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Original Research Article

Increased Level of the Plasminogen Activator Inhibitor Type-1 Is Associated with Severity of NAFLD

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Abstract

Background: Non-Alcoholic Fatty Liver Disease (NAFLD) has been the most common cause of chronic liver disease worldwide. In NAFLD, elevated Plasminogen Activator Inhibitor-1 (PAI-1) is associated with risk factors for thrombosis and a hypercoagulable state.

Objective: This study explored the relationship between NAFLD as an independent factor for increasing PAI-1 in the presence of metabolic syndrome and insulin resistance.

Methods: This observational study used a cross-sectional design with 80 subjects including 40 patients with NAFLD and 40 without NAFLD at dr. Kariadi General Hospital Semarang who met the inclusion and exclusion criteria. Measurement of plasma PAI-1 levels was done using ELISA method.

Results: A total of 80 patients were included. In the case group, based on abdominal ultrasound, NAFLD was mostly found in the mild category (86%), while the rest were moderate (10%), and severe (4%). Only 25% were found in the Simple Steatosis category, and 75% were suspected of having NASH (NAS = 3-4) and NASH (NAS > 5). There was a significant difference between plasma PAI-1 level and the incidence of NAFLD (p = 0.011). The mean PAI-1 level between the three NAFLD severity categories also showed a significant difference (p = 0.032).

Conclusion: There is an increase in PAI-1 levels in patients with NAFLD. PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD.

Keywords: *NAFLD; NASH; PAI-1; liver fibrosis*
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INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) has been the most common cause of chronic liver disease worldwide. The global prevalence of NAFLD is estimated at 24%; the highest rates are reported from South America and the Middle East, followed by Asia, the USA, and Europe.[1,2,3] The prevalence of NAFLD worldwide increased from 391.2 million in 1990 to 882.1 million in 2017, with the prevalence rate increasing from 8.2% to 10.9% during this same period. In the general population, the prevalence of NASH is estimated at 1.5–6.45%.[4,5]

A study about the prevalence of NAFLD in Asia found that the prevalence of NAFLD cases was projected to increase by 6%-20% during 2019-2030, with related mortality projected to increase between 65% and 100% from 2019 to 2030.[6] Recent meta-analyses and systematic reviews have estimated that the global prevalence of NAFLD is between 25.2% and 29.8%.[7] The prevalence increases among people with obesity, diabetes, and metabolic syndrome, where these patients also experience an increased risk of developing advanced fibrosis and cirrhosis.[8]

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Table 1. General characteristics of research subjects

Characteristics	Group		P
	Case	Control	
Gender			
• Men	20 (50%)	20 (50%)	
• Women	20 (50%)	20 (50%)	1.000
Age	43.30±8.68 ;43;29-59	46.3±8.35; 48;26-60	0.074
Body Mass Index[£]	29.71±5.06 ;29.17;22.49-47.45	22.99±2.55; 23.45;18.3-27.5	**<0.0001**
Waist circumference (cm)[$]	98.2±10.55 ;98;77-134	84.95±7.15; 86.5;64-98	**<0.0001**
Blood pressure (n) [¥]			
• ≥ 130/85 / history of hypertension	11 (27.5%)	6 (15.0%)	0.274
• < 130/85	29 (72.5%)	34 (85.0%)	

[¥] Statistical test with Chi square
[£] Statistical test with Mann Whitney test
[$] Statistical test with T test
Mean±SD;median;min-max

Table 2. Differences in plasma PAI-1 levels with the incidence of NAFLD

Variable	n	Mean ±SD	Median	Min	Max	p
Case	40	112.47±47.55	114.04	93.92	123.67	**0.011***
Control	40	107.22±94.89	108.96	83.75	119.85	

* Statistical test with Mann-Whitney Test

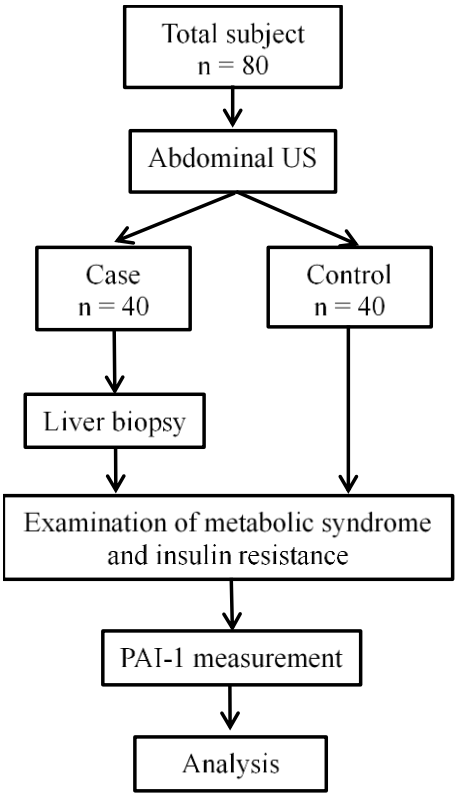

Figure 1. Study Flow Chart

NAFLD is strongly associated with an increased risk of cardiovascular disease (CVD). NAFLD and CVD share several risk factors for metabolic syndrome, including central obesity, hypertension, dyslipidemia, insulin resistance, metabolic syndrome, impaired glucose tolerance, type 2 diabetes, endothelial dysfunction, low plasma insulin-like growth factor-1 (IGF-1) and high plasma hemostatic.[9,10] In NAFLD, inflammation occurs when several pro-inflammatory and oxidative stress cytokines are released, such as CRP, IL-6, TNF- and other reactive oxygen species. Proatherogenic and hypercoagulable-hypofibrinolytic factors were also increased such as fibrinogen, factor VIII, tissue factor and Plasminogen Activator Inhibitor-1 (PAI-1).[11,12]

Elevated PAI-1 is associated with risk factors for thrombosis and liver fibrosis. Thus, there is a relationship between the development of liver fibrosis in chronic liver disease and hypercoagulable conditions, especially triggered by prothrombotic conditions in NAFLD.[12,13] Various studies have attempted to evaluate the role of PAI-1 in the pathogenesis of NAFLD, but they demonstrated conflicting results. Animal studies demonstrate that inhibition of PAI-1 prevents fatty liver.[14] Therefore, we conducted the association between PAI-1 and NAFLD in different severity biopsies proven

Table 3. Differences in Mean plasma PAI-1 levels according to NAFLD severity by NAS

Variable	n	PAI-1 level (ng/ml)				p*
		Mean ±SD	Median	Min	Max	
NASH	30	112,54±7.58	114.74	93.92	123.67	0.032
Simple Steatosis	10	112,28±4.54	111,66	105.62	121.82	
Control	40	107.22±9.48	108.96	83.75	11.85	

* Statistical test with Kruskal-Wallis Test
Statistical Mann-Whitney Test : NASH vs Simple steatosis p = 0,417 , NASH vs control p= 0.013 , Simple Steatosis vs control p= 0.174

Table 4. Differences in plasma PAI-1 levels with fatty liver status based on US

Degree	n	PAI-1 level (ng/ml)				p*
		Mean ±SD	Median	Min	Max	
Severe	4	114.64±7.47	115.40	104.79	122.97	0.046
Moderate	11	113.27±3.76	113.56	105.87	119.47	
Mild	25	111.78±7.92	111.78	93.92	123.67	
Control	40	107.22±9.48	108.96	83.75	119.85	

* Statistical test with Uji ANOVA
Post Hoc test: Severe vs Moderate p = 0.78 ; Severe vs Mild p= 0.527 ; Severe vs Control p= 0.095 ; Moderate vs Mild p=0.623 ; Moderate vs Control p=0.037 ; Mild vs Control p=0.036

in humans. Whether the NAFLD acts as independent risk factor for increasing PAI-1 in the presence of metabolic syndrome and insulin resistance or not, needs a further study.

MATERIALS AND METHODS

This observational study used a cross-sectional design with 80 subjects including 40 patients with NAFLD and 40 without NAFLD at dr. Kariadi General Hospital Semarang who met the inclusion and exclusion criteria. This study was approved by the Health Research Ethics Committee of Dr. Kariadi General Hospital Semarang (number 696/EC/KEPK-RSDK/XII/2017). The case group contained subjects who had fatty liver based on abdominal ultrasound (US) and had undergone liver biopsy to determine the severity of liver fibrosis. The control group contains healthy subjects without fatty liver as evidenced by abdominal US.

Diagnosis of NAFLD was confirmed by the abdominal US GE Logiq S4 done by 2 experienced radiologists, with or without a liver biopsy performed by 1 experienced hepatologist. The inclusion criteria in this study included: adult age more than 14 years old, met the NAFLD criteria from abdominal US examination results which showed fatty liver and without history of alcohol consumption or alcohol consumption did not exceed 70 g/week for women and 140 g/week for men. The exclusion criteria were patients with hepatitis B and C, patients with alcoholic liver disease, taking drugs that can cause fatty liver, have a disorder or history of disease that can cause fatty liver, patients with other liver disorders, arteritis, connective tissue disorders, kidney disease, or thyroid disorders, and patients with weight loss more than 3 kg in the last three months.

The blood samples used in this study were venous blood of patients with NAFLD cases and non-NAFLD controls that had been stored (stored sample). The blood taken was stored as serum/plasma at a temperature of -80^0C. Measurement of plasma PAI-1 levels was done using ELISA method with the Human PAI-1 (Elabscience, United States). Examination of plasma PAI-1 levels was carried out in the GAKI laboratory Faculty of Medicine Universitas Diponegoro which is licensed and standardized. The PAI-1 levels were measured using the following steps : 50 µL of standard and sample incubated for 90 minutes at 37 °C, then mixed with 100 µL Biotinylated detection Ab, incubated again for 1 hour at 37 °C. Aspirate and wash 3 times, add 100 ml HRP and incubate for another 30 minutes at 37°C. Aspirate and wash 5 times then add 90 µL of substrate reagent, incubate for 15 minutes at 37 °C, add 50 µL of stop solution then read immediately at 450 nm. Results are recorded in ng/ml units. The flow of the study is explained in Figure 1

Table 5. Differences in plasma PAI-1 levels with clinical and laboratory parameters

Variable	Plasma PAI-1 level (ng/ml)			p
	Simple Steatosis	Probable & definite NASH	Controls	
Waist circumference category				
Central obesity	112.28±7.25	112.44±8.17	106.61±1.04	0.066*[1]
Without central obesity	-	113.00±3.95	107.83±8.67	0.227[#]
BMI category				
Obesity	111.38±4.24	115.07±6.11	-	0.121[£]
Without obesity	112.89±5.03	110±8.24	107.22±9.48	0.266*
Blood pressure				
≥ 130/85 /history of anti-hypertension drug	-	110.76±9.05	108.66±5.78	0.506[#]
< 130/85	112.02±4.74	113.43±6.81	106.97±10.04	**0.040[¥]**
ALT level[¥]				
Increased (>65 IU/L)	-	111.64±6.32	103.68±1.94	0.751[£]
Normal	112.04±4.74	113.32±8.67	107.41±9.16	**0.039[¥]**
AST level[¥]				
Increased (>37IU/L)	116.03±26.94	111.57±9.14	107.74±12.61	0.537*
Normal	110.67±3.45	113.09±6.73	107.16±9.30	**0.040***
Ratio AST/ALT				
AST/ALT > 1	111.24±3.75	112.76±6.90	108.54±8.68	0.129[¥]
AST/ALT < 1	114.70±6.17	110.48±14.40	103.75±11.03	0.290*
Blood fasting glucose (mg/dL)				
> 100 / history DM type 2	-	109.92±9.59	105.40±10.09	0.355[#]
≤ 100	112.03±4.74	114.05±5.91	107.67±9.44	**0.022***
HDL cholesterol level (mg/dL)				
< 40 (male) or < 50 (female) or history of lipid profile disturbance	110.75±3.83	111.45±7.81	107.85±9.22	0.476[¥]
≥ 40 (male) or ≥ 50 (female)	113.30±5,02	113.07±7,61	104.23±10.89	**0.048***
Fasting TG level (mg/dl)				
≥ 150 / history of lipid profile disturbance	111.98±2.86	112.75±8,85	110.53±5.44	0.328[¥]
< 150	112.99±8.19	112.09±4.41	106.63±9.97	0.257[¥]

* Statistic test with uji ANOVA ; [1] post hoc test variables Central obesity NASH vs control p=0.028
[#] Statistic test with T-test
[£] Statistic test with dengan Mann-Whitney Test
[¥] Statistic test with Kruskal-Wallis Test

The data are described in frequency, percentage, and mean ± SD for categorical data, and presented in tabular form. Patient characteristics were analyzed descriptively, then continued with bivariate analysis to find the

significancy of the relationship between each variable with cross tabs. A correlation test was done to analyze the relationship between the independent variable and the dependent variable.

RESULTS

This study involved 40 subjects with NAFLD as a case group and 40 healthy subjects as a control group, with the same sex ratio between the two groups. Table 1 represents the general characteristics of these 80 patients. Age and blood pressure status in the two groups were not significantly different. Body mass index and waist circumference were significantly higher in the case group than in the control group ($p = <0.0001$). Biochemical parameters such as ALT, AST, triglycerides, cholesterol, insulin, and HOMA-IR scores were significantly different between the two groups ($p<0.001$), while the fasting blood sugar levels of the two groups were not significantly different.

In the case group, there was an incidence of metabolic syndrome (58%) and insulin resistance (60%); compared to the control group, metabolic syndrome was found in only 8%, while the insulin resistance was found in 13%. There was a significant relationship between the incidence of metabolic syndrome and insulin resistance with the incidence of NAFLD ($p < 0.001$).

The distribution of fatty liver status was based on abdominal US examination. US of the liver in all control group subjects showed normal results. In the case group, it was mostly found in the mild category (86%), followed by moderate (10%), and severe (4%). The degree of fatty liver (NAFLD) is based on the Histological Scoring System for Non-Alcoholic Fatty Liver Disease Score and Fibrosis Staging (NASH activity score = NAS). It appeared that only 25% were found in the Simple Steatosis category, and among the group suspected of having NASH (NAS = 3-4) and NASH (NAS > 5), as much as 75%.

The difference in plasma PAI-1 levels with the incidence of NAFLD can be seen in Table 2, which show that there were significant differences between plasma PAI-1 levels and the incidence of NAFLD ($p = 0.011$). Table 3 shows the relationship between plasma PAI-1 levels and the severity of NAFLD based on NAS, there were significant differences between the three NAFLD severity categories ($p = 0.032$). The highest mean value was obtained in the NASH category (112.54 ng/ml), and the highest median value was also in the NASH category (114.74 ng/ml). In the Mann-Whitney test, only the NASH variable and the control showed a significant difference ($p = 0.013$).

Table 4 shows a significant difference between the mean plasma PAI-1 levels and fatty liver status based on abdominal US ($p=0.046$). The PAI-1 value increases along with the fatty liver status, although in post hoc analysis, there were no significant differences between the case groups, but significant differences were found when case groups compared to controls.

Table 5 describes the differences in the PAI-1 levels based on clinical parameters, including the obesity component, the liver function component, and the component in the metabolic syndrome. All variables are divided into two major categories. In the category of blood pressure status in the category with hypertension, there was no significant difference ($p=0.506$) but a significant difference ($p=0.04$) in the non-hypertensive group. In the category of laboratory parameters, there were also significant differences in the categories of normal AST ($p=0.039$), normal ALT ($p=0.04$), normal fasting blood sugar ($p=0.022$), and high HDL cholesterol ($p=0.048$). There was no significant difference between patients with simple steatosis, NASH, and controls in the group with abnormal laboratory parameters.

There were significant differences in the categories of patients without hypertension ($p=0.04$), normal AST ($p=0.039$), normal ALT ($p=0.04$), normal fasting blood sugar ($p=0.022$), and high HDL cholesterol. ($p=0.048$). In the group with abnormal laboratory parameters, there was no significant difference between patients with simple steatosis, NASH, and controls. We also analyzed the mean PAI-1 levels between the case and control groups based on the category of metabolic syndrome status and insulin resistance, but there was no significant difference ($p>0.05$).

DISCUSSION

The characteristics of the subjects in this study illustrate that patient with NAFLD have higher BMI and abdominal circumference than the control group. According to recent consensus, BMI and waist circumference are important measures in determining central obesity and metabolic risk.[15] Fat distribution is a major pathophysiological mechanism for metabolic disease and central obesity. Patients with NAFLD experienced a significant increase in body weight compared to those without.[16] This is also in line with previous studies that the prevalence of NAFLD increased in parallel with the increase in obesity.[17] Tilman Kühn examined anthropometric parameters in NAFLD patients with obesity. The presence of fatty liver showed the strongest correlation with waist circumference.[18]

This study also found significant differences in the components of the metabolic syndrome (fasting blood sugar levels, triglycerides, and serum HDL cholesterol) and components of insulin resistance (serum insulin levels and HOMA-IR index) between the case group and the control group. Insulin resistance is based on the HOMA-IR if the value is >2. An increase in the HOMA-IR indicates impaired insulin sensitivity.[19] NAFLD-induced changes in the secretion of liver proteins, lipids, and other metabolites alter metabolism in the liver, muscle, adipose tissue, and pancreas to induce insulin resistance.[20]

Based on abdominal US, NAFLD was mostly found 86% in the mild category, however, based on histology, only 25% were found in the Simple Steatosis category, and 75% suspected of having NASH (NAS = 3-4) and NASH (NAS > 5). Abdominal US is reported to have a sensitivity of 60-94% in detecting fat, so it can be used to determine the presence of fatty liver. However Abdominal US has the disadvantage of being subjective and poor in sensitivity and specificity in obese patients.[21] Therefore liver biopsy remains the reference standard to determine the severity of NAFLD.[22]

We found that PAI-1 levels were significantly higher in advanced NAFLD, assessed using NAS. Significantly increased PAI-1 levels were mainly found in patients with NASH compared to the control group. PAI-1 is an

important regulator of fibrinolysis, with a known profibrotic function in various tissue types. PAI-1 inhibits the activation of plasminogen to plasmin, which will have an impact on decreasing levels of matrix metalloproteinases (MMP) and increasing extracellular matrix (ECM) deposits so that the process of liver fibrogenesis occurs. Elevated serum PAI-1 has been shown to correlate with the degree of fibrosis in liver biopsies in patients with NASH.[12] In a previous study in children, it was found that circulating PAI-1 levels were closely correlated with the amount of hepatic steatosis as measured by magnetic resonance spectroscopy, regardless of adiposity and insulin resistance.[13]

Previous studies revealed that PAI-1 expression is influenced by various pro-inflammatory conditions and is associated with cardiovascular risk. Metabolic syndrome, obesity, and insulin resistance are associated with elevated PAI-1 levels.[23,24] Therefore, in our study, we developed an analysis to determine whether other factors influence PAI levels in patients with NAFLD. Then it was found that obesity, hypertension, elevated liver enzymes, diabetes mellitus, and HDL levels did not affect PAI-1 levels in patients with simple steatosis, NASH, or controls. It can be concluded that PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD.

Elevated PAI-1 can be used as a predictive factor for the occurrence of thrombosis associated with an increased risk of cardiovascular disease (CVD). The active form of PAI-1 specifically inhibits both tissue-type and urokinase-type plasminogen activators. As a result, fibrin breakdown is inhibited, which makes it easier for a thrombus to form. Study about the predictive value of PAI-1 in deep venous thrombosis (DVT) showed that level of PAI-1 in the DVT group was significantly higher than the control group ($P<0.05$).[25] Frischmuth et al. concluded that PAI-1 is associated with increased risk of future incident VTE.[26] Recent studies imply that local thrombotic events play a role in the progression of liver disease since patients with cirrhosis who received low-molecular-weight heparins saw a lower rate of complications.[27]

Significant differences in PAI-1 levels were actually found in patients who did not have hypertension, normal AST and ALT levels, and normal blood sugar levels. It can be caused by several factors that may still be a limitation of our study, we did not further investigate the drugs consumed by the patient. In hypertensive patients, using losartan – an angiotensin II type 1 receptor antagonist (AT1R) for 12 weeks can reduce PAI-1 gene expression, improve fibrosis, reduce liver inflammation, and improve insulin sensitivity. Sodium-glucose co-transporter-2 (SGLT2) inhibitors such as empagliflozin are known to reduce plasma PAI-1 concentrations in patients with type 2 diabetes. [28]

From our results, PAI-1 levels were not affected by the presence of metabolic syndrome and insulin resistance. Although the association between NAFLD and insulin resistance is generally evident in most patients, this is not always the case for genetically determined forms of fatty liver. For example, frequent sequence variation (I148M) in a protein containing the patatin-like phospholipase domain (PNPLA3) is strongly associated with fatty liver disease in the absence of insulin resistance or dyslipidemia, and similar dissociation was reported in individuals with single-nucleotide polymorphisms for acyl-coenzyme A (CoA), diacylglycerol acyltransferase (DGAT) and Lys167 allele in the transmembrane superfamily (TM6SF2).[20]

Our study has several strengths. This is the first study in Indonesia on PAI-1 levels involving patients with and without NAFLD. The findings from our study support the hypothesis that PAI-1 plays an important role in the pathophysiology of NAFLD. Diagnosis and grading of NAFLD in our study used 2 modalities, namely liver biopsy and ultrasound. This study also further analyzed other factors that might influence PAI-1 levels in patients with NAFLD.

