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Analysis of Interleukin-6 level in Serum and Histopathology Changes of Cardiovascular Tissue on Male Wistar Rat with Nonalcoholic Fatty Liver Disease (NAFLD)

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ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic hepatopathy and a global health issue. Interleukin-6 (IL-6) in liver pathology is very complex, and its participation in the development of NAFLD remains unclear. This study aimed to analyze the relationship between NAFLD and IL-6 as one of inflammaging biomarkers along with CVD histopathology.

Methods: This study used a post-test control group design. The subject of this study was 14 male Wistar rats and divided into two groups. The control group was given standard diet and the NAFLD-modelling group was given 8 weeks high-fat-high-fructose diet. Both groups will be terminated at the 8th week for organ histopathological examination.

Results: The IL-6 levels in NAFLD rats (16.14±6.46ng/ml) and non-NAFLD rats (11.28±3.49ng/ml). The blood vessel wall thickness of NAFLD (1.47±0.33 µm) and non-NAFLD rats (1.70±0.28 µm). The diameter of the blood vessels of NAFLD rats (14.06±3.30 m) and non-NAFLD rats (15.66±1.02 m). It was found that there were differences in cardiac histopathology (hemorrhagic (p:0.003), degenerated (p:0.036), and hyperemic(p:0.003)) between NAFLD and non-NAFLD modeling rats. As for IL-6 levels, blood vessel wall thickness, diameter, and heart histopathology (necrosis) there was no difference between NAFLD and non-NAFLD modeling rats (p>0.05).

Conclusion: It has shown that NAFLD rats significantly caused cardiac histopathological changes. The levels of IL-6 tend to be higher in NAFLD rats but not significant, as well as the diameter of coronary artery shows a small tendency in NAFLD rats.

Keywords: Interleukin-6 (IL-6), Cardiovascular Histopathology, Non-alcoholic Fatty Liver Disease (NAFLD), Wistar.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic hepatopathy and a global health issue. It is estimated that NAFLD has affected a billion people worldwide and the highest prevalence has been reported consecutively in Middle East, South America, and Asia. Its global prevalence was estimated to be approximately 25%.[1,3] NAFLD is characterized by the presence of pathologic accumulation of fat in the liver with >5% of hepatocytes containing visible intracellular triglycerides (TGs), or steatosis affecting at least 5% of the liver volume or weight, in the absence of significant alcohol consumption and other specific causes of fatty liver disease, including hepatitis C, lipodystrophy, medications, and inherited metabolic disorders.[1,2]

Cardiovascular disease (CVD) is the leading contributory cause of death in subjects with NAFLD, and more severe forms of liver disease were associated with increased risk of CV morbidity and mortality. Nevertheless, current knowledge on the relationship between NAFLD and cardiac metabolism, structure, and function is still incomplete, and the most effective strategies to reduce the burden of CVD associated with NAFLD remain to be defined.[1]

NAFLD and CVD are both manifestations of end-organ damage of the metabolic syndrome. However, a specific contribution of NAFLD to increase CVD risk is difficult to distinguish from the combination of these shared risk factors. The underlying mechanisms linking NAFLD to CVD are very complex and simultaneously involve several different pathways. Dysfunctional visceral adipose tissue, also an increased accumulation of dysfunctional ectopic fat in the liver and

other organs such as the pericardium, pancreas, kidneys, or skeletal muscle, are closely related to adverse cardiometabolic outcomes. The accumulation of visceral and ectopic fat and the subsequent release of fat-derived toxic metabolites along with activation of inflammatory pathways instigates a cluster of local and systemic pathophysiological changes that ultimately leads to the development of both NAFLD and cardiovascular diseases, possibly via mechanisms beyond overweight and obesity.[3]

