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The Effect of *Blastocystis sp.* Administration on the Differential Leukocyte Count in Male Wistar Rats *(Rattus norvegicus)*

Salma Sayyidatussauqiyah[1], Eka Nofita[2], Selfi Renita Rusjdi[2], Dian Pertiwi[3], Almurdi[3], Miftah Irramah[4]

[1]Faculty of Medicine, Andalas University, Padang, West Sumatera, Indonesia
[2]Department of Parasitology, Andalas University, Padang, West Sumatera, Indonesia
[3]Departement of Clinical Pathology, Andalas University, Padang, West Sumatera, Indonesia
[4]Departement of Physiology, Andalas University, Padang, West Sumatera, Indonesia

Corresponding Author: Salma Sayyidatussauqiyah

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ABSTRACT

Blastocystis sp. could release pro-inflammatory cytokines and cysteine proteases, leading to a shift in the modulation of the adaptive immune response and leukocytosis. This study aimed to investigate whether there is a correlation between *Blastocystis sp.* infection and the differential count of leukocytes. This study was pure experimental research with a post-test-only control group design approach, involving 21 male Wistar albino rats *(Rattus norvegicus)* as test animals. The study was conducted at the Animal House, the Biomedical Laboratory, and the Central Laboratory of Medical Faculty, Andalas University, from December 2022 to June 2023. *Blastocystis sp.* in rat feces was observed using a microscope and culture methods, while the differential count examination was performed microscopically using peripheral blood smears. Data analysis was performed using the One-Way ANOVA test and the Kruskal-Wallis test. The research results are in the form of mean values of leukocyte differential count in percentage (%) for each group. The negative control group (K-) with 0 basophils, 0 eosinophils, 28.1 neutrophils, 71.1 lymphocytes, and 0.7 monocytes. Treatment group 1 (P1) with a mean of 0 basophils, 0 eosinophils, 25.6 neutrophils, 60.4 lymphocytes, and 1.1 monocytes. Treatment group 2 (P2) with a mean of 0 basophils, 0 eosinophils, 33 neutrophils, 67 lymphocytes, and 1.4% monocytes. The significance value obtained for neutrophils count were 0.5, lymphocytes were 0.6, and monocytes were 0.1 ($p > 0.05$). The conclusion was that the administration of *Blastocystis sp*. does not significantly affect the differential count of leukocytes in rats.

Keywords: *Blastocystis sp*., Leukocytes Differential Count, *Rattus norvegicus*

INTRODUCTION

Blastocystis sp. is the most commonly found intestinal protozoan in human fecal samples.[1] The infection caused by *Blastocystis sp.* is known as blastocystosis. The prevalence of *Blastocystis sp.* infection in developed countries has been reported to exceed 5%, while in developing countries the rates are significantly higher, ranging from 30% to 60%.[2] This is associated with poor personal and environmental hygiene as well as frequent close contact with infected animals. Transmission of this protozoan occurs via the fecal-oral route, primarily when humans accidentally ingest

Blastocystis sp. cysts that contaminate food or drink.[3]

Not all individuals infected with *Blastocystis sp.* show symptoms.[4] The symptoms that do arise are categorized as intestinal and extra-intestinal. Common intestinal symptoms include diarrhea, abdominal pain, nausea, and vomiting. Other symptoms, such as bloating and constipation, have also been reported in some cases. In addition, extra-intestinal symptoms such as urticaria (hives) have also been associated with the infection.[5]

A study by Li (2013) on white rats inoculated with 10^2 *Blastocystis sp.* cysts showed clinical changes in the form of lethargy without weight loss or diarrhea.[6] To date, the role of *Blastocystis sp.* as a pathogen remains uncertain. Several sources suggest that the pathogenicity of this intestinal protozoan is related to specific subtypes and the number of parasites infecting the host.[7,8] Each subtype exhibits differences in dominant morphology (although not very specific), varying susceptibility to medications, and differences in mechanisms for inducing the host's immune response, making some subtypes more pathogenic than others.[6] Kok (2019) conducted a pathogenesis study and reported the presence of edema in the intestinal mucosa caused by the penetration of *Blastocystis sp*., which stimulates the production of proinflammatory cells. That study also noted the expression of cysteine proteases, which play a role in the infection process by lysing protective tissue proteins such as Secretory Immunoglobulin A (SIgA).[9]