This study has several limitations, including the small number of subjects. Therefore, larger studies need to be conducted to confirm our findings. A second limitation is its correlation analysis which does not allow evidence of a causal relationship between plasma PAI-1 and clinical variables. In this study, analysis of thrombosis status and coagulation studies were not performed.

CONCLUSION

There is an increase in PAI-1 levels in patients with NAFLD. PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD. PAI-1 levels were not affected by the presence of metabolic syndrome and insulin resistance. The level of PAI-1 linked to thrombotic events rises with the severity of NAFLD.

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Original Research Article

Molecular Detection Challenges of Human *Plasmodium knowlesi* infection by Polymerase Chain Reaction

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Abstract

Background: *Plasmodium knowlesi* is the fifth *Plasmodium sp*. causing malaria in humans. There were 545 *P. knowlesi* malaria cases reported in Indonesia in 2010-2021 period. The first case was reported from South Kalimantan, and more cases were reported in Sumatra and Kalimantan Island. The morphology of *P. knowlesi* is difficult to distinguish from other *Plasmodium* species, especially with *P. falciparum* and *P. malariae*. Therefore, molecular identification is still the most promising method for diagnosing *P. knowlesi* infection.

Objective: This study aimed to analyze the molecular detection method of human *P. knowlesi* infection using Polymerase Chain Reaction (PCR) and sequencing techniques.

Methods: DNA was isolated from malaria blood samples. *P. knowlesi* detection was conducted by nested PCR using primer rPLU1 and rPLU5 for nested 1 and Kn1f and Kn3r for nested 2. The PCR products were directly sequenced. The sequences were analysed using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI).

Results: Blood samples from ten malaria patients from Maluku province were collected after informed consent. The *P. knowlesi*-specific PCR amplification resulted in a band of approximately 420 bp in all samples. Sequence analysis showed the highest similarity (89-92 %) with many global *P. falciparum* strains. However, BLAST analysis for part of sequences also showed high similarities with several *P. knowlesi* H strains 18s rRNA from Peninsular Malaysia. Primer analysis using BLAST demonstrated the specificity of Kn3r-nested 2 primer, however, Kn1f primer showed a cross-reactive with other *Plasmodium sp*, including *P. falciparum* and *P. vivax*.

Conclusion: Molecular detection of *P. knowlesi* infection is challenging. A new target gene for primer design and detection method with higher specificity for human *P. knowlesi* examination is needed to develop.

Keywords: *Malaria, PCR, Plasmodium knowlesi, Primer.*
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INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium* sp. and transmitted by *Anopheles* mosquito. In 2020, the World Health Organization (WHO) reported 241 million malaria cases with 627,000 deaths.[1] The Indonesia Ministry of Health reported 254,05 thousand malaria cases in 2020.[2]

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Table 1. Patients Characteristics, clinical symptoms, and microscopic examination

Patient	Sex	Age	Clinical symptom	Microscopic examination
P1	M	23	fever, chills, headache	*P. falciparum*
P2	F	9	fever, chills, headache	*P. falciparum*
P3	M	15	fever, chills, headache	*P. falciparum*
P4	M	40	fever, chills, headache	*P. falciparum*
P5	M	16	fever, chills, headache	*P. falciparum*
P6	M	33	fever, chills, headache	*P. falciparum*
P7	M	48	fever, chills, headache	*P. falciparum*
P8	M	17	fever, chills, headache	*P. falciparum*
P9	F	32	fever, chills, headache	*P. falciparum*
P10	F	27	fever, chills, headache	*P. falciparum*

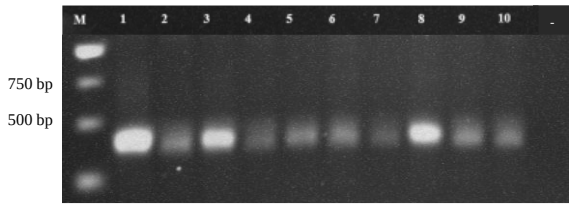


Figure 1. Visualization of the PCR in 1% agarose gel electrophoresis resulted in a single band of ±420 bp. M: 1 kb DNA ladder; 1-10: PCR result from ten samples; -: negative control.

Twenty types of *Plasmodium* infect primates, and five of them can cause malaria in humans, including *Plasmodium knowlesi*. *P. knowlesi* is a type of *Plasmodium* that naturally infects long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*), whose transmission is mediated by the *Anopheles leucosphyrus* group mosquito.[3,4] In Indonesia, natural hosts of *P. knowlesi* are dispersed on the islands of Sumatra, Kalimantan, Java, Bali, Lingga Islands, Riau Islands, Bangka, Belitung, Banyak, Tambelan Islands, Natuna Islands, Simalur, Nias, Matasari, Bawean, Maratua, Timor, Lombok, Sumba and Sumbawa.[5,6] In addition, the vectors are spread on the islands of Sumatra, Java, Bali, Kalimantan, Sulawesi, and Maluku, along with several other small islands in Indonesia.[3,7]

Several Asian countries has reported human *P. knowlesi* infection. The first large cases of human *P. knowlesi* was reported from Sabah, Malaysia in 2004, followed by other countries such as Cambodia, Indonesia, Myanmar, the Philippines, Thailand, Vietnam, Singapore, and Brunei.[8] The first case of *P. knowlesi* infection in Indonesia was found in 2010 in Kalimantan. The case was found in gold miners in the tropical forests of South Kalimantan.[9] The following case of *P. knowlesi* infection occurred in an Australian citizen who had a history of working in the forests of South Kalimantan for 18 months, and subsequently suffered from high fever and other malaria symptoms emerged after returning to his home country.[10] And several cases of human *P. knowlesi* infection were reported from the western part of Indonesia, especially from Sumatra and Kalimantan island.[7]

Peripheral blood smear examination is one of the standard diagnostic examinations for malaria. However, *P. knowlesi* and other *Plasmodium* species are difficult to differentiate since their morphology is similar to *P. falciparum* and *P. malariae*. In the early stages of its life cycle, the trophozoites of *P. knowlesi* resemble the trophozoite form *P. falciparum*. In contrast, the later stages of *P. knowlesi*, including the trophozoites, schizonts, and gametocytes, are more similar to *P. malariae.*[11,12] Rapid Diagnostic Test (RDT) has shown low sensitivity and cannot be used to confirm the cases of *P. knowlesi* infection[13,14] Previous studies stated that the nested Polymerase Chain Reaction (PCR) method has the highest sensitivity and specificity compared to other known methods.[11,12] Therefore, molecular identification in PCR is still the most suitable and applicable technique for diagnosing *P. knowlesi* infection.[11,13,15] This study aimed to analyze the molecular detection method of human *P. knowlesi* infection using PCR and sequencing techniques and the extent of human *P. knowlesi* infection in Indonesia.

MATERIALS AND METHODS

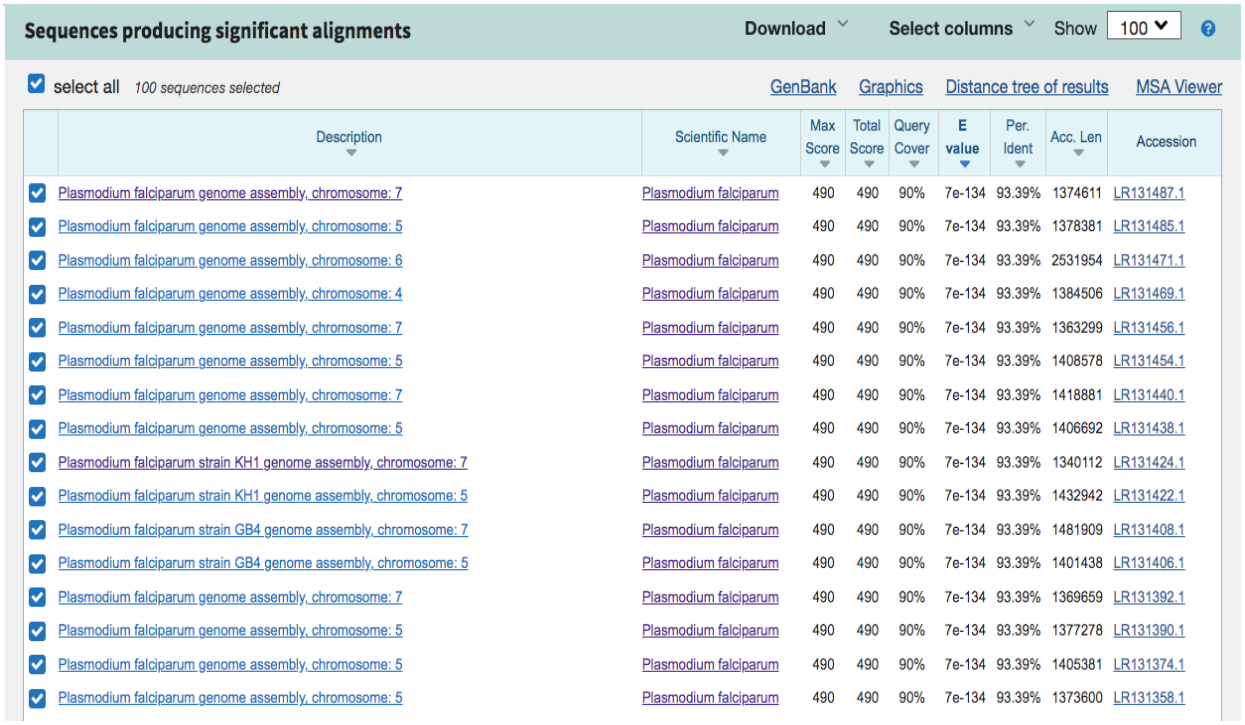
Study Design

The study was an explorative laboratory study and conducted by serial sample collection followed by laboratory research. Samples were collected from the blood of malaria patients from Tiakur Health Center, South-West Maluku Regency, Maluku Province, Indonesia. Blood was dropped on a Whatman filter paper, dried at room temperature (RT), and stored at -20 ^{o}C until it was used.

Patients were recruited based on inclusion criteria, i.e., patients with complaints of headache, fever, chills, nausea, vomiting, and other symptoms of malaria, show *Plasmodium sp.* infection on microscopic examination, and do not take malaria treatment for the last month.

Ethical Approval

The study has been approved by the Ethical Committee of the Faculty of Medicine University of Jember No. 1596/H25.1.11/KE/2022.



Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Plasmodium falciparum genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1374611	LR131487.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1378381	LR131485.1
Plasmodium falciparum genome assembly, chromosome: 6	Plasmodium falciparum	490	490	90%	7e-134	93.39%	2531954	LR131471.1
Plasmodium falciparum genome assembly, chromosome: 4	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1384506	LR131469.1
Plasmodium falciparum genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1363299	LR131456.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1408578	LR131454.1
Plasmodium falciparum genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1418881	LR131440.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1406692	LR131438.1
Plasmodium falciparum strain KH1 genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1340112	LR131424.1
Plasmodium falciparum strain KH1 genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1432942	LR131422.1
Plasmodium falciparum strain GB4 genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1481909	LR131408.1
Plasmodium falciparum strain GB4 genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1401438	LR131406.1
Plasmodium falciparum genome assembly, chromosome: 7	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1369659	LR131392.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1377278	LR131390.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1405381	LR131374.1
Plasmodium falciparum genome assembly, chromosome: 5	Plasmodium falciparum	490	490	90%	7e-134	93.39%	1373600	LR131358.1

Figure 2. BLAST analysis result. The sequences match with several *P. falciparum* sequences in the database.

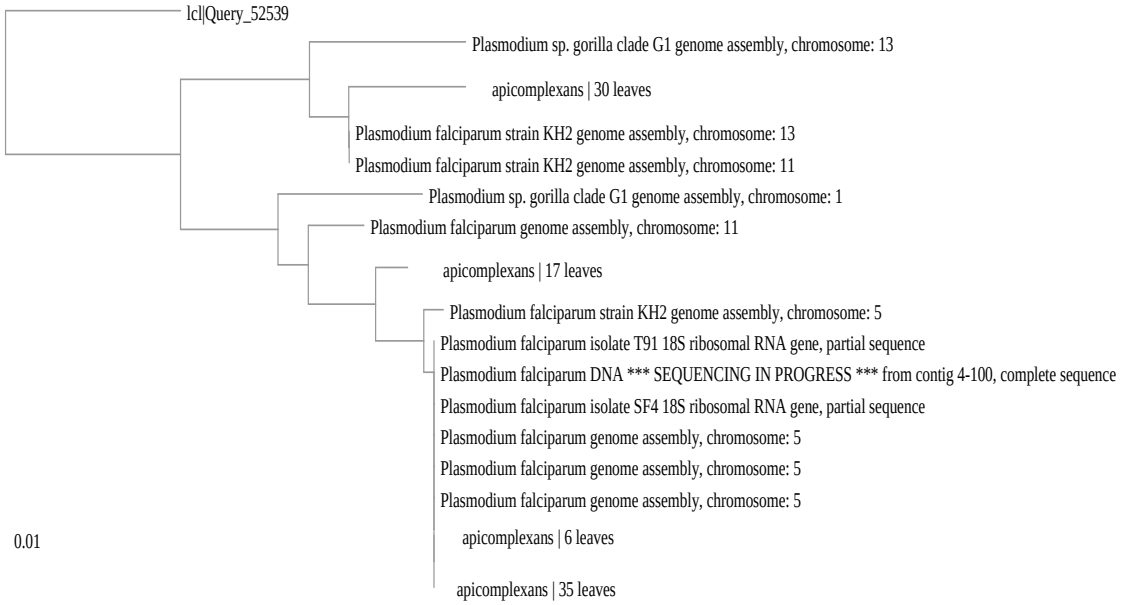


Figure 3. The cladogram of the sequence. The sequence has a proximity with several *P. falciparum* sequences and *Plasmodium sp.* gorilla clade.

DNA Isolation and PCR Amplification

The DNA isolation was carried out according to the mini QIAamp DNA kit protocol (Qiagen, Hilden, Germany). Five pieces of 3 mm-filter paper containing the patient's blood were added with the 180 µl buffer and incubated at 85ºC for 10 mins. Then proteinase-K was added and incubated at 56ºC for 1 h. The sample was washed twice with buffer and centrifuged before the DNA was extracted using a spin column.

The isolated DNA was amplified using MyTaq HS Red Mix (Bioline, London, UK) with nested PCR. The nested 1 PCR used genus-specific primers, i.e., rPLU1 (TCA AAG ATT AAG CCA TGC AAG TGA) and rPLU5 (CCT GTT GTT GCC TTA AAC TTC), with a total volume of 50 µl and was performed with the following settings: initial denaturation at 94°C for 4 mins, followed by 35 cycles of 94°C for 30 secs, 55°C for 1 min, and 72°C for 1 min, and finalized by 72°C for 4 mins. The nested 2 PCR were carried out using species-specific primers, i.e., Kn1f (CTC AAC ACG GGA AAA CTC ACT AGT TTA) and Kn3r (GTA TTA GGT ACA AGG TAG CAG TAT GC), in a total volume of 20 µl with the similar setting with the nested 1, except for annealing

temperature of 62°C. Nested 2 PCR products were analyzed with agarose gel electrophoresis.

Direct Sequencing and Sequence Analysis

The PCR products were directly sequenced in both directions using BigDye® Terminator v3.1 cycle sequencing kit and ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). The nucleotide sequences were analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) tool on the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to perceive the nucleotide sequence's identity.

RESULTS

Ten malaria patients were recruited after written informed consent. The patients were seven males and three females. The patients' characteristics are described in Table 1.

DNA isolation from all samples yielded 1.7-50 ng/µl. Nested PCR amplification resulted in a single band of approximately 420 bp in all samples. The negative control showed no band. Unfortunately, there was no positive control due to difficulty finding a positive sample (Figure 1).

Direct sequencing of PCR product read for approximately 402 nucleotides. The sequence will be deposited at GenBank. BLAST analysis demonstrated that the sequences matched for *P. falciparum* sequences (LR131487.1, LR131485.1, LR131471.1, LR131469.1, LR131456.1), as shown in Figure 2. It has 90% sequence coverage and 93.39% similarity with *P. falciparum* sequences. Sequence analysis using the Neighbour-joining method to design a cladogram demonstrated its proximity to several *P. falciparum* sequences and *Plasmodium sp.* gorilla clade, as presented in Figure 3.

Further analysis using BLAST and multiple alignments was conducted to investigate the relation of the sequences with *P. knowlesi* sequences. The 119 nucleotide upstream sequences from samples (P1_Kn1f_fw, P5_Knf1f_fw, P8_Kn1f_fw) were aligned with *P. knowlesi* strain H (XR_005506393.1; XR_005506386.1; XR_005506366.1), as shown in Figure 4. However, BLAST analysis for those sequences showed 99.16% similarities with many *Plasmodium* species, such as *P. knowlesi, P. delichoni, P. gonderi, P. sp. gorilla clade, P. inui, P. sp—DRC-Itaito, P. vivax, P. gaboni, P.cynomolgi, and P. coatneyi* (supplementary 1).

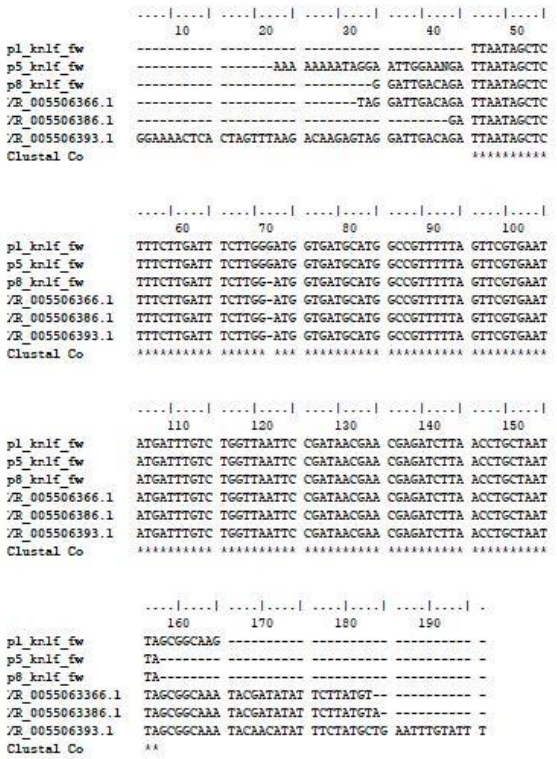


Figure 4. Multiple alignments of 119 upstream sequences from samples (P1_Kn1f_fw, P5_Knf1f_fw, P8_Kn1f_fw) and several *P. knowlesi* strain H (XR_005506393.1; XR_005506386.1; XR_005506366.1).

DISCUSSION

The study isolated DNA from the patient's blood filter paper and yielded DNA in a concentration of 1.7-50 ng/µl. The use of blood filter paper has proven to be effective for DNA isolation to diagnose infection and determine molecular epidemiology.[4,11,16,17] Detecting malaria parasite DNA extracted from archival slides is relatively specific, though the sensitivity varies and does not depend on parasitemia. Previous epidemiological studies reported similar findings of the nested PCR method for malaria using DNA extracted from filter paper.[11,16]

Microscopic examination as the gold standard for malaria diagnosis was applied in this study. The microscopic examination of patients in this study showed *P. falciparum* in all samples. Further determination by nested PCR to detect the possibility of *P. knowlesi* infection using *P. knowlesi*-specific primer, i.e., Kn1f and Kn3r yielded a single band of approximately 420 bp in all samples (Figure 1). The observed-single band of 420 bp in all samples except the negative control led to baffling questions since the reference reported a 290 bp as the *P. knowlesi* amplicon.[14,18] Moreover, direct sequencing of PCR product confirmed *P. falciparum* infection for all samples. Further analysis by designing a cladogram validates the *P. falciparum* sequence and its similarities (Figures 2 and 3).