The role of interleukin-6 (IL-6) in liver pathology is very complex and its participation in the development of NAFLD remains unclear. IL-6 activates several cells, such as immune cells, hepatocytes, hematopoietic stem cells, and osteoclasts. IL-6 was initially considered as hepatoprotector in liver steatosis, capable of reducing oxidative stress and preventing mitochondrial dysfunction. Nevertheless, IL-6 is a key element in acute phase response, mediating the synthesis of several acute-phase proteins, such as C-reactive protein and serum amyloid A. Thus, we cannot exclude the possibility that IL-6 might also play an indirect deleterious role in NAFLD pathogenesis. In addition, IL-6 is considered a predictor marker of insulin resistance and cardiovascular diseases. In patients undergoing bariatric surgery, decreased IL-6 concentrations were associated with weight loss and insulin resistance improvement. Recently, Mas and co-workers showed that diet-induced-NASH was reduced in IL-6 knockout mice as compared to the control group. A positive correlation was found between the expression of IL-6 in hepatocytes and the severity of NAFLD in humans with NASH.[4] This study aimed to analyzes the relationship between NAFLD and IL-6 as one of the inflammaging biomarkers along with CVD histopathology examination.

MATERIAL AND METHODS

Experimental Design

This study was an experimental study on male Wistar rats with a post-test with a control group design. Male Wistar rats will be divided into two groups, the control group consists of rats that were given a standard diet and the group of rats that were given a High Fat High Fructose Diet (HFHFD) for NAFLD modeling. Both groups will be observed for eight weeks. Before the study, Wistar rats were acclimatized for seven days.

Animal Subject

The sample of this study was male Wistar (*Rattus norvegicus*) rats obtained from the Veterinary Laboratory of the Faculty of Medicine, Universitas Hasanuddin. Fourteen male Wistar rats, 8-12 weeks of age (weight of 200-250gr), were divided into two groups. Group A consists of seven rats given standard feed as a control group. Group B consists of seven rats given HFHFD as a group of NAFLD modeling. Both groups will be terminated at the 8th week for blood serum and organ histopathological examination.

Histopathology Preparations

The cardiac and coronary artery tissues of rats were prepared for histological observations using the paraffin method. They were fixed in 10% buffered formalin, processed for paraffin sectioning, and sectioned at 4um. The next step was dehydration using graded alcohol (80%, 90%, and 95%) for 2 hours of each concentration. Then, the tissues were flushed using xylol I and II for 60 minutes and followed with the infiltration process using the paraffin. Furthermore, deparaffinization was done using xylol I and II for 5 minutes of each and graded alcohol (95%, 90%, and 80%) for 2 minutes of each and rinsed with distilled water. The staining process was using Hematoxylin-Eosin. Slides were put into Hematoxylin for 2 minutes and rinsed using water. The next step was to put the slides into eosin for 5 minutes, then graded alcohol (80%, 90%, and 95%) for 2 minutes and xylol I and II for 2 minutes of each. The last step was the mounting process, which is a process that applies the adhesive substance to the preparation and covering it with a cover glass. The thickness of the coronary artery wall was measured from the tunica intima to tunica adventitia, while the diameter of the coronary lumen was measured from one tunica intima to another tunica intima. All slides were observed under an Olympus CX31 microscope equipped with a photomicrograph at 40x magnification for coronary artery tissues, and 400x magnification for heart tissues.[16]

The parameters for cardiac histopathology examination are hemorrhagic, degeneration, and necrosis findings. The assessment was performed based on the average view in 10 fields using 400x magnification. The results were classified into three categories, specifically mild (1-10 per observation field), moderate (11-20), and severe (>20).

Data Analysis

We used independent sample T-Test, Mann Whitney, and Fisher Exact Test to evaluate the significant difference between control and experimental groups. Spearman and Pearson Correlation test to analyze the relationship between variables. We used an alpha value of 0.05 as the significance level. The data was analyzed using SPSS version 21 software.

RESULTS

The overview of Interleukin-6 serum levels and histopathology of cardiovascular tissue examination of Wistar rats can be seen in Table 1. Average serum IL-6 levels were 13.71±5.59 ng/mL, the average wall thickness of coronary artery was 1.58±0.32 µm, and the average lumen diameter of artery coronary was 14.86±2.49 µm. Cardiac histopathology examination showed grade 3 hemorrhages (42.9%), grade 0 degeneration (50%), grade 0 hyperemia (42.9%), and grade 0 necrosis (85.7%).

The analysis results showed that there were differences in cardiac histopathology among NAFLD and non-NAFLD rats which included hemorrhage (p:0.003), degeneration (p:0.036), and hyperemia (p:0.003). As for IL-6 serum levels, coronary artery wall thickness and lumen diameter, and cardiac histopathology (necrosis percentage) were no differences between NAFLD and non-NAFLD modeling rats (P>0,05) as seen in table 2.

