplement, contains a diverse array of essential and non-essential amino acids that play crucial roles in the immune system, including glutamine. In fact, casein has been found to have the highest levels of glutamine compared to whey and soy protein supplements [16,17].

Protein-calorie malnutrition has been found to impact monocyte function in both human and animal models. Fortunately, immune function can be restored to normal levels after a period of re-nourishment, typically around 4 weeks. In the case of mice, protein-calorie malnutrition has been shown to increase their susceptibility to influenza infection. However, when provided with a balanced protein diet, the immune system is stimulated, leading to a reduced likelihood of mice contracting influenza. Additional studies have revealed that malnourished mice exhibit a delayed response to Leishmania chagasi infection, primarily attributed to a reduced production of INF-γ [18,19].

It can be concluded that the components of the immune system contain a significant amount of protein. The level of protein present in the body is directly related to the protein intake from the diet. Therefore, research is needed to investigate whether adding protein to the diet can influence the immune system's performance in handling malaria infections and whether it has an effect on the host's parasitemia levels. This study will provide deeper insights into the potential impact of protein intake on the immune system's response to malaria infections, thus enhancing our understanding and efforts to manage and combat this infection effectively.

2. Material and methods

The research followed an experimental with a Posttest-only Control Design. It was carried out at the Laboratory of Animal and the Laboratory of Parasitology, located in the National Research and Development Center for Biomedical and Basic Health Technology, Ministry of Health, Jakarta, Indonesia. The data collection and analysis took place over a five-day period in November 2019. Clinical Ethics Research Committee of the Faculty of Medicine, University of Lampung, Indonesia, has granted approval for this study

For the research, female Swiss Webster mice (*Mus musculus* L) were used as the subjects. They were obtained from The Animal Laboratory in National Research and Development Center. Five mice were assigned to each of the five treatment groups. The sum of subject are determined by a feeder formula. The inclusion criteria for the mice were; being 8-11 weeks old, weighing 20-40g, and in generally good health (active, eating and drinking normally, and no fur loss or defect). Exclusion criteria were: a significant weight loss (>10%) during the adaptation period in the laboratory and any mice that died during the research. Simple random sampling technique was used to randomized the subject.

Each of the subject received a specific dose (per-weight) of cassein powder dissolved in water, depending on their assigned treatment group. Additionally, all subjects were given chloroquine at a dose of 0.05 mg/g(Weight)/d to ensure their viability throughout the research. There were five treatment groups, labeled as Group 1 through Group 5, with each group receiving a different dose of cassein as follows; Group 1 serve as control and not given any dose of casein; Group 2 0.015 g/gBW/d; Group 3 0.025 g/gBW/d; Group 4 0.035 g/gBW/d; Group 5: 0.05 g/gBW/d. The research results were based on the subjects' parasitemia levels on the final day, expressed as parasites/μL of blood

Plasmodium berghei was obtained from The Laboratory of Parasitology in National Research and Development Center. Inoculation of the *Plasmodium berghei* was done intraperitonealy to two donor mice outside the test subject. Two days was needed to make the parasitemia level sufficient enough in both of the donor mice's blood to infect the test subjects. Bassed on the parasitemia level of the donor mice, we can calculate that each subject will be given approxiamlly 10^7 infected red blood cells from the donor mice. *Peter's 4-days Suppresive test* method was used. Treatment commenced three hours after inoculation to test subject, marking day-0, and was administered daily at the same hours until day-4. Blood smear of each test subject was evaluated in day-5 to to determine the parasitemia level [20–22].

The final outcome underwent one-way ANOVA analysis to examine the significance of the diverse outcomes in different treatment groups, aiming to identify potential correlations between the variables.