The study result corroborates the previous studies on the difficulties of *P. knowlesi* detection.[9,19,20] PCR amplification followed by sequencing confirmed the *P. falciparum* infection as the result of microscopic examination. Another study reported mixed infection of *P. vivax* and *P. falciparum* or a single infection of *P. falciparum* in microscopic examination of thick and thin blood smears, but positive for *P. knowlesi* infection upon a molecular examination.[21] Therefore, suspected cases of *P. knowlesi* infection require a molecular examination to confirm the diagnosis.

Primer pair rPLU1 and rPLU5 for nested 1 are genus-specific primers, and Kn1f and Kn3r are species-specific primers designed based on the small subunit rRNA genes. BLAST analysis for Kn1f demonstrated that the ten most similar sequences with 100% coverage and 100% identity are *P. falciparum* (MN852862.1), *P. knowlesi* strain H

(LR701173.1), *P. falciparum* isolate Jr-01 (CP101625.1) *dan P. vivax* isolate P023VOO34 (OM033598.1). Meanwhile, Kn3r showed 96% coverage and 100% identity with all *P. knowlesi* clones (KY404060.1, MN535379.1), indicating its specificity. This result is similar to previous primer designs for *P. knowlesi* detection, such as Pmk8 and Pmkr9, with Pmk8 being highly specific while Pmkr9 cross-reacts with *P. vivax* S-type strain and other *Plasmodium species,* especially those related to *P. vivax.*[9] Another method using 5-plex malaria immunoassay containing pan-*Plasmodium* lactate dehydrogenase (pLDH), *P. falciparum*-LDH, *P. vivax*-LDH, *P. falciparum*-histidine-rich protein-2 (HRP2), and C-reactive protein showed cross-reactivity of *P. knowlesi* and *P. vivax.*[22] Furthermore, a new detection method using qPCR assay based on 18S rRNA gene with fluorescence-labeled probing showed high sensitivity compared to the conventional PCR method. However, the study was conducted in a malaria-endemic area, and further validation is needed for field settings, especially for mass screening and surveillance programs.[23]

The study also investigated the evolutionary relationship of *Plasmodium species* due to the high cross-reactivity of the sequences, especially between *P. knowlesi* and *P. vivax*. It found that the 119 upstream sequences have 99.16% similarities with many *Plasmodium species*. This result is similar to the previous studies.[18,24] This result supported our finding on the potential cross-reactivity of Kn1f primer with many *Plasmodium species* due to its similarity.

Molecular detection for *P. knowlesi* infection is still challenging. Several targets for detection, such as 18S ribosomal RNA and cytochrome-b (cytb gene), were found to be less specific and need sequencing for definite confirmation. Therefore, it is crucial for developing sensitive and specific *P. knowlesi* detection using new target genes and detection methods.

CONCLUSION

This study highlights the challenges in detecting *P. knowlesi* at the molecular level. Previous molecular targets were found to have limited specificity, indicating a need for new target genes and detection methods. Developing such methods is crucial for preventing potentially severe cases of human malaria caused by *P. knowlesi* and designing effective control strategies.

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Original Research Article

Potential Effects of *Ipomoea reptans Poir.* Extract on LDL, HDL levels and liver Histopathology

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Abstract

Background: *Ipomoea reptans Poir*. extract has been known to have antioxidant, antihypertensive, antidiabetic and anti-obesity activities. The content of the secondary compounds antioxidant in the extract is believed to play an important role in this mechanism also often reported that have regulating cholesterol levels.

Objective: This study aims to investigate the antioxidant activity of *I. reptans* extract on changes in LDL and HDL and liver histopathology in animal models of hypercholesterolemia that treated with *I. reptans* extract.

Methods: Twenty-five wistar rats were randomly divided into 5 groups: normal group (N), hypercholesterolemic group (Chol), *I. reptans* extract group at a dose of 200 mg/kg BW, 300 mg/kg BW and 400 mg/kgBB (IE 200; IE 300, and IE 400). Rats in group Chol and IE were induced by cholesterol in the form of a mixture of quail egg yolk and animal oil for 21 days. Then, continued with therapy with *I. reptans* extract in the IE group only according to their respective doses for 14 days. LDL and HDL levels were measured using enzymatic colorimetric, and liver organs were also taken for histopathological analysis with hematoxylin-Eosin (HE) staining.

Results: The results showed that the average LDL levels in the N, Chol, IE 200, IE 300 and IE 400 groups were 8.56 ± 1.36, 29.9 ± 1.05, 25.22 ± 4.72, 9.12 ± 0.72, and 9.22 ± 0.77 mg/dL, while the average HDL levels in the were 38.1 ± 2.24, 15.92 ± 4.39, 33.4 ± 5.91, 36.92 ± 0.47, and 42.82 ± 3.27 mg/dL respectively. Liver histopathology in the IE 300 group showed reduced fatty degeneration compared to the Chol group.

Conclusion: It can be concluded that the administration of *I.reptans* extract in hypercholesterolemia rats was able to ameliorates LDL and HDL levels, and improve liver histopathology.

Keywords: *Hypercholesterolemia; Ipomoea reptans; LDL; HDL; Liver Histopathology*

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INTRODUCTION

Hypercholesterolemia is a non-communicable disease that can cause chronic illness to death in a person. It is characterized by having low-density lipoprotein (LDL) cholesterol levels above normal or high-density lipoprotein (HDL) cholesterol in the blood below normal. Researchers calculated that increases in LDL cholesterol in 2017 caused nearly 4 million deaths, accounting for one third of deaths from ischemic heart disease and stroke. These deaths markedly decreased in Western countries while significantly increasing in Asian countries[1]. In Indonesia, it was recorded that the percentage of the population aged >15 years had LDL levels above normal by 73.8%, total cholesterol above normal by 28.8%, and low HDL levels by 24.3%.[2]. People with high blood cholesterol are at increased risk of heart disease (the leading cause of death) and stroke (the fifth leading cause of death), atherosclerosis, coronary heart disease, diabetes mellitus, and liver[3].

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The condition of hypercholesterolemia causes an increase in excess fat production in the liver so that accumulation (steatosis) occurs, leading to a more serious and even fatal condition, namely nonalcoholic steatohepatitis (NASH) and even death[4].

Currently, medicinal plants have also become a concern and not a few are exploring these plants from the medical community. This is because medicinal plants are easier to obtain and tend to have fewer side effects[5,6]. One of the plants that is very easy to cultivate, has a very affordable price and is often even consumed by Indonesian people is ground kale (*Ipomoea reptans Poir.*). This plant is often cooked and used as a companion vegetable for daily consumption by Indonesians.

In a previous study, it was concluded that the aqueous extract of *I.. reptans* family, *Ipomoea aquatica*, at a dose of 400 mg/kg had activity in controlling dyslipidemia in albino rats. Treatment with the extract was reported to be able to improve lipid profiles by lowering cholesterol and LDL levels and significantly increasing HDL levels[7]. Furthermore, in 2020, *I. reptans* extract was reported to have antioxidant, antidiabetic and anti-obesity activities[8]. The high antioxidant activity *in I. reptans* extract cannot be separated from the role of the polyphenol and flavonoid compounds contained therein. According to Laka, K. et al, flavonoids and polyphenols act as antioxidants and are proven to be able to suppress the activity of the HMG-CoA reductase enzyme so that total cholesterol biosynthesis can also be suppressed. Antioxidant activity is also able to inhibit LDL oxidation[9,10].

The role of antioxidant activity of *I. reptans* extract against hyperglycemia model rats has been extensively studied and its efficacy is known. However, until now, the role of *I. reptans* extract on cholesterol levels and improvement of liver histopathology in conditions of hypercholesterolemia has not been known. In this study, we will discuss and examine the effect of *I. reptans* extract on LDL, HDL levels and histopathological features of the liver of experimental white rats (*Rattus norvegicus*) which were made hypercholesterolemia with high cholesterol diet.

MATERIALS AND METHODS

Research Design

This research was an *in vivo* experimental study using mice as experimental animals. Male Wistar strain albino rats *(Rattus norvegicus*), body weight ranging from 150-200 grams (2-3 months) was used in this research. A total of 25 rats and habituated for 2 weeks in the Lab. Pharmacology, Pharmacy Study Program, Universitas Binawan and randomly divided into 5 groups, including: (1) Normal / N group, no intervention was given before necropsy; (2) The hypercholesterolemia/Chol group, the rat group which was given high cholesterol diet and was not treated; (3-5) Group of rats treated with *I. reptans* extract at doses of 200 mg/kgBW, 300 mg/kgBW, and 400 mg/kgBW / IE 200, IE 300, and IE400. In this group, rats that were fed a diet high in cholesterol, after hypercholesterolemia, were treated through a sonde with *I. reptans* extract according to the dose in each group. The use of experimental animals in this study has approval from the Animal Care and Use Committee of Universitas Brawijaya (Ethical Clearance Number: 055-KEP-UB-2022).

Place and Time Research

This research was conducted for 10 months from February 2022 to November 2022. The implementation of this research was in collaboration with several related laboratories. In the early stages of the research, the preparation of ground kale extract (*Ipomoea reptans poir.)* was carried out at the Balittro Test Laboratory, Bogor. While research activities using animal models, carried out in the Lab. Pharmacology, Pharmacy Study Program, Universitas Binawan. Preparation and photos of tissue histopathic preparations were carried out at the Primate Animal Study Center, IPB.

Population and Samples

The population in this study were Wistar strain white rats (*Rattus norvegicus*) with hyperglycemia which had been treated with *I. reptans* extract. While the specimens used were blood serum and liver organs from experimental animals in all groups.

Preparation of *I. reptans Extract*

Fresh leaves of *I. reptans* as much as 8 kg were washed and air-dried to obtain dried *I. reptans* leaves. Then the dried leaves of *I. reptans* were blended/mashed to obtain *I. reptans* powder. Dried *I. reptans* powder was macerated with 70% ethanol solvent, then the macerate was concentrated using a vacuum rotary evaporator and filtered to obtain a thick extract.

Thick extract was screened for phytochemicals to determine the active compounds in it. Phytochemical screening was carried out by adopting several methods that have been common and have been widely used in previous studies. The identification of alkaloid compounds was carried out using the Dragendorf test and Wagner's test. Saponin compounds were tested using the foam method. The presence of tannins and phenols in the extract was tested using gelatin and salt. The flavonoid compounds in the extract were tested using Shinoda's test/ Mg-hydrochloride reduction test. The identification of triterpenoid and steroid compounds was carried out by Salkowski's test and Libermann-Burchard's test. Meanwhile, to identify the presence of glycosides, the Keller-Killani test was carried out

Administration of High Cholesterol Diet

Giving high-fat feed was carried out using the method in previous studies with a few modifications. A mixture of 2 grams of goat oil is mixed with 1 gram of boiled quail egg yolk[11] which is dissolved in water up to 2 ml and given through a gastric tube. The administration was carried out in the Chol, IE200, IE300, and IE400 groups for 21 days.

Measurement of HDL and LDL Levels

HDL and LDL levels determination was carried out using enzymatic colorimetric measurements. The animals underwent cardiac puncture to collect blood. Blood specimens were transferred into anticoagulant-free vials and allowed to stand for 30 min to clot. Afterwards, the vials were centrifuged at 300 × g for 10 min and the

resultant serum was used for further analysis of HDL and LDL. Serum lipid levels were colorimetrically measured by routine procedures, using commercial kits from Roche and measured using a spectrophotometer. All analyzes were performed according to the manufacturer's instructions in triplicate[12–14].

Histopathological Examination

Rats from each group was randomly selected and anesthetized with ketamine. Then liver samples were taken and stored in 4% PFA. Histopathology slides were stained manually with Hematoxylen-Eosin (HE) staining. The stages of HE staining are deparaffinization, rehydration, and staining. The first staining uses hematoxylen, ±10 minutes for the color penetration of the preparation. After that it was rinsed and put in alcohol eosin dye for 5 minutes. The preparations were then immersed in distilled water to remove excess eosin. Then dehydrated with ethanol series. Next, clearing was carried out, using xylol and air-dried, then mounting (gluing) was carried out with entellan[12,15]. Histopathological slides from staining were observed microscopically at 200x magnification using Nikon Eclipse 80i DS Fi1 light microscope. The results of the tissues photograph obtained were compared to each treatment group and analyzed descriptively.

Data Analysis

The research data were analyzed using the Shapiro-Wilk normality test and the Levene's homogeneity test. LDL data is normally distributed so that it was analyzed using One-way Anova comparative test and continued with the LSD Post Hoc test. For HDL results, abnormal data is obtained so that the analysis process is carried out using Kruskal-Wallis and Mann-Whitney tests.

RESULTS

Phytochemical Test Results of *I. reptans* Extract

Based on the results of phytochemical tests, several secondary metabolites were found in the 70% ethanol extract of *I. retpans*. Secondary compounds that were positively identified in this extract included alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, and glycosides. From the results of the phytochemical screening, it was found that the *I. reptans* extract was positive for the seven secondary compounds, and negative for the steroid content as shown in Table 1.

Tabel 1. Results of Phytochemical Screening Test of *I. reptans* Extract

No.	Secondary Compound	Results
1	Alkaloids	+
2	Saponins	+
3	Tannins	+
4	Phenolics	+
5	Flavonoids	+
6	Triterpenoids	+
7	Steroid	-
8	Glycosides	+

(+) : contained in the extract

Improvements in LDL and HDL Levels Hypercholesterolemi Animal Models Treated with *Ipomoea reptans Poir* Extract.

High cholesterol induction was carried out only in the chol group, IE200, IE300 and IE400 for 21 days. On the 22nd day, LDL and HDL measurements were carried out to ascertain whether the rats had hypercholesterolemia. The results showed that before administration of the extract, LDL levels increased significantly ($p<0.05$) in the chol, IE200, IE300 and IE400 groups respectively by 32.12 ± 1.83, 31.62 ± 1.22, 32.32 ± 2.17, and 30.28 ± 1.06 mg/dL compared to the normal group (N) with levels of 8.16 ± 0.59 mg/dL. While HDL levels were also significantly reduced ($p<0.05$) in the chol, IE200, IE300 and IE400 groups respectively, namely by 14.78 ± 1.49, 14.44 ± 1.41, 15.58 ± 1.54, and 13.44 ± 2.73 compared to the normal group 37.76 ± 1.86 mg/dL (Table 2).

After the rats experienced hypercholesterolemia, therapy with *I. reptans* extract was carried out for 14 days. Measurements of LDL and HDL levels were carried out in all groups at the end of the study. Rat blood serum samples were taken and tested for LDL and HDL levels using colorimetry according to each Kit. In LDL levels data, the results showed that post-induction of cholesterol with quail egg yolk and animal oil for 3 weeks caused LDL levels in the Chol group to increase significantly ($p<0.05$) post-induced with a mixture of cholesterol and animal oil with an average of 29.9 ± 1.05 mg/dL compared to the normal group (N) which is equal to 8.56 ± 1.36 mg/dL. Interesting results were obtained after being treated with *I. reptans* extract where, therapy with a dose of 200 mg/kg, IE200, did not cause a decrease in post-cholesterol induction LDL levels. Meanwhile, when treated with higher doses, namely doses of 300 mg/kg and 400 mg/kg, IE300 and IE400, LDL levels decreased significantly ($p <0.05$) compared to the Chol group (Figure 1), with an average LDL of 9.12 ± 0.72 mg/dL and 9.22 ± 0.77 mg/dL successively (Table 2).

For the results of HDL levels, the normal group which was not induced by cholesterol (N) had HDL levels of 38.1 ± 2.24 mg/dL. The decrease in total and LDL cholesterol levels was also followed by an increase in HDL levels (Figure 1), wherein the IE 200, IE 300 and IE 400 treatment groups had HDL levels that approached the normal group, namely 33.4 ± 5.91 mg/dL, 36.92 ± 0.47 mg/dL and 42.82 ± 3.27 mg/dL respectively. Statistically, this value increased significantly ($p<0.01$) compared to the HDL level in the chol group, which was 15.92 ± 4.39 mg/dL. Meanwhile, the normal group had HDL levels of 38.1 ± 2.24 mg/dL (Table 2).

Table 2. Average of LDL and HDL Levels of Hypercholesterolemia Rats Before and After Therapy

	LDL (mg/dL)		HDL (mg/dL)	
	Before therapy (22nd day)	**After 14 days therapy (35th day)**	**Before therapy (22nd day)**	**After 14 days therapy (35th day)**
N	8.16 ± 0.59[a]	8.56 ± 1.36[a]	37.76 ± 1.86[a]	38.1 ± 2.24[a]
Chol	32.12 ± 1.83[b]	29.9 ± 1.05[b]	14.78 ± 1.49[b]	15.92 ± 4.39[b]
IE200	31.62 ± 1.22[b]	25.22 ± 4.72[b]	14.44 ± 1.41[b]	33.4 ± 5.91[a]
IE300	32.32 ± 2.17[b]	9.12 ± 0.72[a]	15.58 ± 1.54[b]	36.92 ± 0.47 [a]
IE400	30.28 ± 1.06[b]	9.22 ± 0.77[a]	13.44 ± 2.73[b]	42.82 ± 3.27[a]

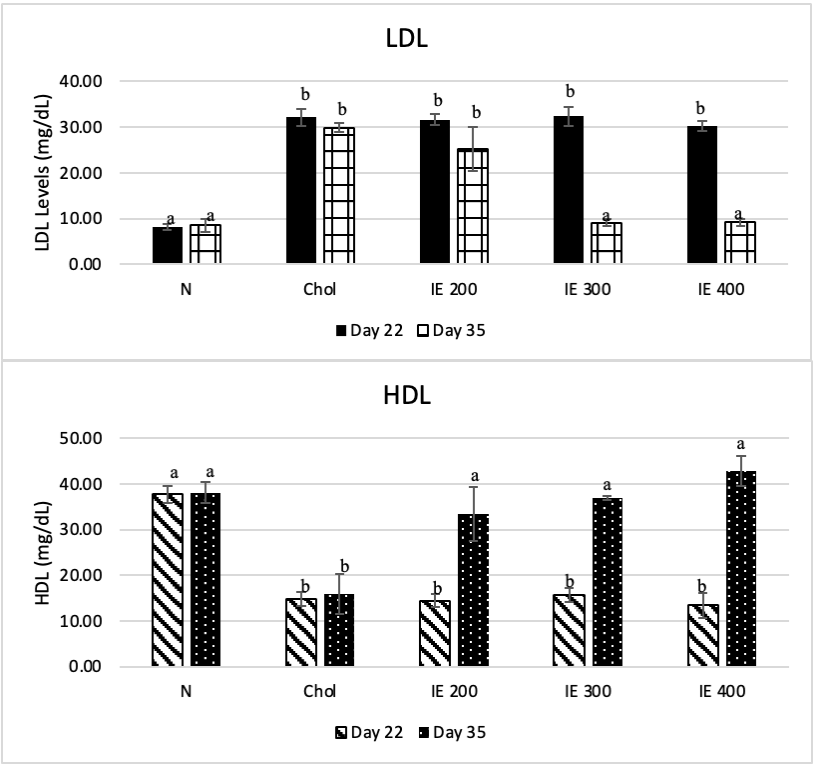

Figure 1. LDL and HDL Level Results between Before and After *I.reptans* extract Therapy

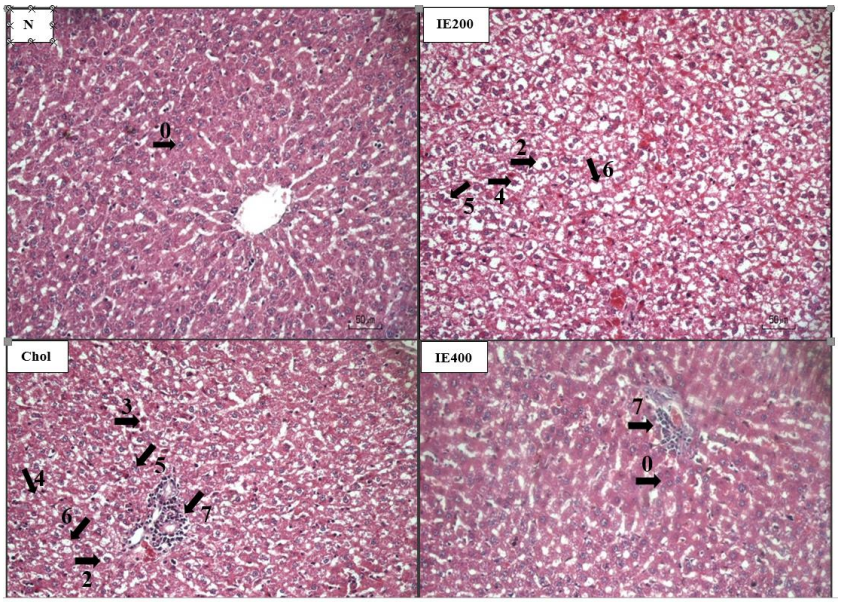

Figure 2. Histopathology of Rat Liver with Hematoxylin-Eosin Staining (200x Magnification).
Description: Normal group (N), Cholesterol group (Chol), the group treated with I. reptans dose of 200mg/kgBB (IE200) and 400 mg/kgBB (IE400); normal cells (0), microvesicular (1), ballooned cells (2), hyperchromatin cell nuclei (3), karyorrhexis (4), karyolysis (5), cells without contents (6), inflammatory cell infiltration lymphocytes (7).