According to the correlation test, IL-6 level was negatively correlated with the lumen diameter of the coronary artery (p=0.038; r=-0.559), which indicates that the lumen diameter would decrease along with the increase of IL-6 level with a strong correlation. However, the thickness of coronary artery walls and cardiac histopathology findings (hemorrhagic,

Table 1. Overview of Interleukin-6 Serum and Cardiac and Coronary Artery Tissue Histopathology Examination.

Variable	n	%	Mean±SD	Min-Max
Cytokine				
IL-6			13.71±5.59	7.00-30.00
Artery Coronary Histopathology Examination				
Wall thickness (µm)			1.58±0.32	1.11-2.09
Lumen Diameter (µm)			14.86±2.49	9.79-18.96
Cardiac Histopathology Examination				
Hemorrhage				
0	6	42.9		
1	1	7.1		
2	1	7.1		
3	6	42.9		
Degeneration				
0	7	50.0		
1	2	14.3		
2	3	21.4		
3	2	28.6		
Hyperemia				
0		42.9		
1		7.1		
2		21.4		
3		28.6		
Necrosis				
0		85.7		
1		14.3		

Table 2. Differences in IL-6 levels, Cardiac and Coronary Artery Histopathology Examination of Wistar Rats with NAFLD modeling.

Variable	NAFLD		P Value
	Yes (7)	No (7)	
Cytokine			
IL-6 (ng/mL)	16.14±6.46	11.28±3.49	0.106*
Coronary Artery Histopathology Examination			
Wall Thickness	1.47±0.33	1.70±0.28	0.184*
Lumen Diameter	14.06±3.30	15.66±1.02	0.259*
Cardiac Histopathology Examination			
Hemorrhage			
0	0 (0.0%)	6 (85.7%)	0.003**
1	0 (0.0%)	1 (14.3%)	
2	1 (14.3%)	0 (0.0%)	
3	6 (85.7%)	0 (0.0%)	
Degeneration			
0	1 (14.3%)	6 (85.7%)	0.036**
1	1 (14.3%)	1 (14.3%)	
2	3 (42.9%)	0 (0.0%)	
3	2 (28.6%)	0 (0.0%)	
Hyperemia			
0	0 (0.0%)	6 (85.7%)	0.003**
1	0 (0.0%)	1 (14.3%)	
2	3 (42.9%)	0 (0.0%)	
3	4 (57.2%)	0 (0.0%)	
Necrosis			
0	6 (85.7%)	6 (85.7%)	1.000***
1	1 (14.3%)	1 (14.3%)	

* Independent Sample T Test
**Mann-Whitney
***Fisher Exact Test

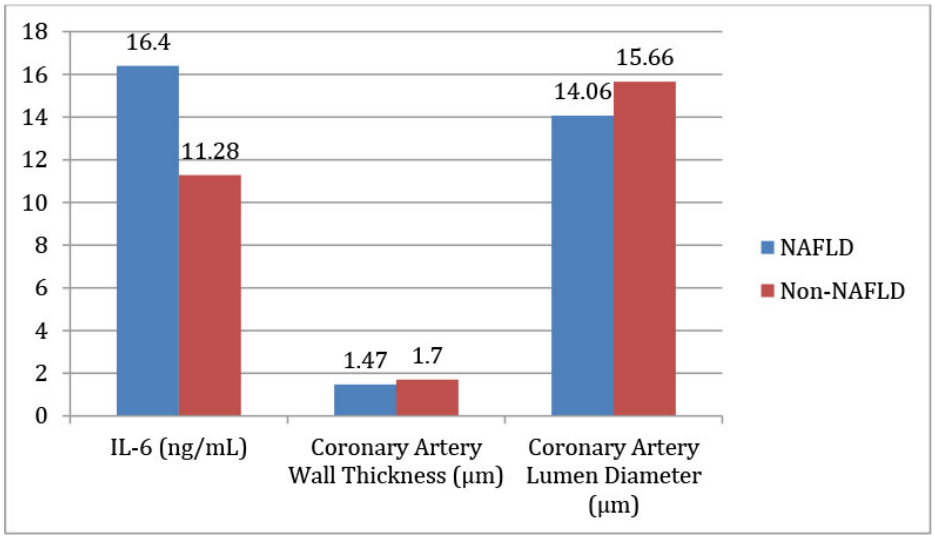

Figure 1. IL-6 Serum and Coronary Artery Histopathology Examination of Wistar Rats with NAFLD.