3. Results

Table 1 Parasitemia level from each subjects

Subject		Weight (g)	Casein dose (g)	Chloroquine dose (mg)	Parasitemia level (Parasite /µL of blood)	Mean Parasitemia
Group 1	1	30	0	1,5	57	53.2
	2	34	0	1,7	70	
	3	31	0	1.55	60	
	4	34	0	1.7	44	
	5	37	0	1.85	35	
Group 2	1	29	0,435	1.45	33	77.6
	2	30	0,45	1.5	61	
	3	35	0,525	1.75	61	
	4	29	0,435	1.45	103	
	5	33	0,495	1.65	130	
Group 3	1	35	0,875	1.75	148	152.8
	2	31	0,775	1.55	55	
	3	35	0,875	1.75	264	
	4	28	0,7	1.4	122	
	5	31	0,775	1.55	175	
Group 4	1	34	1,19	1.7	180	186.2
	2	33	1,155	1.65	206	
	3	30	1,05	1.5	112	
	4	33	1,155	1.65	236	
	5	33	1,155	1.65	197	
Group 5	1	33	1,65	1.65	125	226.6
	2	35	1,75	1.75	97	
	3	31	1,55	1.55	183	
	4	37	1,85	1.85	230	
	5	35	1,75	1.75	498	

The data from Table 1 provide the parasitemia level on the last day of the reserach. Based on the data presented in Table 1, we calculated the mean parasitemia level for each treatment group, as shown in Figure 1. The Shapiro-Wilk method was used to confirm that the data are normaly distributed, allowing us to conduct a one-way ANOVA analysis. The results revealed a p-value of 0.019. Subsequently, a post-hoc analysis using Least Significant Difference (LSD) was performed, and the findings are presented in Table 2.

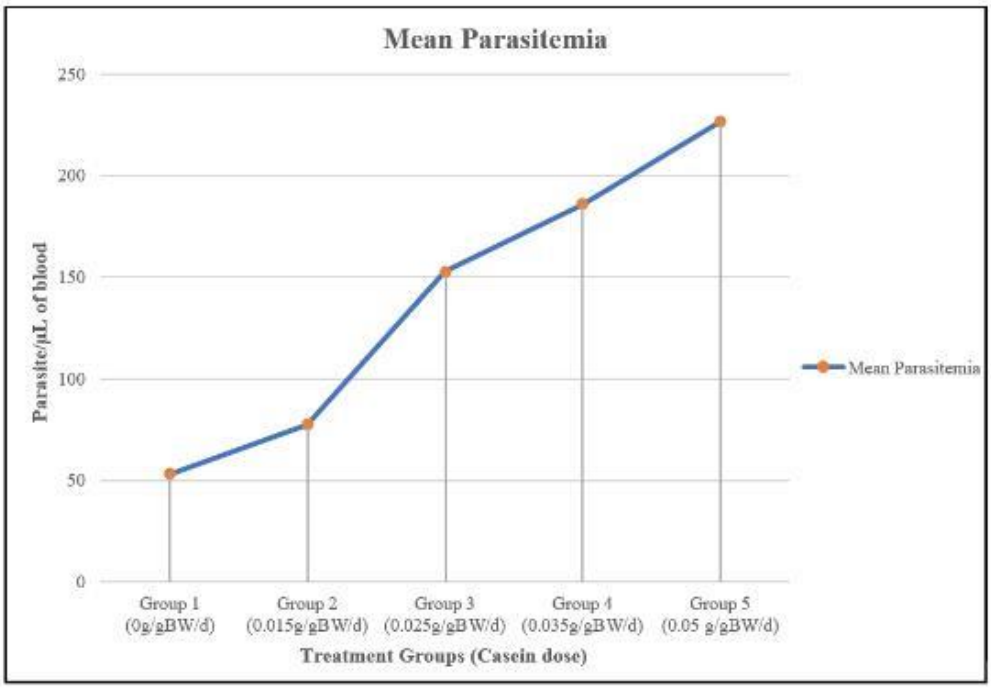

Figure 1 Mean level of Parasitemia from each groups

Table 2 Post hoc (Least significant difference)

Groups		Mean Difference	p-value
Group 1	Group 2	-24.4	0.651
	Group 3	-99.6	0.076
	Group 4	-133	0.021
	Group 5	-173.4	0.004
Group 2	Group 1	24.4	0.651
	Group 3	-75.2	0.173
	Group 4	-108.6	0.054
	Group 5	-149	0.011
Group 3	Group 1	99.6	0.076
	Group 2	75.2	0.173
	Group 4	-33.4	0.537
	Group 5	-73.8	0.180
Group 4	Group 1	133	0.021
	Group 2	108.6	0.054
	Group 3	33.4	0.537
	Group 5	-40.4	0.456
Group 5	Group 1	173.4	0.004
	Group 2	149	0.011
	Group 3	73,8	0.180
	Group 4	40.4	0.456

4. Discussion

Based on the presented results, we can conclude that there is a positive correlation between additional protein intake and the level of parasitemia. Interestingly, these findings contradict the theoretical framework of the research, which proposed a negative correlation. We suggest that protein intake could potentially modulate the immune system to combat the parasite. It is possible that certain variables not accounted for in the study might have influenced the results, leading to this unexpected outcome. Further investigation is needed to elucidate the factors contributing to this discrepancy.