Effects of *Ipomoea reptans Poir* Extract on Histopathology of Hypercholesterolemic Rat Livers

Based on the results of histopathological photos of the liver in the normal group (N) it was found that the liver cells were still normal, but with cholesterol-induced treatment in the Chol group with no therapy there was hepatocyte degeneration in the liver cells. Irregular and dilated sinusoids are seen, this is characterized by the occurrence of microvesicles, the formation of ballooned cells, and hyperchromatin cell nuclei. It can also be seen that some of the characteristics of cells experiencing necrosis include the breakdown of the pyknotic nucleus into many small basophilic particles (karyorexis), experiencing lysis (karyolysis). There is even lymphocytic infiltration of inflammatory cells. In the treatment group (IE200) it was seen that the liver cells still had fatty or degenerated hepatocytes, but there was no lymphocytic inflammatory infiltration. While the IE400 picture shows good changes, where fat degeneration is reduced, the number of normal cells appears to be more dominant and there is improvement in inflammation (Figure 2).

DISCUSSION

This study confirmed that continuous feeding of high-cholesterol diets increased LDL levels, lowered HDL levels, followed by fattening of rat liver tissue. There is an increase in LDL and a decrease in HDL in hypercholesterolemia, due to the accumulation of cholesterol in the blood due to the induction of hypercholesterolemia. Previous studies have stated that increased LDL levels and decreased HDL levels are due to excess cholesterol, which causes cholesterol build up in the body. Furthermore, cholesterol accumulation followed by free radical activity causes oxidative damage to several tissues. High cholesterol levels in the blood cause VLDL to form LDL, resulting in increased LDL in the blood. LDL levels that continue to increase make HDL depressed and cannot get rid of the excess cholesterol in the blood, so that HDL decreases[16]. This situation is in accordance with the results of this study, where there were significantly different increases in LDL levels and decreases in HDL levels in both the Chol group compared to the normal group. In previous studies, it was stated that hypercholesterolemia resulted in disturbances of lipoprotein metabolism, which included increased LDL levels and decreased HDL levels. Excess fat and cholesterol cause cylomicron to be converted into LDL by the enzyme lipoproteinlipase, this is in accordance with the statement of previous researchers who said that cholesterol binds to fat and protein and forms lipoproteins according to their respective compositions[16].

Treatment of *I.reptans* extract in hypercholesterolemic rats with three different doses of HDL levels showed a significantly different increase, but only at doses of 300 mg/kgBW and 400 mg/kgBW showed a significant difference in LDL levels compared to the Chol group. In previous studies, it was reported that *I.reptans* extract had inflammatory and antioxidant activity. Supported by previous research, the results of phytochemical screening that contained flavonoids in the form of quercetin, tannins, and saponins which function as antioxidants that can be used to lower cholesterol levels in the body[9]. These bioactive compounds can increase the synthesis of bile acids, also act as antioxidants and anti-inflammatories.

I. reptans extract therapy can increase bile acid secretion which will increase fat metabolism, as a result, excess fat will be excreted through the large intestine in the form of feces. Removed fat will lower cholesterol levels in the blood, LDL formation will also not be excessive. Antioxidant action in *I. reptans* extract serves to reduce the activity of LDL oxidation which occurs due to the accumulation of cholesterol in the blood. Antioxidants will increase HDL levels by increasing hepatic Apo A1 mRNA which plays a role in initiating Apo A1 synthesis, where Apo A1 is the main component of HDL. Apo A1 can also suppress LDL multiplication, so that LDL oxidation does not occur[17].

Inflammation that occurs due to cholesterol induction was also seen in the histopathology of the liver where the liver cells were seen to be fatty and cell infiltration occurred in the Chol group (Figure 1). Inflammatory cell infiltration is caused by the oxidation of LDL. Oxidation of LDL initiates an acute inflammatory process that causes vasodilation, so that monocytes in the blood enter through the gap between the endothelium and fat degeneration also occurs. This damage is caused by the induction of hypercholesterolemia which triggers free radicals and results in an inflammatory reaction and causes fattening of the hepatocyte cells[18]. Excessive cholesterol disrupts the metabolic process, so that cholesterol accumulates in the liver. Cholesterol that enters the liver cannot be transported entirely by lipoproteins to the liver from the bloodstream throughout the body[19]. This situation increases total cholesterol and LDL cholesterol levels, increased LDL will trigger free radicals and cause an inflammatory process.

I. reptans extract therapy which has a strong antioxidant content shows a good effect on the histopathological picture of the liver. It can be seen that there was no infiltration in the IE200 group but there was still fat. The best results were obtained when treated with *I.reptans* extract at a dose of 400 mg/kg where it was seen that there was a reduction in cells that were experiencing fat and a reduction in areas of inflammation. The presence of antioxidant action from *I.reptans* extract in the form of quercetin, tannins, and saponins, can inhibit LDL oxidation by reducing ROS and increase HDL by increasing Apoprotein A1, so that the inflammatory process and LDL oxidation are reduced resulting in improvements in the histopathology of the liver of hypercholesterolemic rats.

From histopathology results indicate that induction of cholesterol for 21 days with quail egg yolk and animal oil causes hypercholesterolemia in rats which causes histopathological damage to the rat's liver where there is fattening to fatty degeneration in hepatocyte cells, necrosis and inflammation (Figure 2). This results in line with previous research that high cholesterol leads to hepatocellular degeneration[18,20,21]. The irregular location of the sinusoids may also be caused by lipid peroxidation and oxidative stress which then increases the production of hydroxyneale (HNE) and malondialdehyde (MDA) which increases liver fibrosis through activation by stellate cells which causes an increase in the production of transforming growth factor-beta (TGFβ)[22–24]. Therapy

using *I.reptans* extract at a dose of 200 mg/kgBW was not enough to improve the fat degeneration that occurred, but at a dose of 400 mg/kgBW the fat degeneration could improve but was not optimal (there was still inflammatory infiltration of inflammatory cells). This may be due to the process or duration of therapy which is only carried out for 14 days, and a longer duration of therapy is needed to obtain more optimal results in further research.

CONCLUSION

Based on the results and discussion above, it can be concluded that *I.reptans* extract therapy at doses of 300 mg/kgBW and 400 mg/kgBW significantly reduced LDL levels and increased HDL levels in the blood. Improvement in liver histopathology as indicated by reduced inflammatory and fatty cells was obtained after therapy with *I.reptans* extract at a dose of 400 mg/kg BW only.

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Original Research Article

The Activity of Liposome-Parijoto Formula Through p53 Expression in HepG2 Cell Line

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Abstract

Parijoto, one of Melastomaceae family, has been known to have cytotoxic activity in HepG2, a hepatocellular cancer cell line, but with low activity. However, the ethyl acetate fraction of Parijoto gave the highest antioxidant and cytotoxic activity in 4T1. Then, purification and liposome formulation need to be carried out to increase the cytotoxic activity of Parijoto extract. Objective**:** This research aimed to study the cytotoxic activity and p53 gene expression of LEA (Liposom-Ethyl Acetate of Parijoto Fraction) in HepG2.

Method: Extraction has been done by maceration, followed by partition using n-hexane, ethyl acetate, and methanol. LEA formulation was carried out by thin-layer hydration with modification and the formula was sized using a bath sonicator. Cytotoxic activity test of LEA and extract was performed in five serial concentrations (3,9 µg/mL–250 µg/mL), while the positive control doxorubicin performed in 3,9 µg/mL – 250 µg/mL by MTT assay. P53 gene expression was analyzed by using PCR-electrophoresis.

Result: Results showed that LEA increased the cytotoxic activity (IC50 = 28.40 µg/ml). Furthermore, based on the electrophoresis study, LEA induced the p53 expression while the extract only did not.

Conclusion: Liposome formula from ethyl acetate fraction of Parijoto extract (LEA) was able to increase cytotoxic activity and p53 gene expression was possible through the apoptotic mechanism. This shows that this formula is a promising strategy to improve the bioavailability of herbal medicines as cytotoxic agents.

Keywords: *Liposome; Parijoto; p53; HepG2*
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INTRODUCTION

Hepatocellular carcinoma (HCC) is primary and mortality liver cancer that causes the second death in worldwide[1]. Pathogenesis of HCC is closely associated with chronic hepatitis that is caused by several factors such as infection with a virus (Hepatitis B or C), chemical exposure (Aflatoxin), alcoholic lifestyle, and other conditions (diabetic disease, obesity). Those factors will increase the risk of liver cirrhosis which will develop to be liver cancer[1]. Nowadays, treatment of HCC has been done by curative methods (orthotopic liver transplantation, surgical resection, and local destruction) and palliative methods (trans arterial chemo-embolization, systemic chemotherapy, interferon, and hormonotherapy). Those treatments still have limitations such as the impact on the long-term survival of patients who need the adjuvant after/before curative treatment[2]. Therefore, there is a need to look for new active ingredients and strategies to improve cancer treatment with low side effects.

A previous study demonstrated that Parijoto extract (Medinilla speciosa) is known to have low cytotoxic activity on the HepG2 cell line, which means it has low bioavailability[3]. Moreover, the ethyl acetate fraction from Parijoto extract showed the highest antioxidant activity that correlated with cytotoxic effect in 4T1 cell line[4].

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Hence, the extract bioavailability will be increased by purification and lipid-based encapsulation of ethyl acetate fraction to form liposome-ethyl acetate (LEA). The liposome is a vesicle enclosed by phospholipids which is an analog to the cell membrane. This vesicle can improve the bioavailability and solubility of water-insoluble drugs which is poorly-permeable. However, liposome is more promising to deliver hydrophobic drugs than peptide and protein drugs for oral drugs, especially for phenolic compounds[5]. Melastomaceae family has been known has several phenolic compounds, such as ellagitannin (Medinillin A, Medinillin B[6], ellagic acid[7]) and flavonoids which influence their activity as antioxidant and cytotoxic. Therefore, the active compounds will be able to be encapsulated into liposomes that are expected to increase their bioavailability.

A molecular study of HCC pathogenesis revealed that there is a genetic change in a signaling pathway which is mediated by p53, Ras/ERK, PI3K/AKT, and wnt/B-catenin. Another study on p53 showed that its role was in the regulation of the cell cycle, apoptosis, and genomic stability. However, p53 was mutated and plays a key role in the signaling pathway of PI3K/AKT, TGF-B and B-catenin to metastasis in HCC cells[8]. Hence, it is necessary to investigate the cytotoxic activity of liposome-Parijoto and its molecular mechanism through the p53 gene expression

MATERIALS AND METHOD

Extraction and purification

Extraction of Parijoto (Medinilla speciosa) fruit was carried out by three days of maceration using ethanol 70% and then evaporated using rotary evaporation. The macerate was separated using n-hexane, ethyl acetate, and methanol. Each fraction was evaporated and the ethyl acetate fraction was encapsulated to form the LEA.

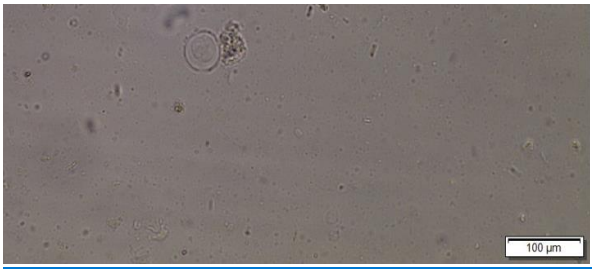

Figure 1. Optical microscope image of LEA formula (400x resolution)

LEA formulation and characterization

LEA was made using the thin-layer hydration method. Lipids were prepared by dissolving 1:1 b/b soya lecithin: cholesterol in chloroform, then evaporated using a dehydrator until the thin layer was performed. Hydration was carried out by mixing the ethyl acetate fraction solution in 1% methanol followed by water addition using a magnetic stirrer for 40 minutes. Then, sizing was done by bath sonicator for 10 minutes. The characterization of LEA was carried out by the microscopic study.

Cytotoxic activity

Cell lines ($1x10^5$ cells/well) were cultured into a 48-well plate and incubated overnight. The serial concentration of LEA samples was in the range of 3,9 µg/mL to 250 µg/mL and the positive control doxorubicin was 3,9 µg/mL – 250 µg/ml were put onto cells and incubated overnight. A 100 ul MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) on PBS (Phosphate Buffer Saline) 5% was put onto cell solution followed by incubation for 4 hours until the formazon formed. The reaction has been stopped by adding DMSO (Dimethyl sulfoxide) 10-20% in a protected light for 5 minutes and incubated. The absorbance was read using a microplate reader in 595 nm. The % of inhibition was calculated according to this formula:

((sample abs-medium abs))/(cell control abs - medium abs)) x 100%

The IC50 was calculated based on linear regression of log concentration vs %inhibition.

P53 gene expression study

Cell lines were cultured in 48-cell wells and incubated overnight. The IC50 concentration of LEA was put into the well and incubated overnight. RNA isolation was carried out according to the Invitrogen™ TRIzol™ Plus RNA Purification Kit (Roche, Swiss) protocol for the cells. cDNA synthesis was done using Trancriptor High Fidelity cDNA Synthesis Kit (Roche, Swiss). PCR reaction was used according to the FastStart High Fidelity PCR System Kit (Roche, Swiss) condition (Pre-denaturation 95°C, 1 minute; Denaturation 95°C, 15 seconds; Annealing 58°C, 15 second; Elongation 72°C, 30 second; 35 cycles). The primer used in this research can be seen in Supplementary File 1. The visualization was done by using electrophoresis in 2% agarose

RESULTS

LEA characterization

LEA formula showed Giant Unilamellar Vesicles (GUVs) (Figure 1) with one compartment/lamellarity after sizing with a bath sonicator. Furthermore, the average particle sizes ranged between 3,73 to 16,64 µm.

Cytotoxic activity

Results obtained the LEA formula has a potent cytotoxic activity when compared with the extract. Moreover, it gave a lower concentration to inhibit cell proliferation in HepG2 from IC50 value (Table 1). However, this finding suggested that the cytotoxic activity in the HepG2 cell line was greatly improved by lipid encapsulated in liposome formula

p53 gene expression

p53 gene expression could be seen in Figure 2. Our results showed that the LEA formula induced the p53 gene expression while the extract did not. This finding demonstrated that the LEA formula influenced cell proliferation by inducing p53 gene expression which influenced the apoptotic program.

Table 1. The IC50 value of the samples in HepG2

	Doxorubicin	LEA	Extract	Doxorubicin
IC50 value (µg/mL)	0,23	28,4	>250*	11,12

* The extract did not show 50% inhibition of HepG2 until 250 µg/mL[3].

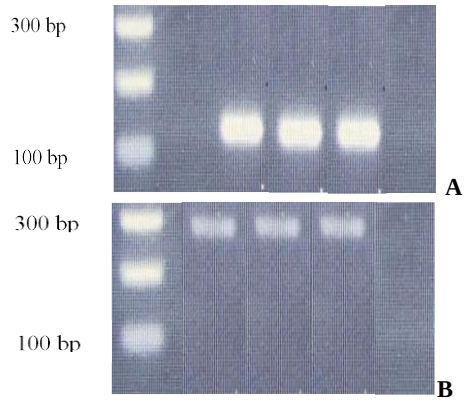

Figure 2. The expression of p53 in HepG2. A.GADPH (150 bp). B.p53 (300 bp). left to right: untreated cell, positive control (Doxorubicin), LEA; extract. Treated cell was induction accroding to IC50 value of LEA. There was no expression of GADH and p53 when the cell treatment to 28,4 ug/ml extract.

DISCUSSION

Mutations in the p53 gene cause cell malignancy and unrestricted DNA replication, resulting in uncontrolled cell proliferation and cancerous tumors. p53 activity is impaired by multiple mechanisms in HCC, hence contributing to HCC genesis. The HCC-inducing extrinsic factors which etiologically associated with p53 are AFB1 (Aflatoxin B1), vinyl Chloride, NAFLD (Non-Alcoholic Fatty Liver Disease), Iron, HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus) infection[9]. The results of our study showed that the IC50 of the LEA formula increased sixty times higher than the extract. It indicated that the fractionation and encapsulation of the LEA formula could increase the bioavailability of the extracts. Similar results were shown in the research of Mabrok, 2002 which states that Apigenin (flavonoid from Apium graveolens) that is encapsulated into chitosan and albumin-folic acid can improve its hydrophobicity, stability, and bioavailability to target the cancer cells. The treated HePG-2 cells with Ap-CH-BSA-FANPs demonstrated the induction of apoptosis by increasing p53 gene expression, arresting the cell cycle, increasing caspase-9 levels, and decreasing both the MMP9 gene and Bcl-2 protein expression levels[10].

Parijoto, the Melastomaceae family, contains flavonoids[11] and some ellagitannin[7]. Furthermore, these flavonoids and ellagitannins can exert cytotoxic activity in various cancer cell lines. Granado-Serrano et al., in 2006 reported quercetin-induced apoptosis in the HepG2 cell line and evaluated the modulation and expression of Bcl-x and Bax. Bcl-xL has been identified as a caspase substrate and the product of Bcl-xL cleavage, Bcl-xS, has a pro-apoptotic function[12]. Moreover, hydroxygenkwanin along with kaempferol showed both cytotoxic and antioxidative potential against HepG2 cell lines[13]

Yohida et, al, reported that Medinilla magnifica, which has the same genus as Medinilla speciosa, was composed by medinillin A and B, the ellagitannin compounds. These compounds give cytotoxic activity through a p53-dependent pathway. Punicalagin, one of ellagitannin from Punica granatum, has been shown to impact the proapoptotic protein such as Bax, caspace 3 and 9, and the tumor suppressor p53 in human cervical cancer cell lines[14]. Furthermore, the other ellagitannin such as 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (PGG), showed its antiproliferative activity in humanSK-HEP-1 hepatocellular carcinoma cells which caused by the suppression of NF-kB activation and G0/G1-phase arrest via an IκB-mediated mechanism. Moreover, PGG was reported to induce atypical senescence-like S-phase arrest in HepG2 and Huh-7 human hepatocarcinoma cells. Furthermore, it also induced the senescence-associated β-galactosidase activity, inhibited proliferative capacity, and influenced the autophagy process by activating the MAPK8/9/10 on two model studies (in vitro and in vivo) of human HepG2 liver cancer[15].

CONCLUSION

The fractionation and lipid-based encapsulated formula influenced the increasing cytotoxic activity of Parijoto extract. The induced p53 expression was a key role in the apoptotic program. This formula was a promising strategy to improve the bioavailability of herbal extracts as cytotoxic agents.

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Original Research Article

BALB/c Mice as Animal Model in Dengue Infection Research: Role of Endothelial Activation

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Abstract

Introduction. There were various challenges in using experimental animals model for dengue infection studies aside from the fact that dengue infection only naturally affects humans and does not manifest clinical signs as in humans. Various experimental animals have been used in dengue research, but the mouse model is more widely used since it is easier to obtain although sometimes they do not show clinical symptoms but may still measure the immune response. BALB/c mice are immunocompetent mice that have the potential to be used in dengue infection research. Endothelial cell activation plays a role in the pathogenesis of dengue virus infection which contributes to plasma leakage. One of the biomarkers of endothelial cell activation is soluble intercellular adhesion molecule 1 (sICAM-1).

Method. An analytic observational study was conducted using BALB/c mice aged 8 weeks and weighed 40 grams. Selected BALB/c mice were randomly assigned to serotype 2 dengue virus containing 2.1 x 10^6 pfu/ml intraperitoneally, given only once. A total of 11 mice were injected with dengue virus serotype 2 and 11 mice were not injected with dengue virus. On the second day of virus injection, non structural (NS) 1 antigen dengue examination was carried out to prove that the BALB/c mice were indeed infected with dengue virus. In BALB/c mice that were proven to be infected with dengue virus, sICAM-1 levels were examined in serum after 7 days of infection. Mice that were not injected with dengue virus were also examined for sICAM-1.