Table 3. Correlation between IL-6 serum and Cardiovascular Histopathology Examination in NAFLD rats.

Groups	Correlattion	
	r	p
IL-6 vs Coronary Artery Wall Thickness	-0.001	0.997*
IL-6 vs Coronary Artery Lumen Diameter	-0.559	0.038*
IL-6 vs Hemorrhage in Cardiac Histopathology	0.264	0.362**
IL-6 vs Degeneration in Cardiac Histopathology	0.474	0.087**
IL-6 vs Hyperemia in Cardiac Histopathology	0.200	0.493**
IL-6 vs Necrosis in Cardiac Histopathology	-0.231	0.428**

*Pearson Correlation
**Spearman Correlation

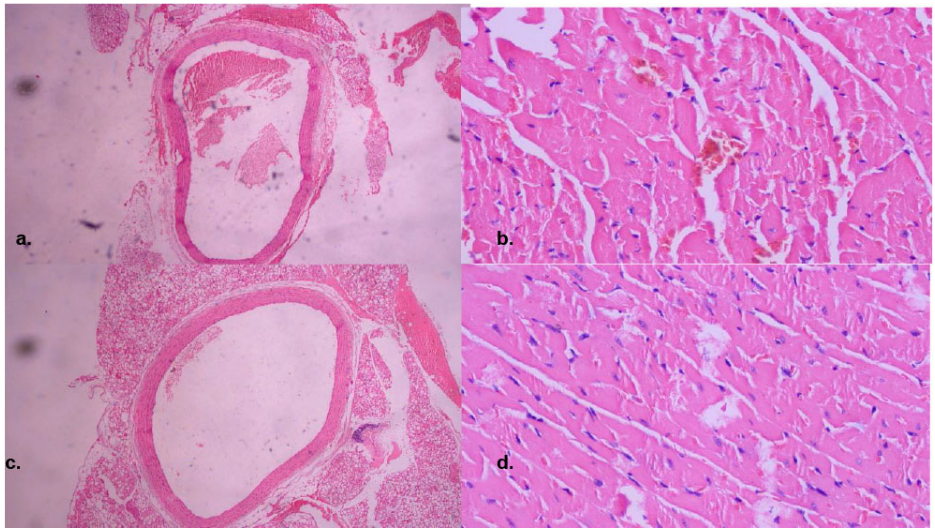

Figure 2. Cardiac and Coronary Artery Histopathology Examination of Wistar Rats NAFLD modeling: a. Non-NAFLD coronary artery (40x magnification), b. Non-NAFLD Cardiac Histopathology (400x magnification, no sign of Hemorrhage, Degeneration, Hyperermia and Necrosis), c. NAFLD coronary artery, d. NAFLD Cardiac Histopathology (400x magnification).

degeneration, hyperemia, and necrosis) did not correlate with IL-6 levels (p>0.05) as seen in table 3.

DISCUSSION

The mechanisms by which NAFLD increases CVD risk are very complex and involve simultaneously different pathways at different functional and structural levels, namely the metabolism, cardiovascular system, and liver function. Insulin resistance is recognized as the main determinant of NAFLD pathogenesis. However, the mechanism of how NAFLD is responsible for accelerated CVD remains unclear.[5]

Increased circulating markers for systemic inflammation are associated with NAFLD. IL-6 levels were increased along with histological severity, though not consistent. The hepatic expression of IL-6 was also related to NAFLD, though lost significance results may occur when adding metabolic risk factors.[6] The present study evaluated that the IL-6 levels were higher in NAFLD rats compared to the control group, even though the results are not significant. This study was in line with Mohamed AA, et al. study, which reported that NAFLD patients have higher IL-6 levels compared to their control group in particular and did not have significant results.[4] This finding shows that IL-6 levels has some tendency to increase as an inflammatory mediator in NAFLD rats.