Infection with intestinal protozoa triggers an immune response in the body and causes a shift in the distribution of leukocyte types, such as eosinophilia in giardiasis and lymphocytosis in amebiasis.[10,11] A study by Cifre (2018) on blastocystosis reported a shift in adaptive immune response modulation, with high titers of Immunoglobulin G (IgG) and a significant increase in proinflammatory cytokines.[12] Blastocystis sp. has been reported to play a major role in altering the gut microbiota, which is linked to an increased risk of chronic gastrointestinal inflammation, such as ulcerative colitis.[13] So far, no literature has specifically discussed the effect of *Blastocystis sp.* infection on the differential leukocyte count. Therefore, the researchers were interested in conducting this study, which aims to provide new data on the effect of Blastocystis sp. administration on the leukocyte differential count in male Wistar rats *(Rattus norvegicus).*

MATERIALS & METHODS

This study is a true experimental study using a post-test only control group design. The research was conducted at the Animal House of the Faculty of Medicine, Andalas University. Fecal examinations of the rats were carried out at the Veterinary Hospital Laboratory of the West Sumatra Provincial Livestock Service, and the differential leukocyte counts were performed at the Central Laboratory of the Faculty of Medicine, Andalas University. The study was conducted from January to June 2023, following ethical approval from the Faculty of Medicine, Andalas University, with ethics approval number 92/UN.16.2/KEP-FK/2023.

The experimental animals were acclimatized to the Animal House environment of the Faculty of Medicine, Andalas University, for 7 days in individual rat cages. Each cage housed one rat and was placed in a well-ventilated room, protected from direct sunlight. The cages were cleaned at least three times per week to ensure the rats were comfortable and free from infection risk.

The study population and samples consisted of 21 male Wistar rats aged 4–6 weeks, weighing 100–250 grams, and confirmed to be infection-free based on fecal examinations. The rats were then randomly divided into three groups: Negative control group (K−): rats given only a standard diet ad libitum without Blastocystis sp. Inoculation, treatment group 1 (P1): rats inoculated with Blastocystis sp. at a dose of 10^4/ml, and treatment group 2 (P2): rats

inoculated with Blastocystis sp. at a dose of 10^5/ml.

After the acclimatization period, cultured Blastocystis sp. was orally administered to the rats in the treatment groups using an inoculation gavage according to the designated doses. The rats were then monitored regularly every two days. On day 21 post-inoculation, after the rats were anesthetized via inhalation, blood samples (1 ml) were collected from the orbital sinus of each rat. The blood samples were mixed with 3 mg of EDTA anticoagulant, followed by preparation of peripheral blood smears (PBS) stained with Giemsa. The differential leukocyte count was then examined microscopically at the Central Laboratory of the Faculty of Medicine, Andalas University. The resulting leukocyte count data were analyzed using One-Way ANOVA if the data were normally distributed. If not, the non-parametric Kruskal-Wallis test was used as an alternative.

RESULT

1. Results of the evaluation of body weight, feces consistency, and motor activity of the rats

Group	No	Weight (gr)		Feces Consistency		Motor Activity	
		Early	End	Early	End	Early	End
K−	1	136	211	Solid	Solid	Active	Active
	2	142	209	Solid	Solid	Active	Active
	3	192	233	Solid	Solid	Active	Active
	4	190	241	Solid	Solid	Active	Active
	5	190	236	Solid	Solid	Active	Active
	6	188	241	Solid	Solid	Active	Active
	7	107	121	Solid	Solid	Active	Active
P1 (10^4/ml)	1	215	247	Solid	Semi solid	Active	Active
	2	219	245	Solid	Solid	Active	Active
	3	201	(Died)	Solid	(Died)	Active	(Died)
	4	132	213	Solid	Solid	Active	Active
	5	116	193	Solid	Solid	Active	Active
	6	120	199	Solid	Solid	Active	Active
	7	120	197	Solid	Solid	Active	Active
P2 (10^5/ml)	1	201	248	Solid	Semi solid	Active	Active
	2	180	241	Solid	Solid	Active	Active
	3	189	245	Solid	Solid	Active	Active
	4	168	238	Solid	Solid	Active	Less
	5	150	224	Solid	Solid	Active	Less
	6	140	193	Solid	Solid	Active	Active
	7	137	198	Solid	Solid	Active	Active

The data in Table 1 showed changes in the rats' body weight, with an increase observed over the 3-week period prior to the termination procedure. However, the other two indicators—stool consistency and motor activity—did not show any significant changes.