Results. All of BALB/c mice injected with dengue virus were proven to be infected, as indicated by the detection of NS1 antigen in their serum. The mean serum level of NS1 antigen was 88.35 ng/ml (mean 95.34 ng/ml and standard deviation 21.94). The level of sICAM-1 in BALB/c mice infected with dengue virus (mean = 1.34) was significantly higher than mice that were not infected (mean = 0.79), with a p-value 0.045

Conclusions. BALB/c mice were proven to be infected with dengue virus by detecting NS1 dengue virus antigen in the serum. The sICAM-1 levels in the group of BALB/c mice infected with dengue serotype 2 were significantly higher than the BALB/c mice that were not infected with dengue virus.

Keywords: *BALB/c mice; NS1 antigen dengue; sICAM-1*
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INTRODUCTION

Dengue virus infection is still a major public health concern in many countries, particularly in tropical areas like Indonesia.

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Dengue infection is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the West Pacific. In the last few decades, the incidence of dengue virus infection has dramatically increased, leading to significant morbidity and placing a socioeconomic burden on society. The clinical spectrum of dengue infection varies significantly from asymptomatic, undifferentiated fever, dengue fever, dengue hemorrhagic fever, dengue shock syndrome (DSS), and atypical manifestation (unusual manifestation/ expanded dengue syndrome). The pathological process that occurs is plasma leakage from the intravascular and the presence of hemorrhage.[1,2]

Manifestations of intravascular plasma leakage are strongly associated with changes in vascular function including the role of endothelium and other supports such as perivascular muscle cells, extracellular matrix, basement membrane, and glycocalyx. Dengue infection activates endothelial cells although the morphological changes are relatively minimal. Several biomarkers indicating endothelial cell activation were found to increase during dengue infection such as soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule (sVCAM-1).[3] Endothelial cells infected with the dengue virus were found in autopsy samples of Dengue Shock Syndrome (DSS) patients as well as in animal study models. it is suspected that these endothelial cells directly contribute to pathogenesis through increased viremia, cytokine secretion, complement modulation, and serving as targets of humoral and cellular immune responses. Dengue virus and TNFα together stimulate endothelial cell apoptosis through the production of reactive oxygen species (ROS). Through the role of vascular endothelial cells in the pathogenesis of dengue infection, a therapeutic approach by improving the vascular barrier through endothelial cells that can reduce plasma permeation is an option.[4]

The use of experimental animals in dengue infection research has faced many challenges, due to dengue infection does not naturally infect non-human species. Various animals have been tested in dengue research in terms of pathogenesis research, anti-viral, and development of dengue vaccine. Mice are the animal species most frequently used in dengue trials. Immunocompetent mice are naturally resistant to dengue virus but infection can occur with high levels of virus inoculation (10^6-10^8 pfu/ml). Previous study showed after administration of dengue virus serotype 2 as much as 10^8 pfu/ml intravenously to C57BL/6 mice, virus content in serum, spleen, liver, and brain can be detected which is marked by the spread of the virus and viral replication in various tissues.[5,6] Immunocompetent mice were also infected with dengue virus serotype 2 with a titer of 10^8 pfu/ml intravenously. Viremia was detected by RT-PCR on the second day after the virus injection.[7] Experimental studies using BALB/c mice that were injected intraperitoneally with dengue virus serotype 2 resulted in liver damage characterized by a progressive increase in SGOT and SGPT that peaked on the seventh day. Furthermore, dengue virus antigen was detected in hepatocyte cells by RT-PCR. BALB/c mice are susceptible to serotype 2 dengue virus infection, there are also structural and histological changes in the lungs of infected mice.[8,9] On the second to the eleventh day since infection with dengue virus serotype 2, BALB/c mice developed viremia. Dengue virus antigen was detected in the hepatocyte and the capillary endothelium of the central lobular vein area.[10]

OBJECTIVE:

This study aimed to support that BALB/c mice can be used as animal models in dengue virus infection research and to prove the existence of endothelial cell dysfunction in BALB/c mice infected with dengue virus serotype 2

MATERIALS AND METHODS

The research design was analytic experimental using BALB/c mice. Male BALB/c mice were reared until they were 8 weeks old and weighed 40 grams Mice were kept in a comfortable environment with 12 hours cycles of light and darkness and a room temperature of 25°–30°C. The cage was 30 x 40 cm in size with a 20 cm height, and there were 4 to 6 mice in each cage. Mice were fed daily with poultry feed with an amount of approximately 10-15 grams, or as much as the mice could consume. Then the randomization of the BALB/c mice was carried out to be used as a sample. At the end of the study, mice were euthanized as accepted in animal ethics. The mice were anesthetized with combination of ketamin, zylasine, and acetopromasin (mouse cocktail). Mouse cocktail were injected intramuscularly into the thigh muscle as much as 0.02 cc (200 microliters). Mice were euthanized by dislocating their neck bones then freezing them before burning them in an incinerator. Dengue virus serotype 2 isolates were obtained from the serum of dengue patients from the Denpasar site, AFIRE study by Ina Respond (Indonesia Research Partnership on Infectious Diseases). Isolate production was carried out at the Ministry of Health's Research and Development Laboratory, Jakarta. BALB/c mice were randomized, 11 mice were injected with dengue virus serotype 2 and 11 mice were not injected with dengue virus. In the selected BALB/c mice, dengue virus serotype 2 with a viral titer of 2.1 x 10^6 pfu/ml was administered intraperitoneally, given only once. On the second day of virus injection, NS1 dengue examination was carried out to prove that the BALB/c mice were indeed infected with dengue virus. Serum sICAM-1 levels by ELISA method were evaluated on the seventh day after infection using ELISA method in both groups. ELISA method product by BIOENZY catalogue number BZ-08188140-EB. Standard curve range 0.05ng/ml – 20ng/ml with sensitivity 0.026ng/ml.

RESULTS

All mice injected with dengue virus serotype 2 proved to be infected as indicated by the detection of NS1 dengue in the mice serum with varying levels as shown in the table 1:

Figure 1. BALB/c mice were injected with dengue virus serotype 2

Figure 2. Serum specimens were collected from BALB/c mice

Table 1. NS1 dengue level in the mice serum

Subject	NS1 **dengue level (ng/ml)**	Subject	NS1 **dengue level (ng/ml)**
1	96,932	**7**	98,864
2	77,121	**8**	101,780
3	97,538	**9**	58,438
4	95,985	**10**	99,735
5	102,159	**11**	96,439
6	94,621		

The mean serum NS1 dengue level was 88.35 ng/ml (median 95.34 ng/ml and standard deviation 21.94). There were no clinical symptoms in all mice that infected with dengue virus serotype 2.

The sICAM-1 levels in mice dengue infected was higher than the sICAM-1 levels in non-infected mice. The serum sICAM-1 levels in the two groups (the group infected with dengue virus and those not infected) appeared different as shown in the following graph:

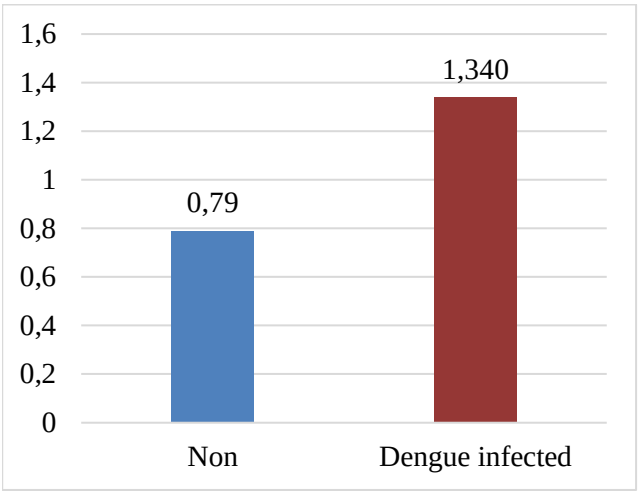

Figure 3. Graph of the serum sICAM-1 levels in mice non-dengue infected and mice dengue infected

DISCUSSION

Various animal models have been tested in research on dengue virus infection both in terms of pathogenesis, host immune response, development of dengue vaccines and anti-virus, but none of animal models have demonstrated clinical dengue symptoms as in humans.[6] Animal models that have been used in research on dengue infection include mouse models, human primate models and shrew models. Each animal model has its own advantages and limitations, such as level of viremia or viral replication, clinical manifestations and immune response, so the selection of an animal model depends on the purpose of the dengue infection research.[5,11] In recent years, studies on dengue infection have increasingly used mouse models because they are easier to obtain and can measure the immune response, however not all dengue-infected mice show clinical manifestations. Mice were infected with dengue by intraperitoneal, subcutaneous, intradermal or intravenous administration of dengue virus with an infective dose of 10^6 to 10^8.[5,6] The mouse model used could be immunocompetent mice or immunodeficient mice.[12,13]

BALB/c mice are immunocompetent mice which are also used in dengue virus research because they are proven to stimulate an immune response. [12,13] Various studies have shown that BALB/c mice are the most susceptible to dengue virus serotype 2 infection so that they can be used as a model in dengue research, especially in pathogenesis. In the study of dengue virus infection in BALB/c mice, viremia was found on the second to eleventh day, in addition to dengue virus antigens being detected in hepatocytes and capillary endothelial cells in the central lobular vein area.[10] When dengue virus was administered to mice, virus detection in serum, spleen, liver and brain indicated virus dissemination and virus replication in various tissues.[5,6] When dengue virus serotype 2 was intraperitoneally injected into BALB/c mice, viremia and liver damage

were found, which were marked by progressive increases in SGOT and SGPT. In addition, dengue virus antigen was found on hepatocyte cells, Kupffer cells, and hepatocyte cells, indicating the presence of viral replication in these cells. Liver damage in the form of injury to hepatocyte cells, steatosis, edema and necrosis. This liver damage can be seen histologically, liver lesions appeared within 2 days after infection and peaked on day 7 of infection.[8,9,14] In BALB/c mice infected with dengue through intraperitoneal injection of serotype 2, the level of viremia was very low and there were no clinical manifestations but there was injury to the liver in the form of increased SGOT and SGPT and the presence of viral antigens histopathologically.[8] Liver damage in BALB/c mice infected with dengue virus was also suspected due to the role of the immune response as evidenced by the increased expression of pro-inflammatory cytokines early in infection followed by with an anti-inflammatory response.[15] Another study with BALB/c mice injected with dengue virus serotype 4 intravenously showed histopathological changes similar to those in humans and dengue virus was detected in the liver, heart, lung, and serum.[16] In this study all BALB/c mice that had Intraperitoneal injection of dengue virus serotype 2 were detected NS1 dengue antigen on the mice serum on the second day after injection of the virus. The findings of this study support the theory of BALB/c mice as an experimental animal model for research on dengue virus infection.

Endothelial cell activation plays an important role in the pathogenesis of dengue infection which triggers an inflammatory response to infection. In a study conducted by Cardier et al, it was proven that in the acute phase there was activation of microvascular endothelial cells as indicated by an increase in ICAM-1. Endothelial cell activation is mediated by TNFα released by infected monocyte cells. This study also proved that dengue infection stimulates the apoptosis of microvascular endothelial cells. [17] Intercellular Adhesion Molecule-1 is part of the immunoglobulin consisting of transmembrane (ICAM-1) and soluble protein (sICAM-1), expressed by various cells such as leukocytes, hepatocyte cells and endothelial cells in response to inflammation. In a case control study conducted by Conroy, sICAM-1 levels were higher in dengue infection than healthy controls and higher in DHF and DSS than in dengue without complications. This study describes the role of inflammation and endothelial cell activation in the pathogenesis of dengue infection.[18] ICAM-1 levels in the serum of dengue patients are dynamic according to the course of dengue infection where the highest levels are found in the critical phase.[19]

The level of sICAM-1 as a sign of endothelial activation was higher in the group of BALB/c mice infected with dengue when compared to the BALB/c mice that were not infected with dengue. This finding is evidence that dengue virus infection increases endothelial cell activation. Endothelial cell activation plays a role in the pathogenesis of dengue infection and sICAM-1 is a marker of endothelial cell activation. Excessive endothelial cell activation causes an increase in plasma permeation through activation of NF-kB in addition that endothelial activation also causes an increase in the production of pro-inflammatory cytokines. Increased levels of sICAM-1 can be used as a marker to predict the progression of infection to a more severe disease.[20] MIF (Macrophage Migration Inhibitory factor) and TM (thrombomodulin) concentrations increase in plasma of dengue hemorrhagic fever patients and the potential effect of MIF on coagulation through TM and ICAM -1 in endothelial cells and monocyte cells. Severe dengue infection stimulates the expression of MIF which then stimulates endothelial cells and monocyte cells to express TM and ICAM-1.[21]

Activation of vascular endothelial cells in the group of mice infected with dengue virus was higher in mice without infection, this can be proven by the higher levels of sICAM-1 in BALB/c mice with dengue infection Endothelial cell activation plays an important role in the pathogenesis of dengue infection which triggers an inflammatory response. Endothelial cell activation is mediated by TNFα which is released by infected monocyte cells.[15] Excessive activation of endothelial cells through NF-kB activation, further increases the production of various cytokines which causes increased capillary permeability. Moreover, the dengue virus is also said to directly infect endothelial cells and cause apoptosis and endothelial cell dysfunction which contributes to plasma vascular leakage.[22] We need the next research to reduce endothelial activation in dengue viral infection.

CONCLUSIONS

BALB/c mice were proven to be infected with dengue virus by detecting NS1 dengue virus antigen in the serum. The level of sICAM-1 as a marker of endothelial activation in the group of dengue-infected BALB/c mice was significantly higher when compared to BALB/c mice that were not infected with dengue.

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Original Research Article

Effect Of Zinc Administration in Preventing Ethambutol-Induced Optic Neuropathy in Wistar Rats Model

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Abstract

Background: Ethambutol-induced optic neuropathy (E-TON) is one of the most common optic neuropathies, especially in developing countries with an increasing rate of tuberculosis (TB). Various outcomes of visual impairment that may lead to permanent visual loss makes it imperative to prescribe supplementation to prevent E-TON. Zinc supplementation is suggested as one of many to prevent E-TON since ethambutol is a zinc chelator.

Purpose: To analyze the apoptosis rate of retinal ganglion cell (RGC) in Wistar rats given ethambutol and zinc supplementation.

Methods: A true experimental research, post-test only control group design using wistar rats was done. Subjects were divided into 2 groups, treatment group was given ethambutol and zinc and control group was given ethambutol only for 30 days. Assessment of apoptosis rate from hematoxylin eosin staining specimen was observed by two pathologist using histopathological scoring. Differences between two groups were statistically analyzed using Mann Whitney U test, (significant $p < 0.05$).

Results: The score of apoptosis rate in treatment group was 28.6% and in control group was 71.4%. There was a significantly different in apoptosis retinal ganglion cell between two groups ($p = 0.015$).

Conclusion: The rate of retinal ganglion cell apoptosis was lower in rats treated ethambutol with zinc than ethambutol only.

Keywords: *E-TON; zinc; retinal ganglion cell*
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INTRODUCTION

Ethambutol-induced optic neuropathy (E-TON) is one of the most common and recognized drug-induced optic neuropathies, especially in developing countries with increasing rate of tuberculosis (TB). Treatment for TB is divided into two phase, intensive and continuation phase. Ethambutol is one of the medications used in the intensive phase.[1,2]

According to World Health Organization (WHO), the incidence of TB in 2016 is 10.4 million (CI 8.8-12 million) or equivalent to 120 cases in 100.000 population. The incidence of ethambutol-induced optic neuropathy has been reported to range from 1.0% to 22%. The reported incidence of E-TON varies between 18% in patients receiving more than 35 mg/kg per day, 5% to 6% with 25 mg/kg per day, and less than 1% with 15 mg/kg per day of ethambutol. No 'safe dose' of ethambutol has been reported, 0.3% developed E-TON with dose of < 15 mg/kg/day.[3–5]

Reports of E-TON in Indonesia were varied in each area. Indrayani et al recorded 5 of 30 patients (16.7%) with TB on ethambutol developed E-TON in Sanglah Hospital Bali. Juzmi et al reported 3 of 119 patients developed E-TON in Wahidin Sudirohusodo Hospital Makasar.

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Table 1. RGC apoptosis between treatment group and control group

Group	Microscopic Apoptosis			Total	p
	No apoptosis	< 25%	25 – 50%		
Treatment	28.6%	71.4%	0%	100%	0.015*
Control	0%	42.9%	457.1%	100%	
Total	14.3%	57.1%	28.6%	100%	

Description: * significant if *p value* < 0,05

Munandar from Cicendo Hospital Bandung, reported 33 patients developed E-TON with various visual disturbances. Based on these datas we can conclude that the incidence is low, but because of permanent visual impairment impact of E-TON, it not only need a health concern but also an economic and social problems.[6–8]

The toxic effect of ethambutol has been recognized as an apoptosis of retinal ganglion cell (RGC) in the exitotoxic glutamate pathway due to zinc chelator in the forming of cytoplasmic vacuole, protein kinase C (PKC) activation, and increase of malondialdehyde (MDA) which results in decrease of endogen antioxidants superoxide dismutase (SOD). Ethambutol binds to cuprum (Cu) and zinc (Zn) in the RGC and optic nerve fibers. Ethylene diimino dibutanol acid is an ethambutol metabolite that strongly binds to Cu and Zn which inhibits the electron transport chain and disrupted oxidative phosphorylation secondary to decreased available copper in human mitochondria or inhibit lysosomal activation due to the chelation of Zn. [4,9–14]

The exact mechanism of this ocular neurotoxic effect proved by in vivo and in vitro in rodent's animal studies have demonstrated ethambutol toxicity in the retinal ganglion neurons of rodents. One of the principal theories for its toxicity has been the zinc-chelating effect of ethambutol and its metabolite. Many postulated biochemical pathways that mediate the toxic damage include downstream effector caspase- 3 and caspase-6,8 and an excitotoxic glutamate pathway.[9,15]

The best management for E-TON is to cease the use of ethambutol.[5-7] Previous studies reports Zn therapy in E-TON results in good prognosis. Zn therapy in E-TON may prevent visual field impairment, improve contrast, and prevent progression. These studies reported the effect of Zn after E-TON has developed, but none have suggested supplementary Zn in patients receiving ethambutol before E-TON occurs. Our goal is to research this process.[3,16–18]

There is currently no effective treatment for E-TON. However, if detected early and with prompt discontinuation of ethambutol, between 30 and 64% of patients show some improvement in their visual disturbances over a period of several months.[5,19]

MATERIALS AND METHODS

This study was experimental study with post-test only randomized controlled group design using Wistar rats that given ethambutol. Subjects were divided into 2 groups, treatment group was given ethambutol 32 mg/200 gr rat's body weight (BW), equivalent to 25mg/kg human BW and Zn 1.5mg/200 gram rat's BW equivalent to 80 mg in human, and control group was given ethambutol only. Both groups were treated for 30 days. Doses have been converted in accordance to Laurence and Bacharach. This study included 14 Wistar rats, male, age 2-3 months, 200-300 gram of weight. Rats with eye infection and rats that showed no activity within treatment period were excluded.

Rats were acclimatized for 14 days and then divided into 2 groups. After 30 days of treatment period, rats were enucleated, and histopathology examination with hematoxylin eosin was prepared and interpreted by two pathologists. Retinal ganglion cell apoptosis was determined by measuring cell morphology and cell count based on score classification as follows; score 0: no apoptosis, score 1: <25% cell apoptosis, score 2: 25-50% cell apoptosis, score 3: 50-75% cell apoptosis, score 4: >75% cell apoptosis.[20] Differences between two groups were statistically analysed using Mann Whitney U test, (significant p <0.05).

RESULTS

Seven eyes from seven rats of each group were obtained. No rats died or dropped out during treatment period. RGC apoptosis reliability results were examined by two anatomical pathologist using Cronbach's Alpha and had a 84% Kappa (K), that's means the best inter-rater agreement. (Figure. 1)

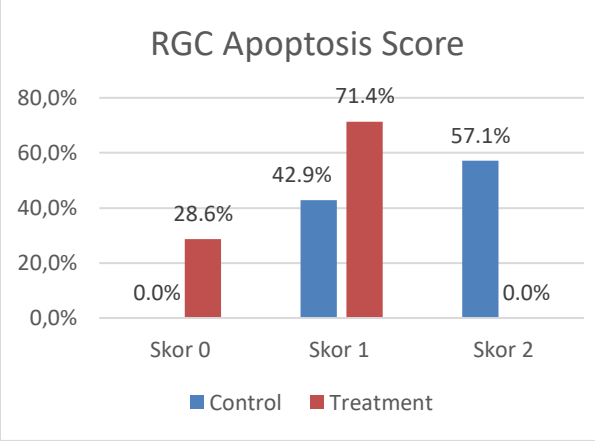

Figure 1. RGC Apoptosis between treatment group and control group

RGC apoptosis in the control group showed none in score 0, 42.9% in score 1, and 57.1% in score 2. Treatment group had 28.6% in score 0, 71.4% in score 1, and nil in score 2. RGC apoptosis between treatment group and control group showed statistical significance (p<0.05), in which RGC apoptosis was lower in the treatment group compared to control group (table 1).