Analysis on cardiac histopathology examination shows a significant difference in hemorrhagic (p: 0.003), degeneration (p:0.036), and hyperemia (p:0.003) between NAFLD and control group rats, hence no significant difference on coronary arteries histopathology examination, such as vascular wall thickness and lumen diameter (p>0.05). This result was in line with an experimental study by Aisyah S, et al in 2014 that also have histopathological changes of heart tissue of Wistar rats using cooking oil as their induced diet. They stated that abnormal accumulation of intracellular lipid causes cell toxicity as known as lipotoxicity. Lipids penetrate through the cell membrane with fatty acid transporter proteins and cellular damage resulting in fat infiltration. Fat cells penetrate through cell membranes and the accumulation of intracellular lipid cells

occurs between parenchymal cells of an organ, one of which is cardiac muscle cells. It is likely a result of the transformation of tissue cells at the interstitial junction into lipid cells.[17]

The present study showed a significant difference in cardiac histopathology examination in NAFLD rats, such as hemorrhagic, degeneration, and hyperemia. According to Cai et al, NAFLD promotes CVD development through some mechanisms, including lipid and glucose metabolism, immunologic homeostasis, and oxidative stress. The induced ROS will overwhelm the antioxidant system in the liver and spill over into the circulation, which leads to amplified oxidative stress in the systemic circulation.[10] Increased production of circulating oxidizing species can damage cellular components and modify circulating metabolites (e.g., lipid proteins and fatty acids) to generate dysfunctional metabolites and pathogenic molecules, which contributes to endothelial dysfunction, impaired vasoreactivity, and atherogenicity potential. The liver is the main organ producing homocysteine in the methionine cycle. Elevation of circulating homocysteine levels was reported in NAFLD, which induces oxidative damage in cardiomyocytes and endothelial layers.[11]

A systematic review and meta-analysis of 34 studies showed that NAFLD was associated with an increased risk for CVD incidents and specifically for coronary artery disease and hypertension. However, it was not associated with CVD-related mortality and overall mortality when compared to patients without NAFLD. A meta-analysis of 16 cohort studies with median 7-year follow-up revealed that patients with NAFLD were 64 times more likely than patients without NAFLD to have fatal or nonfatal cardiovascular events, such as myocardial infarction, stroke, angina, or coronary revascularization. An additional analysis of 1051 Framingham Heart Study participants revealed that patients with increased liver fat on multidetector computed tomography at baseline experienced higher incident cardiovascular risk factors, including hypertension (odds ratio [OR], 1.42; $P < 0.001$) and T2DM (OR, 1.43; $P < 0.001$).[7]

We did not find a significant difference between coronary artery wall thickness & diameter and their average value on NAFLD rat's coronary walls were thinner but the lumen diameters were narrower than non-NAFLD rats. Multiple levels of evidence support the idea that NAFLD is a major mediator of subclinical atherosclerosis and atherosclerotic CVD events. It is important to detect the carotid artery intima-media thickness and plaque presence as the risk of coronary heart disease. Targher et al, reported that the NAFLD severity among 85 patients that were classified based on liver histopathology was strongly associated with early carotid atherosclerosis, regardless of other classic CVD risk factors.[9]

Based on the correlation test, we found that the IL-6 levels were negatively correlated with the coronary artery lumen diameter ($p=0.038$; $r=-0.559$), where the diameter would decrease with IL-6 levels enhancement with strong correlation power. This result would be in line with a meta-analysis including 27 studies that concluded that NAFLD was independently associated with subclinical atherosclerosis even after adjusting for traditional risk factors such as age, sex, BMI, smoking, LDL cholesterol, insulin resistance, and metabolic syndrome. Furthermore, studies reported that carotid plaques were present more frequently in NAFLD patients.[13,14,15]

CONCLUSION

There is a relationship between NAFLD and cardiovascular disease, which is characterized by significant changes in the histopathological examination of the heart. In addition, the examination of IL-6 serum can be used as a marker of cardiovascular disease risk in NAFLD patients. Expanded sample and time experiment needed in further studies.

AUTHOR CONTRIBUTIONS

All authors contribute equally in the writing of this article.

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

RESEARCH ETHICS

Ethical clearance was approved by the Committee of Health Research Ethics Universitas Hasanuddin, under letter number 175/UN4.6.4.5.31/PP36/2021.

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