2. Average differential leukocyte count of the rats

Variable	Group	Average ± DS* (%)	P value
Neutrophil	K-	28,1428 ± 16,9059	0,583
	P1	25,5714 ± 14,0814	
	P2	33,00 ± 7,2801	
Lymphocyte	K-	71,1429 ± 17,4301	0,614
	P1	60,4286 ± 27,2388	
	P2	67,00 ± 5,3541	
Monocyte	K-	0,7143 ± 1,1127	0,144
	P1	1,1429 ± 0,69007	
	P2	1,4286 ± 0,5345	

DS: Deviation standard

3. Normal values of the differential leukocyte count in rats[14]

Leukocyte type	Normal range(%)
Basofil	0-0.5
Eosinofil	1-4
Neutrofil	12-38
Limfosit	60-75
Monosit	1-6

The results of the leukocyte differential count were recorded in the Schilling hemogram, followed by the calculation of the average leukocyte types for each group (see table 2). Basophils and eosinophils were not detected at all in this study. The neutrophil, lymphocyte, and monocyte counts showed variations in the average values across the groups. However, the average counts of neutrophils, lymphocytes, and monocytes in all three groups remained within the normal range (see table 3). A normality test was then performed on the data, which showed that only the neutrophil count data were normally distributed, with a p-value > 0.05. Next, a homogeneity test was conducted, yielding a p-value of 0.322 ($p > 0.05$), indicating that the variances of the neutrophil count data among the groups were equal. A One-Way ANOVA test was subsequently performed, resulting in a p-value of 0.583. Since the lymphocyte and monocyte count data were not normally distributed, an alternative non-parametric Kruskal-Wallis test was conducted, resulting in p-values of 0.614 and 0.144, respectively. The data analysis results indicated that there were no significant differences in neutrophil, lymphocyte, and monocyte counts among the groups of rats, as all p-values were greater than 0.05.

DISCUSSION

In this study, basophils and eosinophils were not detected at all. This result may have occurred because the differential leukocyte count was performed manually using peripheral blood smears (PBS), which might have led to these two types of leukocytes not being included in the sample, or because the presence of basophils and eosinophils was below the microscopic detection threshold. Another possible cause was the use of Giemsa stain, which can dissolve the granules in basophils, making them microscopically undetectable. The Wright-Giemsa combination stain has been proven to be more effective for basophil detection. In addition to technical limitations, eosinopenia (a decrease in eosinophil count) might have been triggered by an increase in catecholamine synthesis during acute inflammation.[14]

This study did not show any effect of *Blastocystis sp.* administration on the differential leukocyte count in the negative control group (K−), treatment group 1 (P1), or treatment group 2 (P2). These results differed from the study conducted by Mutlag (2019), which reported leukocytosis, particularly a significant increase in neutrophils and monocytes in patients with blastocystosis. The increase in leukocyte count associated with *Blastocystis sp.* infection often occurred in patients with gastrointestinal symptoms. *Blastocystis sp.* could trigger increased production of pro-inflammatory cytokines in the host's intestinal epithelial cells.[15]

The absence of an increase in differential leukocyte count in this study was consistent with the findings of Kosik et al. (2021), who reported that in *Blastocystis sp.* infections, the counts of each leukocyte type remained within the normal range.[16] Several studies on the immune evasion mechanisms of intestinal protozoa have shown that these protozoa can form immune-resistant cysts, alter their surface antigens to avoid antibody recognition, and even induce immunosuppression in the host. Tissue invasion by *Entamoeba histolytica* in patients with amebiasis was known to modulate macrophage and T cell activity, as

the parasite could prevent phagocytosis by macrophages through the monocyte locomotion inhibitor factor, thereby halting the immune response.[17] Other studies on *Blastocystis sp.* infection have shown that this protozoan can suppress nitric oxide (NO) secretion, thereby preventing apoptosis-like cell death in *Blastocystis sp.*, ultimately leading to suppression of the immune response. The impaired immune response could explain the absence of leukocyte increase in *Blastocystis sp.* infection.[18]