Histopathology examination of RGC with HE staining compared in treatment group. Figure 2a represents normal RGC (score 0) with normal, homogenous cells, without apoptotic body. Figure 2b shows wrinkled/smaller, disparate, along with <25% karyorhexis and apoptotic body within 5 high power field.

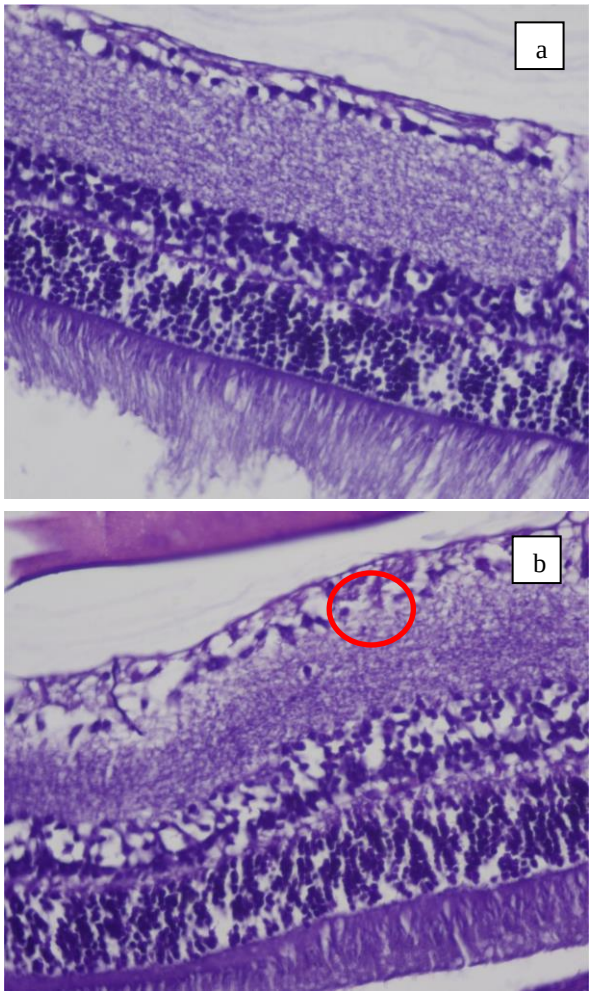

Figure 2. RGC in treatment group, (a). without apoptosis /score 0, (b). apoptosis < 25% / score 1

Figure 3 represents histopathology examination of RGC with HE in the control group. Figure 3a shows wrinkled, smaller, disparate RGC, along <25% of karyorhexis and apoptotic body within 5 high power field. Figure 3b depicts 25-50% / score 2 and show cell loss.

DISCUSSION

This study presents statistically significant differences in RGC apoptosis between the treatment group and control group (p = 0.015). RGC apoptosis in treatment group is lower compared to control group. Forty-two-point nine percent of treatment group had apoptotic score 1 and 57.1% had apoptotic score 2.

Based on the theory as a metal chelator, ethambutol interferes with oxidative phosphorylase dan inhibits lysosomal activation due to Zn chelators, resulting in decreased mitochondrial function by interfering with complex I (Fe/Zn) and complex IV (Cu). Other studies suggest the damaging effects caused by ethambutol are due to formation of cytoplasmic vacuoles in RGC which in turn will decrease intracellular Zn. [9–14]

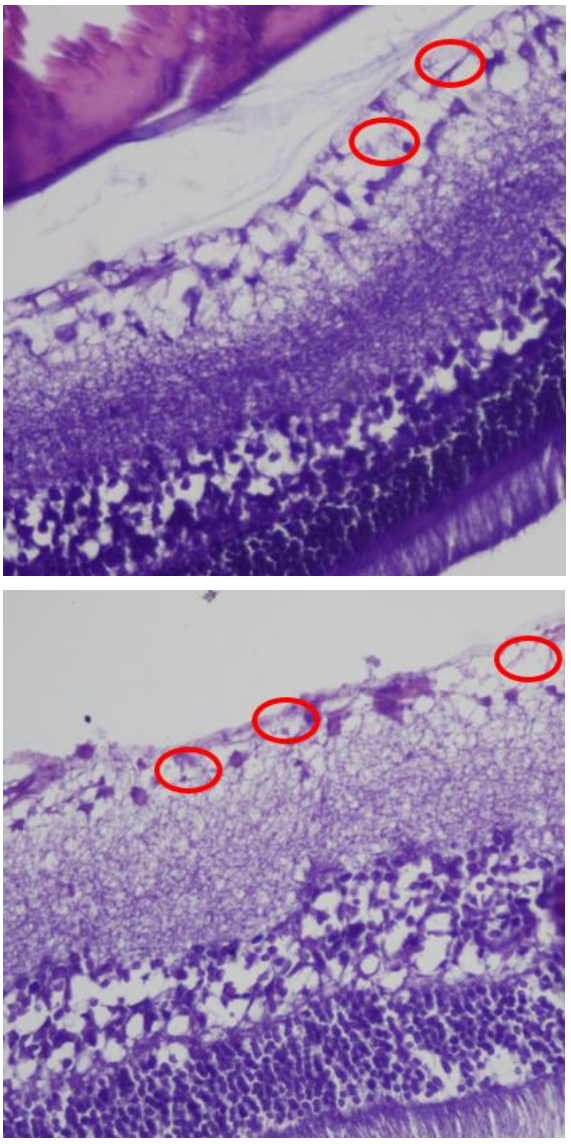

Figure 3. RGC control group, (a) apoptosis <25 % /score 1, (b) apoptosis 25-50% /score 2

Reported cases of E-TON in the population is between 0.1-22%. Based on the dosing that is equivalent to this study (25mg/kgBW), the incidence of E-TON is reported to be 5-6%. However, 100% of rats receiving ethambutol in this study suffered mild-moderate RGC apoptosis. Subclinical E-TON may progress in the early stages in humans. Although incidence of E-TON is reported to be very low, subclinical alterations can be detrimental. Studies evaluating visual evoked potential (VEP) and optical coherence tomography (OCT) reports increased VEP latency, decrease in retinal nerve fiber layer (RNFL) and ganglion cell inner plexiform layer (GCL-IPL) thickness upon follow-up despite no complaints or visual deterioration. Mandal et al. reported the incidence of symptomatic E-TON with a dose of 15-20 mg/kg/day is < 2%. However, 46% had increased VEP latency and decrease in RNFL and GCIPL thickness in asymptomatic patients. This is consistent with a study by Yan Sheng et al that suggest RGC damage after ethambutol treatment although no visual disturbance was

present. Regrettably, functional examination cannot be performed in E-TON rat models.[21,22]

This study reports no apoptosis amongst 28% of wistar rats that received ethambutol and Zn, mild apoptosis (<25%) in 71.4% rats. This signifies the effect of Zn in reducing the incidence of RGC apoptosis. A study by Hasan suggested declining Zn serum in TB patients under ethambutol treatment may return to its normal limits by cessation of ethambutol and consumption of healthy foods. This is due to ethylene diimino dibutanol which is an ethambutol metabolite that strongly binds to RGC cells. Free ZN (Zn^{2+}) is required as a cofactor for cytochrome c oxidase, the main enzyme for transport chain and cellular oxidase metabolism in the mitochondria. Furthermore, Zn^{2+} serves as a disregulator for RGC and glial cells in the events of nerve injury. Binding of Zn^{2+} will cause disruption of ATP production and ultimately damage the ATP-dependent axonal transport system. [10,22,23]

This study still reports apoptosis in groups receiving ethambutol and Zn (treatment group) though to a lesser degree. This is due to apoptosis occurring in normal cells to maintain hemostasis. Typically, cells will carry out a regeneration process along with cell death called apoptosis.

Zinc supplementation may ameliorate declining Zn serum caused by this mechanism. Daniati suggested Zn supplementation in E-TON patients may prevent visual field abnormalities. Similarly, Rindawati reported a significant difference in contrast sensitivity between patients with and without ethambutol. A study by Yulianti et al shows that Zn supplementation serves as a neuroprotector against toxic effects of ethambutol upon VEP examination. Another study by Fitirah presented decrease of RNFL thickness in the temporal quadrant but none in the nasal, superior, and inferior quadrant that measured using spectral domain optical coherence tomography (SD-OCT).[16–18,24]

Yudapratiwi reported no significance in RGC density between treatment and control groups in rats given ethambutol, 75.5% and 76% respectively. However, histopathology findings presents an orderly arranged cell structure amongst groups given Zn supplementation. A difference in research results is due to interpretation readings where 1 field of view with 400x magnification was performed while our study examined 5 field of view which represents a broad view of RGC. Both Yudapratiwi and our study used the same dose, equivalent to 25mg/kgBW in humans for 30 days.[25]

Zn supplementation in TB patients provides many benefits, not only does it improve RGC structure, boosts the immune system, but also accelerates the conversion to negative sputum after 2 months of Zn supplementation. Given the many advantages of Zn supplementation, it is favourable to prescribe it as a prevention to E-TON, specifically subclinical E-TON.[24-26]

Limitation of this study was indistinction between ethambutol related apoptosis or normal apoptosis that occurs at the cellular level.

CONCLUSION

Analysis of both groups presents lower RGC apoptosis in rats given ethambutol and Zn. Further research is necessary to evaluate mediating and confounding factors that may influence RGC apoptosis. Additional studies on humans in association with internists to provide a larger sample is essential. The authors expect Zn to be a obligatory supplement in patients receiving ethambutol.

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The authors do not have a proprietary interest in any of the materials used in this study. This research was approved by the Health Research Ethics Commission, Faculty of Medicine, Universitas Diponegoro according to recommendations of the Association for Research and Vision in Ophthalmology (ARVO).

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Case Report

Plasma Exchange as A Rescue Therapy in Weil's Disease with Severe Hyperbilirubinemia, Acute Renal Failure, and Multidrug-Resistant Organism Co-Infection: A Rare Case in Critical Care Setting

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Abstract

Background: Leptospirosis is a zoonotic disease caused by spirochete *Leptospira interrogans*. Additional therapy modality is required in critical Weil's Disease, which does not improve despite standard therapy with antibiotics and renal replacement therapy (RRT). Therapeutic plasma exchange (TPE) has been reported to show positive outcomes in patients with sepsis. However, TPE has not been widely used in treating severe leptospirosis. We report a case of Weil's disease with severe hyperbilirubinemia, acute renal failure, and co-infected with multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA), successfully treated with TPE, intermittent RRT, and definitive antibiotics.

Case Presentation: A 25-year-old male developed Weil's disease with severe hyperbilirubinemia and acute renal failure treated in the ICU. He received empirical antibiotics and intermittent RRT but deteriorated and experienced a septic shock. His serum bilirubin increased to 43.34mg/dL, and the blood culture result showed MDR-PA co-infection. Further, he underwent a single TPE and received definitive antibiotics, resulting in rapid clinical improvement as well as liver and renal function recovery.

Conclusion: Therapeutic plasma exchange as a rescue therapy can be considered for further intensive care support in severe leptospirosis which has not shown improvement despite standard treatment with RRT and antibiotics. However, the use of TPE requires well-designed clinical trials to further establish its efficacy. Leptospirosis can coexist with other pathogens which is adding the therapeutic challenge.

Keywords: *Leptospirosis; Weil's disease; plasma exchange; renal replacement therapy; multidrug-resistant organism*

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INTRODUCTION

Leptospirosis is a zoonotic disease caused by spirochete *Leptospira interrogans*. According to official Ministry of Health (MoH) records, leptospirosis caused an estimated 895 human cases in Indonesia in 2018, with a case fatality rate of 17.8%.[1] The annual morbidity of leptospirosis in the population was recently calculated to be 39.2 per 100,000.[2] The clinical spectrum of leptospirosis ranges from subclinical infection to fulminant infection resulting in septic shock and severe multiorgan failure. Leptospirosis shows a biphasic disease course. The first phase is the septicemic phase in which leptospira can be found in blood and cerebrospinal fluid.

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(Albert Frido Hutagalung)

This phase lasts 4 to 7 days with non-specific symptoms including fever, headache, myalgia, and abdominal pain. An immune phase follows, during which the leptospires are excreted in the urine, and anti-Leptospira immunoglobulin M (IgM) antibodies develop in the blood. At this stage, the patient re-develops the initial symptoms. Five to 10 percent of patients develop Weil's disease, a severe form of leptospirosis that manifests as renal system failure, hepatomegaly, liver impairment, and/or an alteration in the levels of consciousness.

Severe hyperbilirubinemia and acute renal failure have been associated with high mortality in leptospirosis patients.[3] High serum bilirubin can cause toxic damage to kidney and liver cells then worsening multiorgan failure. Treatment of Weil's disease is an antimicrobial agent and supportive care including adequate hydration, advanced respiratory support, and early initiation of renal replacement therapy.[4] Additional therapy modality is required in critical Weil's Disease, which does not improve despite standard therapy with antibiotics and renal replacement therapy (RRT). There have been reports of the use of plasma exchange in sepsis and leptospirosis patients that showed positive outcomes. However, TPE has not been widely used in treating fulminant leptospirosis.

Leptospira may coexist with other pathogens leading to complicated diagnosis, extending the length of treatment, and increasing mortality, therefore identifying the presence of co-infected pathogens is very crucial.[5] Here in, we report a case of Weil's disease with severe hyperbilirubinemia, acute renal failure, and co-infected with multidrug-resistant *Pseudomonas aeruginosa (*MDR-PA), successfully treated with TPE, intermittent RRT, and definitive antibiotics.

CASE REPORTS

A 25-year-old male presented to our emergency departments with a 5-day history of fever and bilateral calf pain. He complained of headache, jaundice, nosebleed, tea-colored urine, and black discoloration of stools. He lives in a flood-prone area and worked as a waiter. He denied recent travel, recent antibiotic exposure, or sick contacts. He took no regular medications.

On examination, the patient was mildly tachycardic (120 beats per minute) with a blood pressure (BP) of 114/80mmHg, respiratory rate of 20 breaths per minute, and temperature of 36.8°C. The patient was alert and oriented. Physical examination findings were icteric sclera, subconjunctival bleeding, the skin appeared jaundiced, the lungs were clear to auscultation, and the abdominal examination revealed no abnormality.

Initial laboratory study results showed anemia (8.5mmol/L), leukocytosis (15,000µL), thrombocytopenia (50,000µL), hypokalemia (3.30mmol/L), and hyponatremia (123.0mmol/L). His serum bilirubin was 41.07mg/dL (direct 24.52mg/dL and indirect 16.55mg/dL), aspartate transaminase (AST) was 20.5IU, alanine transaminase (ALT) was 59.3IU, serum alkaline phosphatase (ALP) was 78.0IU, gamma GT was 86.7µL. Serum creatinine was 10.08mg/dL and blood urea was 346.6mg/dL. Coagulation studies were normal. The leptospiral strip test for IgM antibodies was positive. Hepatitis B surface antigens and anti-human immunodeficiency virus antibody titers were all negative. Results of abdominal ultrasonography and chest radiography were normal, and electrocardiography showed sinus tachycardia at 120 beats per minute.

The patient was diagnosed with Weil's disease and empirical antimicrobial treatment was started with ceftriaxone. Within 12 hours of admission, he became hypotensive with a BP of 81/56mmHg, his temperature rose to 38.4°C, respiratory rate of 40 breaths per minute, his leukocyte increased to 38,600µL, and serum bilirubin 43.34mg/dL. He developed septic shock with an APACHE II score of 25 points (55% estimated non-operative mortality). Therefore, the patient was transferred to the ICU and intubated. Despite receiving adequate intravenous fluids, packed red cell (PRC), and thrombocyte concentrate (TC) transfusions, noradrenaline infusions were started at 0.2 mcg/kg/min for circulatory support. Hemodialysis was started for acute renal failure and sepsis.

Until 3rd day of admission, the patient had undergone second hemodialysis in which blood urea, and creatinine were decreased, but his serum bilirubin and clinical condition had not improved. On the 5th day of admission, the patient's mental state begins to deteriorate (E3M5VETT), and the use of noradrenaline increased to 0.4mcg/kg/min. By the 6th day of admission, plasma exchange was initiated based on clinician judgement. We are working with the Indonesian Red Cross Society to conduct TPE in our ICU. He underwent a single session of TPE with 1.5-liter exchanges using a 5% human albumin solution (body weight 40 kg, hematocrit 40%). After plasma exchange sessions, serum bilirubin was reduced to 14.6mg/dL. His mental state and respiratory function both improved significantly. Respiratory ventilator support and vasopressors were gradually withdrawn (lowered to 0.025mcg/kg/min), and the patient became oriented and alert. Two days after TPE, the patient was extubated and transferred to the intermediate care unit.

Nonetheless, the patient's leukocytes had not improved significantly. Blood cultures and antibiotic susceptibility tests were performed, and the antibiotic was changed to meropenem and amikacin while waiting for the results. After 1 day in the intermediate care unit (9th day since admission), the patient's blood pressure dropped to 75/40mmHg, respiratory rate up to 35breaths per minute, leukocytes rose to 43,700µL, and the patient fell into a septic shock. APACHE II scored 24 points (40% estimated non-operative mortality). The patient was readmitted to the ICU and re-intubated. The culture result showed that he was co-infected with multidrug-resistance *Pseudomonas aeruginosa* (MDR-PA), which is resistant to ceftriaxone, meropenem, cefepime, cefotaxime, ampicillin-sulbactam, amoxicillin clavulanic acid, and erythromycin. The antibiotics were changed according to culture and antibiotic susceptibility tests results using piperacillin-tazobactam 4.5g iv/6hr. After the antibiotic was changed, the leukocytes gradually decreased, and the patient showed clinical improvement. APACHE II score also showed improvement to 18 points (25% estimated non-operative mortality). The piperacillin-tazobactam administration was continued for up to 7 days accompanied by other supportive therapy

and routine hemodialysis. The final laboratory study results were notable for leukocytes 10,500µL, blood urea of 32.4mg/dL, creatinine of 1.21mg/dL, and total bilirubin of 2.02mg/dL The patient was subsequently discharged and scheduled for intermittent hemodialysis.

DISCUSSION

Our patient developed severe sepsis with acute renal failure and multi-organ failure 5 to 6 days after the initial symptoms of Leptospira infection, and this is coinciding with the immune phase of the disease. The patient lives in a flood-prone area which is a risk factor for leptospira infection. The recommended antimicrobial agent is benzylpenicillin (1.5 million units intravenously every 6 hours), ceftriaxone (1–2g IV once daily), or cefotaxime (1g IV every 6 hours) given for a period of 7 days.[3] The patient in this case was given ceftriaxone as the empirical antibiotic. Severe cases with multi-organ involvement should be followed-up in an intensive care unit.