The first variable under consideration is the mice's ability to obtain an additional intake of food. According to a relevant article, the daily dietary intake for a mouse is calculated at 10% of its total body weight. For the subjects in this research, with an average weight of 32.64g (rounded to 33g), a single mouse has the capacity to ingest approximately 3.3g of food per day. In the case of treatment group 1, the weight of the diluted casein was about 1g. Consequently, the administration of full protein treatment causes a shift in the normal dietary intake of the mice, leaving only 2.3g available for the mice to consume from their daily food. The mice's daily food consists of 60% carbohydrates, 25% protein, and 15% fats. The percentage of protein provided by the treatment in group 1 reaches 30.3% of the subject's dietary intake, leaving only 69.7% for carbohydrates, fats, and another additional proteins from their regular food. Due to carbohydrates being the most abundant macronutrient in the mice's daily food, the treatment with cassein reduces the amount of consumed carbohydrates as we increase the cassein dosage. We observed subjectively that the mice in the treatment group consume less of their daily food compared to the control group. This variable could be better controlled using larger subjects such as Sprague Dawley mice, which have a larger gastric capacity, allowing for more flexible manipulation of their dietary proportions. However, due to the limited availability of Sprague Dawley mice in the lab, it poses a constraint in this research [6,23,24].

Numerous prior studies have reported on the adverse effects of protein deficiency on the immune system. However, our search did not yield any relevant articles specifically addressing the impact of excessive protein intake on the immune system. Nevertheless, we came across a study suggesting the crucial role of carbohydrates in immune function. Carbohydrates serve as a primary energy source for various metabolic processes in the human body, including those related to the immune system. According to this article, carbohydrates influence the activation, proliferation, and differentiation of key immune components, such as Lymphocytes and Natural Killer Cells, which play pivotal roles in combating parasite infections. Although the existing literature highlights the negative consequences of protein deficiency on immunity, further investigation is required to elucidate the specific effects of excess protein intake on the immune response, and the significant role of carbohydrates in supporting immune function warrants further exploration in the context of parasite infections.[19,25].

Another aspect worth considering relates to the Peter's 4-day suppressive test method, which is commonly used to assess treatments for therapeutic purposes. In this research, we are particularly interested in examining the effects of additional protein intake as an adjuvant therapy in the context of malaria infection. Another potential investigation involves long-term dietary manipulation to observe the implications of sustained additional protein supplementation on the immune system's ability to combat parasite infections. However, this approach may not provide conclusive evidence regarding the efficacy of protein intake as an adjuvant therapy during a malaria infection. This research primary focus revolves around exploring the potential benefits of additional protein intake as a supplementary treatment in the course of malaria infection.

5. Conclusion

Drawing upon the results and discussions above, we can conclude that there is a recognizable effect of additional protein intake on the level of parasitemia in *Plasmodium berghei* infected Swiss Webster mice under acute conditions. The effect demonstrates a positive correlation, which may spark a new questions and chances for future research.

Compliance with ethical standards

Acknowledgments

All authros would like to to express gratitude to the laboratory staff at the Laboratory of Animal and the Laboratory of Parasitology, National Research and Development Center for Biomedical and Basic Health Technology, Jakarta for their support ini this research. The first author also would like to express his heartfelt gratitude to his mother for unwaveringly supporting him throughout his years of studies.

Disclosure of conflict of interest

The authors declare no conflict of interest

Statement of ethical approval

Clinical Ethics Research Committee of the Faculty of Medicine, University of Lampung, Indonesia, has granted approval for this study

Statement of informed consent

No personal information was incorporated in this study, and neither were human subjects involved. The animal participants were provided a well-maintained, hygienic, and comfortable habitat, adhering to the ethical guidelines.

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