The mucosal immune system of the gastrointestinal tract also played a role in host defense against *Blastocystis sp*. infection.[19] Various gut microbiota colonies helped strengthen mucosal defenses against pathogen colonization by increasing antimicrobial peptide secretion, mucus production, and tightening intestinal epithelial cell tight junctions.[16] The epithelial cells in the mucus layer were able to produce Immunoglobulin A (IgA) and secretory IgA (SIgA), which could neutralize and limit pathogen invasion by agglutinating antigens, wrapping them in mucus, and eliminating them through the intestinal lumen.[20] A high level of microbial diversity was usually associated with good health and a lower risk of inflammation. This indicated that the gut microbiota acted as a competitive inhibitor against *Blastocystis sp.* in the mucosal layer, preventing the parasite from breaching the defense barrier and triggering systemic immunity that would increase leukocytes.[21]

This study did not inoculate *Blastocystis sp.* based on specific subtypes, as the infected fecal samples examined contained more than one subtype. The results obtained may have been influenced by the possibility that the *Blastocystis sp.* inoculated did not have pathogenic potential. The interactions between subtypes could also have affected the study's results. *Blastocystis sp.* has a wide host range, with about 23 subtypes identified so far.[22] Several studies concluded that not all *Blastocystis sp.* subtypes can cause symptoms. The difference in subtypes related to variations in virulence, cell growth, drug susceptibility, and host range.[23] Research on *Blastocystis sp.* pathogenicity reported that ST1-4 and ST6 were the subtypes most often found in symptomatic patients. ST3 was the most commonly detected subtype, followed by ST1 and ST2. It was known that proteases in ST3 could degrade host proteins to evade immune responses, which was an important virulence factor. Other studies found associations between ST3 *Blastocystis sp*. infections and idiopathic urticaria.[24,25] ST1 was the subtype most often found in patients with diarrhea.[16] Research on experimental animal models showed that ST7 was more resistant to antiparasitic agents and had a greater ability to counter nonspecific immune responses compared to ST1 and ST4.[23]

The pathogenicity of *Blastocystis sp.* was also influenced by the morphology of the parasite. Studies on *Blastocystis sp.* forms showed that not all forms were pathogenic, due to phenotypic differences between pathogenic and non-pathogenic *Blastocystis sp.* The most studied phenotypic disparity was the predominance of the amoeboid form in pathogenic strains. The virulence of the amoeboid form was supported by its frequent detection in stool samples from patients with severe diarrheal symptoms.[25] Rajamanikan and Govind (2013) demonstrated a link between protease activity and the abundance of amoeboid forms in cultures from patients with gastrointestinal symptoms due to blastocystosis.[26] The amoeboid form resembled macrophages with pseudopodia, playing a key role in *Blastocystis sp.* adhesion to the mucosal wall before deeper invasion into the intestine.[27] The absence of inoculation with a specific form of *Blastocystis sp.* could have affected the parasite's pathogenicity and, consequently, the host immune response. *Blastocystis sp.* is an intestinal protozoan with diverse classifications, found as both a commensal and a pathogen. To date, studies on *Blastocystis sp.* pathogenicity are ongoing.

The pathophysiology of *Blastocystis sp.* depends on the subtype, morphology, geographical prevalence of the parasite, and the host's immune response capacity. This study did not specifically inoculate *Blastocystis sp*. based on subtype or morphology, thus it is possible that the *Blastocystis sp.* used was not pathogenic. The effective mucosal barrier in the rats' digestive tracts may have inhibited *Blastocystis sp.* invasion, preventing activation of internal immune responses and thus no increase in the differential leukocyte count was observed.

CONCLUSION

Based on the research conducted on the effect of Blastocystis sp. administration on the differential leukocyte count in white rats, it can be concluded that the average leukocyte differential count in the negative control group (K−), treatment group 1 (P1), and treatment group 2 (P2) remained within the normal range, except for a decrease in eosinophil levels. Furthermore, no significant differences were found in the leukocyte differential counts among the negative control group (K−), treatment group 1 (P1), and treatment group 2 (P2).

Declaration by Authors

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