In addition to antimicrobial therapy, renal replacement therapy (RRT) or hemodialysis is the necessary supportive therapy for fulminant leptospirosis with sepsis and acute renal failure. In patients with sepsis, acute renal failure has been associated with prolonged ICU and hospital stays in survivors.[6] Early initiation of RRT has been shown to greatly reduce the mortality associated with Weil's disease.[7] Acute renal failure in leptospirosis typically presents as nonoliguric and hypokalemic.[4] This patient was admitted with high serum creatinine, hypokalemia, and oliguria after several days of hospitalization. Therefore, this patient had been programmed for hemodialysis from the first day of hospital admission. RRT in leptospirosis patients has been known to remove the inflammatory cytokines produced as a reaction to the spirochete.[7] Figure 1 shows the improvement of serum creatinine after the patient underwent routine hemodialysis. However, until the second hemodialysis, the patient's serum bilirubin and clinical condition has not shown improvement

The presence of jaundice is characteristic of Weil's disease, however highly bilirubin values such as those present in our patient are not frequently presented in the literature. Based on the literature review conducted by Edmond et al., there are only four publications on PubMed where bilirubin levels exceed 30mg/dL.[8] Extreme hyperbilirubinemia can have multiple cellular toxic effects, including effects on cellular respiration, membrane integrity, transport function, and direct oxidative damage to tubular cell membranes with subsequent release of vasoactive mediators thereby contributing to persistent renal failure.[9]

TPE as rescue therapy was used as part of our treatment in cases of Weil's disease that did not respond to standard critical care after considering the benefits and risks in the individual patient. The bilirubin level was one of the major parameters on which our TPE decisions were based. There are no definite criteria or evidence-based strategy regarding when to start or stop, the frequency, and duration of the TPE procedure.[10,11] A mild criteria for hyperbilirubinemia with a total serum bilirubin level >5 mg/dL should be considered to perform TPE.[11] The number of TPE sessions is determined based on patient improvement.[11,10] The target of bilirubin reduction after TPE is also unclear. Based on previous reports, TPE could reduce bilirubin levels by 46.53%, 21.20%, and 37.69%.[12]

The guidelines of the American Society for Apheresis (ASFA) until the 8th edition in 2019 recommend TPE in septic patients with multiorgan failure in category III (optimum role of apheresis therapy is not established, decision making should be individualized) grade 2B (weak recommendation, moderate-quality evidence).[13] Although the role of plasmapheresis in the treatment of leptospirosis has not been defined, TPE has been reported as an adjunctive therapy for patients with severe leptospirosis in recent years. Tse et al. reported dramatic improvement in a 39-year-old man with severe leptospirosis complicated by acute renal failure treated with single plasma exchange as

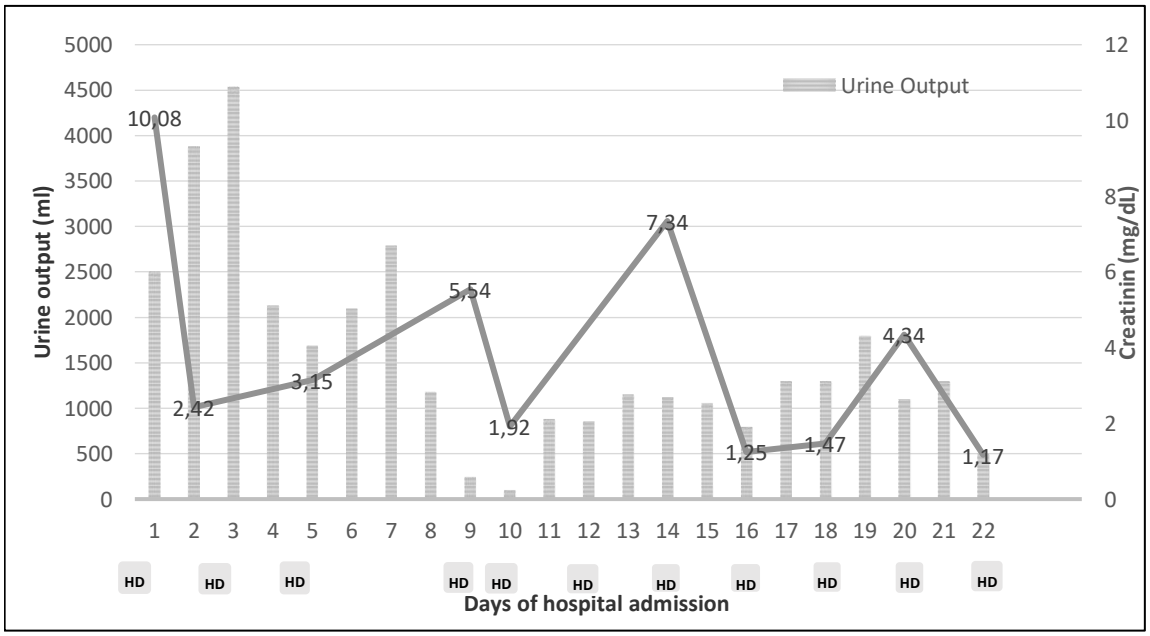

Figure 1. Changes in creatinine serum and urine output

adjunctive therapy.[14] Taylor et al. described a 67-year-old man with severe leptospirosis successfully treated with plasma exchange and showed a significant reduction of serum bilirubin levels.[15] Liu et al., stated that PE is superior in removing bilirubin than the other methods and could reduce blood ammonia.[12] A retrospective single-center pediatric study conclude that TPE and hemodialysis significantly reduced bilirubin and ammonia.[10]

TPE is contraindicated in several conditions, including hemodynamically unstable patients, patients who have an allergy to fresh frozen plasma or replacement colloid/albumin, patients who are allergic to heparin, and unavailability of central line access or large bone peripheral lines. Hypocalcemia and the use of angiotensin-converting enzyme (ACE) inhibitors in the last 24 hours are relative contraindications for TPE. The common complications that can occur during or post-plasma exchange procedure are hypocalcemia or hypomagnesemia, transfusion reactions, fluid and electrolyte imbalance, hypotension, bleeding due to hypofibrinogenemia and thrombocytopenia.[16]

In this report, patients received a single TPE with a centrifugation technique (HAEMONETICS MCS+ 09000 - 220 - E). During the TPE procedure, the patient was hemodynamically stabilized with a ventilator and drug support. Total plasma volume (TPV) was calculated as follows: estimated plasma volume (in liters) = 0.07 × weight (kg) × (1 – hematocrit).[17] The volume of replacement fluid is equal to the volume extracted. We chose 5% human albumin solution (HAS) as a replacement fluid for various reasons. It is essential to use colloid over crystalloid when the volume extracted is more than 1000ml. When compared with fresh frozen plasma (FFP), HAS is easier to obtain, relatively inexpensive, and has minimal side effects such as infection and incompatibility.[18] As demonstrated in Figure 2, the initiation of plasma exchange led to an immediate improvement in serum bilirubin (>50%).

In the septicemic phase of leptospirosis, tissue damage occurs due to systemic inflammation, while in the immune phase, tissue destruction occurs due to immune complex mechanisms. In addition, the use of antibiotics causes the organism to release an excessive amount of endotoxins upon death, which also aids in the immunological pathogenic process (Jarisch-Herxheimer reaction).[19] Plasma exchange eliminates the harmful substances in the bloodstream, such as bilirubin, ammonia, endotoxins, and proinflammatory cytokines which will minimize the tissue damage caused by the mechanism above. TPE also replaces the albumin and helps to maintain the biochemical and homeostatic balance in the body. Removal of serum bilirubin has led to reducing toxic insults to kidney and liver cells. Consequently, improving hyperbilirubinemia with TPE and treating the underlying condition would be the most effective option.[12,20]

As seen in Figure 3, the leukocyte trend of the patient did not show a significant improvement since the start of antibiotic therapy. We started with empiric antibiotic therapy for leptospirosis with ceftriaxone. When the patient showed no improvement in clinical and leukocyte parameters, we took the blood culture and antibiotics susceptibility test. Due to limited facilities, culture findings take longer than expected to be available. While waiting for the culture results, the antibiotics changed to meropenem and amikacin which can cover both anaerobes and pseudomonas.[21] After 5 days, the culture results showed that the patient was co-infected with MDR *Pseudomonas aeruginosa* and had a lot of antibiotic resistance, so we changed the antibiotic to piperacillin-tazobactam as a potent β-lactam/β-lactamase inhibitor.[22] After using piperacillin-tazobactam, the patient's clinical condition and leukocytes gradually improved.

Leptospira may coexist with other pathogens, resulting a complicated diagnosis, extend the length of treatment, and increase mortality, therefore identifying the presence of co-infected pathogens is very crucial.[5] MDR pathogen is a significant cause of infections in hospitals, especially in ICU. This patient experienced MDR-PA hospital-acquired infections (HAI) which certainly poses a therapeutic challenge because effective antimicrobial therapy is very limited.

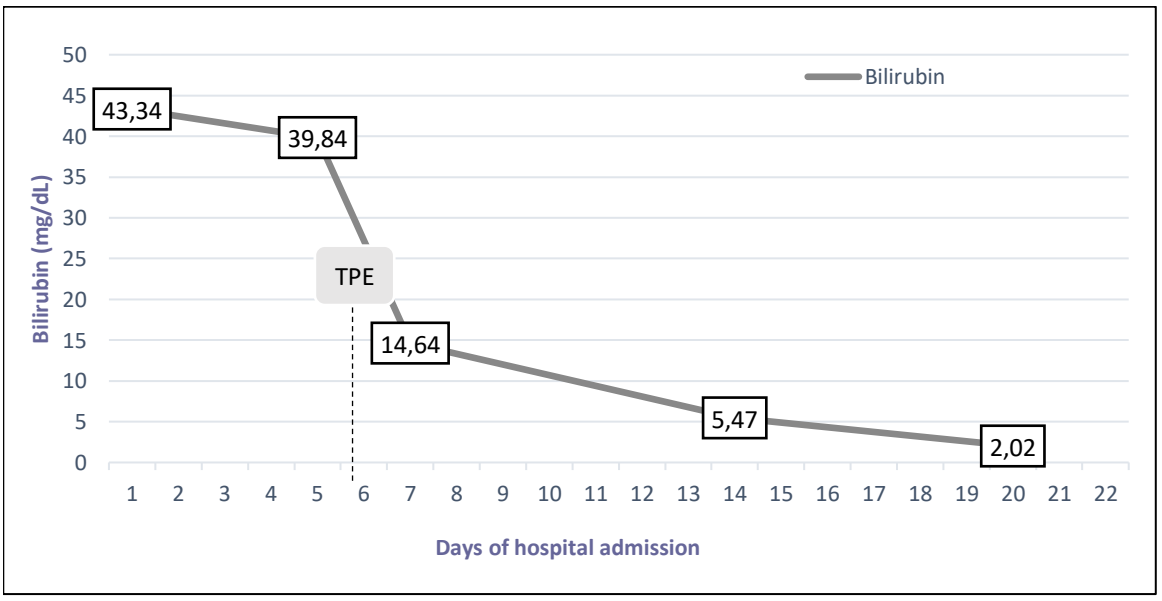

Figure 2. Changes in bilirubin level

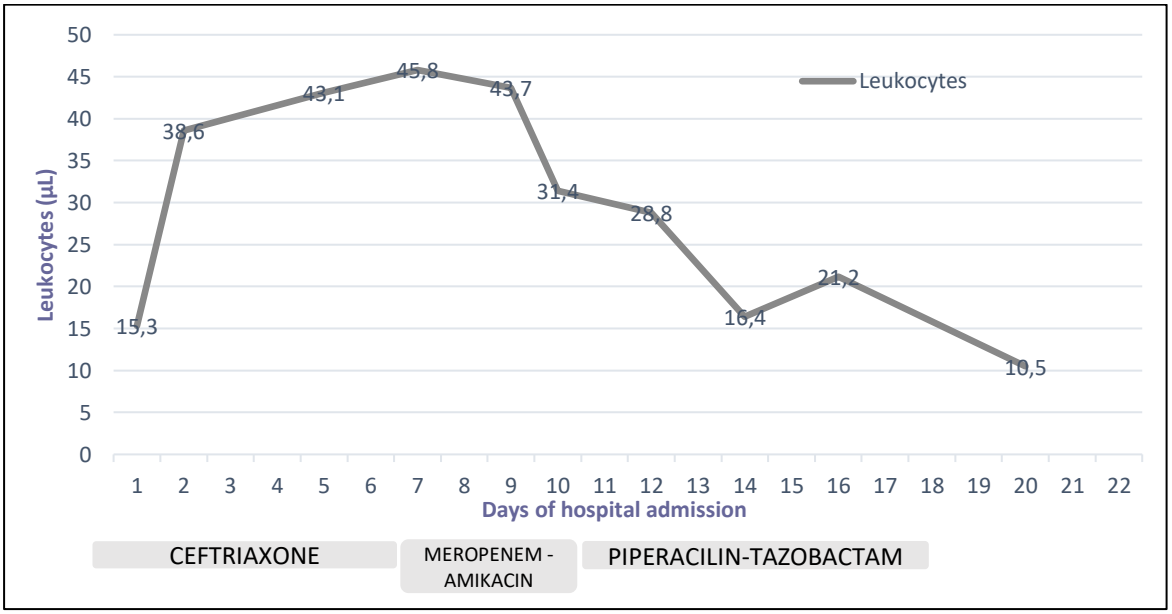

Figure 3. Changes in antibiotics and leukocyte trend

Several factors can explain the cause of this infection such as the use of invasive medical devices (e.g., endotracheal tubes, feeding tubes, and urinary catheters), the use of intravenous antibiotics within the past 60 days, and the duration of hospitalization. The presence of sepsis/septic shock and multiorgan failure are independent factors associated with increased mortality in MDR infections.[23,24,25]

CONCLUSION

In summary, our patient developed Weil's disease with severe hyperbilirubinemia, acute renal failure, and co-infected with multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA). Further, he underwent a single TPE and received definitive antibiotics, resulting in rapid clinical improvement as well as liver and renal function recovery. Based on our experience, we suggest to considered TPE as a rescue therapy for further intensive care support in severe leptospirosis which has not shown improvement despite standard treatment. Co-infection with other pathogens needs to be considered in patients with long hospital stays, especially for those who use invasive medical devices. Improving hyperbilirubinemia with plasma exchange and treating the underlying condition would be the most effective option. However, the use of TPE requires well-designed clinical trials to further establish the efficacy.

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Review Article

Mechanism of Immune System Dysfunction, Apoptosis and Oxidative Stress on Endometriosis

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Abstract

Endometriosis is a gynecological disease that still requires advanced knowledge to develop the best treatment to improve the patient's best quality of life. It is characterized by the formation of endometrial tissue outside the uterus and occurs approximately in 5-10% of women of childbearing age. Endometriosis with poor prognosis can be at high risk of endometrial cancer, ovarian cancer and lead to infertility. In-depth efforts are needed to achieve a clear knowledge of the molecular etiology of endometriosis. Here, there are three dysfunctional molecular mechanism that occur in endometriosis; (1) immune system dysfunction, (2) impaired apoptotic signaling, (3) and increased oxidative stress. These three dysfunctional molecular mechanisms are thought to promote endometriosis development and worse prognosis. Therefore, determining the molecular pathogenesis of endometriosis will be useful in the development of diagnostic and therapeutic methods.

Keywords: *Endometriosis; Immune Dysfunction; Apoptosis; Oxidative Stress*
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INTRODUCTION

Endometriosis is a chronic inflammation disease. The most symptom is pelvic pain, occurring in 75% of endometriosis women.[1] Approximately 30-50% women with endometriosis having infertility and women with endometriosis have high risk to be infertile.[2] Determining the molecular mechanism that occurs in endometriosis is needed for better knowledge regarding diagnostic and therapeutic methods of endometriosis. It has been reported that estrogen level increased in women with endometriosis; additionally genetic factor, endocrine, lifestyle, and immune system dysfunction are also contributing to endometriosis etiology. The appearance of endometrium tissue outside the uterus is reported due to the releasing of endometrium cells, then migrate to the pelvic through the fallopian tube during menstrual.[3] The released endometrial cells can bind also to several adherent tissues such as ovarian tissue, peritoneum, intestine, and uterus, then proliferate to become endometrial lesions.[4]

The immune microenvironment of the women with endometriosis is found in many inflammations, angiogenesis, and endocrine signal. The molecular profile shows the differences condition of immune response and inflammation gene expression between normal endometrium and endometriosis patients, proven by the existence of leukocyte signal dysregulation and cytokine receptor interaction on endometriosis.[5] The increasing level of activation macrophage, T cell, B cell is found on women with endometriosis, while the NK cell is decreased. Upregulation of Stem Cell Growth Factor b (SCGFB), IL-8, human growth factor dan *Monocyte Chemoattractant Protein 1 (*MCP1) is found on endometriosis while IL-13 as an anti-inflammatory is decreased. Immune dysfunction and cytokine interactions stimulate the formation of endometriosis progression.[6] Overexpression of activation macrophage as a result of chronic inflammation in intrauterine and fallopian tube can eliminate the sperm and effects infertility.[7]

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In addition, unbalanced apoptosis signals and oxidative stress also promote the progression of endometriosis. Delbandi AA *et al* reported that BCL2 level as an antiapoptotic molecule in endometrium lesion is higher than normal endometrium. TGF-beta expression is increased in endometrium lesions, it can reduce the level of BAX protein. The dysfunction mechanism of an apoptotic signal can promote the growth of endometrium lesion.[8]

The etiology of endometriosis remains unclear. Sampson firstly recognized its theory of endometriosis at 1920 which called "retrograde menstruation" theory. Sampson was poured his thought that endometrial tissues has been transported by menstrual blood retrograde to the fallopian tube then peritoneal cavity. Those endometrial cells bind to the peritoneal mesothelial and take nutrition out from the blood, proliferate become endometrial implantation in the peritoneal.[9] Retrograde menstrual also promotes unbalanced ROS production through signaling pathway of Extracellular Signal-Regulated Kinase and Phosphatidylinositol 3 Kinase.[10]

This review article will more discuss the three main molecular mechanisms which are dysregulated and contributed to the progression of endometriosis. Considering that endometriosis development is related to the patient's quality of life and infertility, these three main molecular mechanisms deserve further in-depth study. In addition, intensive research is expected to find molecular target for development of the diagnostic and therapeutic methods.

MATERIALS AND METHODS

The narrative review was written initially by searching the recent publication in PubMed NCBI database and in the Google platform with the keyword "immune dysfunction on endometriosis", "apoptosis dysregulation on endometriosis", "oxidative stress on endometriosis", "dysregulation of immune and endometriosis-associated infertility", "ROS and endometriosis". The references which has been taken also restricted from 2015 to 2021. There were 41 articles that correlated with dysfunction of immune, apoptosis, and oxidative stress on endometriosis were found.

DISCUSSION

The progression of endometriosis may occur due to several signaling dysfunction in cellular and molecular mechanisms. Three signaling dysfunction during endometriosis progression which have been reported in several studies are; immune dysfunction, apoptosis signaling disturbance, and oxidative stress elevation.

Mechanism of Immune Dysfunction on Endometriosis

During menstrual, endometrium cells may migrate to the peritoneal area through retrograde menstrual. On the endometriosis lesion was found a number of activated macrophages, B cell, T cell, and low level of NK cell.[4] The abundance of activated macrophages in the microenvironment of endometrium lesion that grows in the peritoneal membrane facilitates the vein to attack to endometrium lesion. Thus, the endometrium lesion grows continually. Activated macrophage secretes number of cytokines for modulating the normal cell function, but the macrophage dysregulation has occurred, and subsequently it causes neutrophil infiltration that implicated to activate the VEGF for neovascularization. Besides, the macrophage also decreases the IL-24, then proliferation and invasion of endometrium lesion are increased. It is supported with increased TGF-Beta in the microenvironment of endometrium lesion which stimulates macrophage activation on the surface of the Integrin alfa/beta.[11]

The macrophage will activate and converts to M2. It causes the stimulation of cytokine secretion, such as IL-10, IL-1, and IL-6 resulted in increasing of lesion growth and its proliferation. The occurrence of retrograde menstrual increases the polarization M2 then causes the increase of endometrium lesion proliferation through the STAT3 signaling pathway.[12,4] Reported that IDO-1 also has a role in the M2 polarization, endometrium lesion secretes IDO-1 that stimulates the polarization of M2.[13] Fractalkine that secreted from endometrium lesion also contributes to the M2 polarization and increases the MMP-9 expression. The decreasing of MMP-1 and MMP-2 also occurred. All events cause the increase of endometrium lesion invasion and endometriosis progression through MAPKs signaling.[14]

Besides macrophage cell dysregulation, T cell dysregulation has occurred. T cell secretes cytokines and T cell profile has been different compared with the normal endometrium. There are many CD8, CD4, and activated T cells in endometrium lesions. While the Th1 has decreased significantly, Th17 and IL-10 population have increased in the peritoneal fluid of endometriosis individual. It has been associated with the increasing of IL-27, IL-6, and TGF-Beta resulted in increasing the production of Th17 and IL-10. Consequently, increasing in the endometrium lesion invasion and proliferation is occurred.[15] IFN-Gamma, IL-10, IL-4, and IL-2 significantly increases in the microenvironment of endometrium lesion. Those cytokines stimulate the formation of the Th2 subset and inhibit the cytotoxic Th1. IL-4 is the Th2 cytokine that can increase the expression of mRNA 3β-hydroxysteroid dehydrogenase, which is the main enzyme for estrogen production. Therefore, this will increase the endometriosis progression through p38 MAPK, and p42/44 MAPK signaling pathway.[16]

The IL-6 was found abundant in endometrium lesion and its expression stimulated by IL-1β and TNF-α. The abundant IL-6 inhibit the differentiation of Th1 through the of the secretion of IFN-γ and SOCS1 (*Suppressor of Cytokine Signaling-1*). Then, IL-6 promotes the differentiation of Th2 through the activation of the transcription factor, like nuclear factor pf activated T cells or NFAT.[17] In addition to Th2, the naive T cell differentiates into a regulator T cells and suppresses the immune response for proliferating and activating the T cells, macrophage, dendritic cells, and natural killer cells. That immune suppression leads to the infiltration of T-reg on the endometrium lesion. It shows the poor prognosis. An increasing of T-reg of the T cell population in the peritoneal fluid induces the decrease of immune response and inhibits in recognizing and eliminating the endometrium lesion. Consequently, the endometrium lesion will persist, grow and survive.[18]

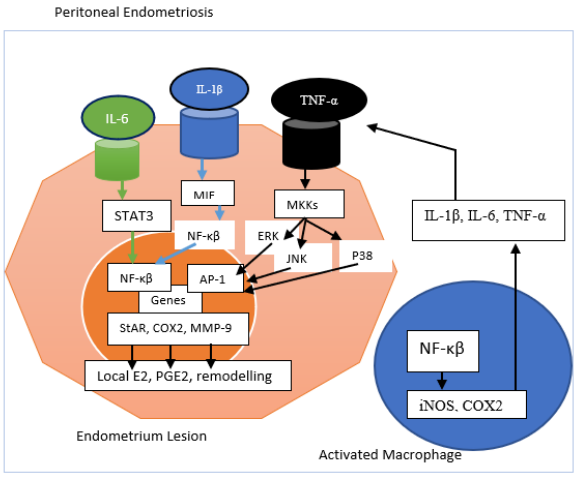

Figure 1. The cytokines interaction induce progression of endometrium lesion. The production of iNOS and COX2 by NF-κβ through the activated macrophage. It secretes the cytokines like IL-1β, IL-6 and TNF-α. Those cytokines bind to their receptors on the endometrium lesion and activated the pathways of STAT3, NF-κβ, MKKs then activated the transcription factor of NFκβ and AP-1 and stimulate the production of local E2, PGE2 and MMP-9 for remodeling and adhesion cell to increase the progression of lesion.

The activated macrophage in the peritoneal has secreted the Nitric Oxide Synthase and COX2 through interferon regulatory factors, NF-κβ and *nuclear factor-2*. Its activation has facilitated by STAT, p38, ERK, and JNK signaling pathways. Besides, the activated macrophage also has secreted many cytokines, like IL-1, IL-6, IL-8, IL-10, IL13, TNF-alfa, growth factor, VEGF, and platelet-derived growth factor or PDGF. TNF-alfa, IL-1β, dan IL-6 bind their receptor in the membrane surface of the endometrium lesion. Those cytokine receptors activate PI3K, MKK, JNK, p38, and IKK pathways for inducing inflammation response and invading the mediators like StAR, COX2, and MMP-9. Through the activation of NF-κβ, and AP1, the forming of estradiol local, PEG2, NCOA-1 isoform, and tissue remodeling has occurred. Those formations will increase the endometrium lesion growth (Figure 1). An interaction complex of estradiol/ESR2/NCOA-1 increases the IL-1β for inducing monocyte differentiation became the macrophage. Therefore, the cytokine interactions in the endometrium lesion and the macrophage role can lead to the initiation, survival, and progression of endometriosis.[4]

The granules of NK cells contained the granzyme, perforin, and IFN-gamma which induced of the lesion apoptosis.[4] But, the NK cell dysregulation has occurred. Some cytokines that can inhibit the NK cells activity have been found in high concentration in the peritoneal fluid of endometriosis as well as the HLA-G and the HLA-E. High concentration of *Immunoreceptor Tyrosine-based Inhibitory Motif Killer Cell Inhibitory Receptors* (ITIM-KIRs) which contain the Immunoglobulin domain such as KIR2DL1, KIR3DL1, EB6, and I-CAM also found in the peritoneal fluid of endometriosis. Those have a role to suppress the NK cytotoxic cell activations. A high level of HLA-G facilitates interacting with immune cells and binding to NK cells. It causes the retrograde menstrual tissues transform and became the lesion .[19]
In addition to T cells, NK cells, macrophage, T-Reg cells, B cells are also dysregulated. Although the T cells were suppressed, the populations of B cells significantly increased, and the B lymphocyte inducer of maturation program or Blimp-1 has been found higher than normal endometrium.[20] The B lymphocyte stimulator or BLys that the role is very important for facilitating the differentiation of the B cells into plasma resulted in antibody. The increased hyperactivity of the B

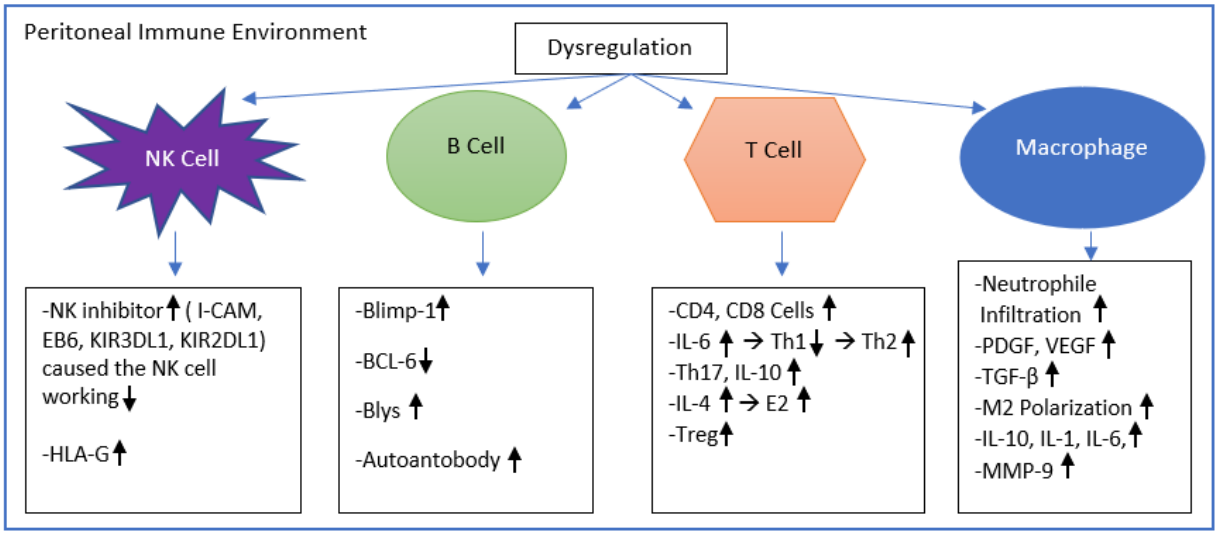

Figure 2. The Summary of Immune Cells Dysregulation in Endometriosis
Neutrophile infiltration occurred, and macrophage dysregulation could secrete an abundance of cytokines; it stimulates PDGF and VEGF increased for neovascularization, increased TGF-β and M2 polarization induces increased cytokines and MMP-9 for increasing the endometrium lesion invasion. T cell profile in the endometrium lesion found increased CD4 and CD8 and induces the increased IL-6, decreased Th1, and increased Th2, it cause attenuated cytotoxicity response, increased Th17, IL-10, IL-4, and T-reg make the lesion to proliferate and attenuate the immune response. Dysregulation of B cell induces the increase of Blimp-1, Blys, and autoantibody and decreased BCL-6; this dysregulation allow the lesion to survive. NK cell dysregulation also occurred; increased I-CAM, EB6, KIR3DL1, KIR2DL1, and HLA-G inhibit the NK cell cytotoxic responses and allow the lesion to grow.

lymphocyte can contribute to the endometriosis progression through the autoantibody which can break the endometrium, but other studies explain the role of hyperactivity of the B lymphocyte in endometriosis is still unclear.[21] Several dysregulations in the immune cells of endometriosis have been summarized in the figure 2.

Endometriosis is a multifactorial disease, many factors can increase the formation of endometriosis; several gene mutations, epigenetic and environmental factors interact to form endometriosis.[22] Several genes involved in immune dysfunction correlated with chronic inflammation.[23] Immune dysregulation can also increase due to the presence of DNA methylation and mRNA expression that regulate inflammation.[22] Increased mRNA expression of the TRPA1 gene and hypomethylation are found in endometriosis. TRPA1 encodes an ion channel protein that regulates pain through neuronal activity.[24] Hypomethylation of the SF-1 gene which plays a role in the regulation of steroid hormone and aromatase biosynthesis is also found in peritoneal and ovarian endometriosis.[25] Increased steroid hormones can cause increased inflammatory cytokine secretion and changes in immune cell activity resulting in immune dysregulation.[26] his is also in line with the increased expression of beta receptor mRNA in endometriosis. Increased beta estrogen receptors can induce COX-2.[27]

Progesterone resistance is also associated with increased inflammation in endometriosis.[26] Hypermethylation of the progesterone receptor A (PGR-A) can lead to decreased PGR-A mRNA expression and is associated with progesterone resistance.[28] Hypermethylation of the progesterone receptor B (PR-B) is associated with progesterone resistance in lesions. endometriosis so that it has the potential to be a biomarker for the diagnosis of endometriosis.[29] Inflammatory mediators can interact with sensory neurons and induce pain signals. PGE2, IL-6, and IL-1β are inflammatory mediators that can induce pain in endometriosis.[30] Increased expression of P2X3 receptor coding genes through the ERK/MAPK activation cascade is also associated with pain in endometriosis lesions.[31] Furthermore, cytokines such as IL-10 and IFN which increase due to the dysregulation of cytotoxic T cells can increase MMP-2 expression.[32] Increased MMP-2 is associated with DNA methylation on MMP-2 and induces increased invasion of endometriosis lesions.[33] Therefore, immune system dysregulation in endometriosis involves immune cell dysregulation and hormonal factor. Furthermore, immune system dysfunction in endometriosis is related to epigenetic factors through inflammation. This factor leading increased the development of endometriosis. Therefore, it can be assumed that immune system dysfunction in endometriosis is the main molecular mechanism factor for the formation of endometrial lesions.

Mechanism of The Signal Apoptosis Dysregulation on Endometriosis

Apoptosis failure for retrograde menstrual tissues and abnormal apoptosis for immune cells are associated with the endometriosis progression.[14] By understanding the molecular mechanism related to the apoptosis dysregulation on the endometriosis tissues and immune cells, then easy to determine the molecular etiology for new therapy agent development. It has been reported that the reduction of apoptosis in the endometriosis ectopic tissues is occurred. It has supported by finding of anti-apoptotic BCL-2 protein in high concentration in endometriosis lesion. The increase of BCL-2 protein accords with the increased of the c-Myc protein which regulate the cell cycle and the increase of TGF-β. While the apoptosis molecule such as BAX has decreased. The apoptosis dysregulation that happened in the endometrium lesion has associated with the retrograde menstrual tissues ability to escape from the immune surveillance lead to increasing of the endometrium lesion growth and proliferation.[34]

Apoptosis signal can be occur in two ways, they are the intrinsic and the extrinsic pathway. The intrinsic pathway runs via mitochondria and the extrinsic pathway carry out via death receptor-mediated mechanism. The dysregulation of intrinsic pathway by suppression its molecular pathway. It is proven by the expression of p53 in the endometrium, the expression of BCL2 protein in the peritoneal are found high.[35,36] Whereas the dysregulation of the extrinsic pathway occurs in the Fas receptor and its ligan. Fas receptor proteins (DR2/CD95/Apo-1) are the protein of membrane type-1 (mFas) that can bind the extracellular domain of FasL (CD95L/CD178/Apo-1L). mFas also binds the cytoplasmic domain which transducing the death signal. The death signal has been mediated by the interaction of Fas/FasL. The death signal has an important role in maintaining the immune surveillance.[36]

FasL has been expressed in the normal endometrium cells and its expression has been stimulated by cytokines released from the macrophage, like PDGF and TGF-β1. The increase of IL-8 in the peritoneal fluid of endometriosis individuals would cause the increasing of the FasL expression in the endometrium cells. Although the FasL expression has increased, it has not induced apoptosis in the endometriosis stromal cells. It has been caused by the decrease of the Fas receptor expression. The ectopic epithelial cells in the endometriosis stimulate an increase in FasL expression and lead to low the Fas receptor expression. The decrease of Fas receptor expression also caused by the apoptosis of cytotoxic T cells which expressed the Fas receptor.[37] Accordingly, the apoptosis dysregulation would cause the endometriosis epithelial cells to avoid the immune surveillance. It can induce the growth of the endometrium lesions.

Apoptosis dysregulation can occurs in TNF-α. Its population was increased by retrograde menstruation. TNF-α binds the TNF Receptor or TNFR for inducing of caspase-8 and caspase-9 expression then mediating the apoptosis of retrograde menstruation tissues. On the endometriosis individual, the number isoform of Nuclear Receptor Coactivator or ESR2 would bind the Apoptosis Signal Regulating Kinase 1 or ASK 1, caspase-8 and caspase-9 then suppress the extrinsic apoptosis signaling in the retrograde menstrual tissues. The increasing of PGE2 on endometriosis would like to increase the ratio of BCL/BAX protein in the mitochondria, accordingly the intrinsic apoptosis signaling has been suppressed. On the endometrium lesion, reported that the level of FasL

has increased then could bind its receptor or Fas receptor in the cytotoxic T cell membranes. Thus, the cytotoxic T cell has died. This has represented the main defense mechanism of endometrium lesion to avoid the cytotoxic T cells during the retrograde menstrual occurred.[4]

The dysregulation of the apoptosis signal also occurred through the NF-κβ and Akt pathways. Normal endometrium has shown that NF-κβ and Akt pathways have been inactivated. It made the stimulation of the Bad/Bax protein dephosphorylation. The Bad/Bax disassociated from 14-3-3 protein has caused the translocation of the Bas/Bax protein from the cytosol to the mitochondria. Then, the interaction between Bad/Bax protein with the Bcl-2/Bcl-XL protein has occurred. That interaction has implicated for the mitochondria to secrete the c-cytochrome into the cytosol and activate the caspase-3 and the enzyme which accelerated the apoptosis of endometrium cells. While endometriosis has shown that NF-κβ and Akt pathway has activated and stimulated the cells stand to survive. Its activation has controlled the Bad/Bax protein phosphorylation. The Bad/Bax binding into 14-3-3 protein has caused the Bad/Bax would survive to release into the cytosol and the interaction between the Bad/Bax and Bcl-2/Bcl-XL has not occurred. Thus, endometrium cells would not has apoptosis.[8]

Mechanism of The Oxidative Stress Dysregulation on Endometriosis

In the normal condition, the homeostasis of ROS and antioxidant has been maintained. An excessive ROS level has induced the oxidative stress, endometriosis, and has implicated to the women's reproductive quality.[38,39] The oxidative stress has broken the lipid, protein, and DNA. The number of ROS production has damaged the cellular function through the NF-κβ transcription factor activation. Its activation has stimulated the expression of many genes related to the initiation process and the endometriosis progression. The erythrocytes, the tissue apoptosis products of endometriosis, the debris cells that were released in retrograde menstrual tissues have migrated to the peritoneum cavity. That migration has been induced by the macrophages which became the main inducer in oxidative stress in the peritoneum cavity. Moreover, excessive iron in the peritoneal fluids of endometriosis individuals can be the cause of the ROS activation by Fenton reaction. The formation of ROS in the peritoneal microenvironment can increase the invasion and the growth of retrograde menstrual tissues.[38]

The number iron would expand the endometrium lesion in the animal model by stimulating the endometrium epithelial cell proliferation. Besides, the number iron would increase the NO production, thus induce the macrophage to fail to phagocytose the endometrium lesion. An excessive iron that cause the formation of oxidative stress is due to the dysregulation of ROS detoxification signaling. Retrograde menstrual which has stimulated by the oxidative stress hyperactivation would activate the ERK, PI3K/AKT/mTOR signaling pathways. That activated signaling pathways would stimulate the adhesion formation, angiogenesis, and endometrium lesion proliferation.[10]

The presence of the iron-rich erythrocytes, the endometrium apoptosis tissues, and the debris cells in the pelvic cavity has stimulated the ROS formation in the mitochondria. ROS hyperactivity then stimulates the increase of the macrophage level in secreting the cytokines like TNF-α and IL-6. Those cytokines could activate the ERK, and PI3K/AKT/mTOR signaling pathways by binding into the receptor in the endometrium lesion membrane, like TNFR, and IL-6 receptor. That signaling pathway is the way for cell adhesions, angiogenesis, and proliferation of endometrium lesion. ROS production which has excessive also could cause cell function failure. It has happened by the alteration of the gene expression profile through the NF-κβ activation and made the upregulation signaling cascade for increasing the inflammation cytokine secretion in the endometrium lesion. As a result, the endometriosis progression has increased.[4]

The oxidative stress in the endometrium lesion microenvironment could cause the activation of p38 MAPK and stimulate the early aging of the fetal tissues. Furthermore, oxidative stress activates the ERK signaling and increases the endometriosis progression. Embryonic stress also can be occurred in endometriosis which stimulated by oxidative stress. The embryonic stress has characterized by the increase of TNF-α and IL-1β that in a high level. High cytokine levels could activate the NF-κβ then the placenta apoptosis can occur. The Forkhead Box O3a or FOXO3a also has activated the NF-κβ pathway through the IKK phosphorylation for degrading the Inhibitor Kappaβ or Iκβ then the NF-κβ transcription factor could translocate into the nucleus and activate the gene expression that related to the inflammation and the apoptosis response. Besides through the directly IKK phosphorylation, FOXO3 also could activate BCL10 for degrading the IKK, then NF-κβ activated. [40,41]

The body has maintained the homeostasis of the oxidant and an anti-oxidant level during pregnancy. TNF-α during that time has increased and the progesterone level alteration has occurred. First, TNF-α has activated the cell signaling pathway complex through the second messenger or cAMP like Keap1-Nrf2, NF-κβ, and MAPK signaling pathways. Then increased the cytokine level and the genes expression alterations related to the antioxidant. When the FOXO3 has increased, that condition could stimulate the binding of Keap1-nrf2 and cause the antioxidant level to decrease. Then, Iκβ has stimulated the NF-κβ released. It could cause apoptosis and cytokine levels to increase. Accordingly, the alteration mechanism of the FOXO family has occurred in increasing oxidative stress, although it has also a role in the reproductive system effects. Another pathway that has included in oxidative stress into apoptosis by FOXO is the JNK pathway. It could make the FOXO dephosphorylation through the cAMP then induced the FOXO1 to translocate into the nucleus and stimulated the apoptosis.[41] Based on the mechanism of oxidative stress dysregulation in endometriosis, several molecules are involved such as TNF-α, NF-κβ, and IL-6 which can trigger inflammation. NF-κβ activation is also involved in the dysregulation of apoptotic signals that prevent endometriotic cell apoptosis. So that it can be strongly suspected that

immune system dysregulation, apoptotic signal and oxidative stress dysregulation are correlated each others, which can be spearheaded by inflammation occurs in immune system dysfunction.

CONCLUSION

The signaling dysregulation that occurs in endometriosis has a role in the development of endometriosis. Three main mechanisms have been reported for dysregulation during the development of endometriosis. Namely immune system signaling dysfunction, apoptotic dysfunction, and dysregulation of oxidative stress signaling. Retrograde menstruation has been a long-held theory in pathogenesis of endometriosis. Retrograde menstrual tissue migrates into the pelvic cavity and outside of the pelvis leading to endometriotic lesions. Thus, three mechanisms have been supported and compatible with the theory of retrograde menstruation.

Some immune cells such as macrophages have increased in the peritoneal fluids then stimulate the secretion of proinflammatory cytokines (IL-6, IL-8), and angiogenesis factors (PDGF, VEGF). NK cells in endometriosis have suppressed cytotoxic T cells so that the lesion has not cleared up. Th2 cells have differentiated into T-reg cells to suppress activation of T cells, macrophages, and B cells in endometriosis. In addition, hormonal and epigenetic factors also induce immune dysregulation through inflammation. Dysregulation of apoptotic signaling has occurred in both the intrinsic and extrinsic pathways which can then stimulate the survival of endometriosis lesion to proliferate. NCOA-1/ESR2 can bind the ASK1, caspase-8, and caspase-9 then suppress the intrinsic apoptotic pathway, whereas activated Akt and NF-κβ can inhibit the intrinsic apoptotic pathway.

Dysregulation of oxidative stress signaling has occurred in endometriosis due to an imbalance in ROS production. Furthermore, this imbalance condition has suppressed macrophage cells to phagocytize endometrial lesions from retrograde menstrual tissue through activation of the ERK pathway, PI3K/AKT/mTOR leading to the development of endometriosis.

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