

Original Research Article

Development of atherosclerotic model through margarine diet induction in Wistar rats

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Abstract

Purpose: To develop and analyze the relationship between dyslipidemia, inflammation, and oxidative stress in the progression of atherosclerosis.

Methods: This study was a randomized controlled trial (RCT) on male Wistar rats (*Rattus norvegicus*) induced with a high-fat diet (margarine supplementation) for 4 weeks. A total of 12 male Wistar rats were randomly divided into control and study groups ($n = 6$ in each group). Control group received standard diet for 4 weeks, while the study group received a margarine-based high-fat diet to induce atherosclerosis over the same period. After 4 weeks, the rats were euthanized under anesthesia. Lipid profiles (total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides), inflammatory biomarkers (TNF- α), oxidative stress markers (reactive oxygen species; ROS), foam cell formation, and tunica intima thickness were assessed and compared using unpaired t-test. Regression analysis was performed to assess the relationship between LDL and TNF- α . $P < 0.05$ was considered statistically significant.

Results: The study group exhibited significantly higher levels of total cholesterol, LDL, and triglycerides, and reduced HDL levels compared to control group ($p < 0.05$). Also, foam cell formation, tunica intima thickness, ROS levels, and TNF- α expression were higher in the study group compared to control group. Regression analysis showed a strong positive correlation between LDL and TNF- α ($r = 0.73$, $p < 0.05$).

Conclusion: Margarine diet induces dyslipidemia and contributes to the progression of atherosclerosis. Therapeutic interventions that target a decrease in lipid levels, as well as modulation of inflammation and oxidative stress, may be potential targets for preventing and treating atherosclerosis.

Keywords: Atherosclerosis, Dyslipidemia, TNF- α , ROS, Margarine, High-fat diet

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INTRODUCTION

Atherosclerosis is a long-term condition marked by lipid buildup, inflammation, and cellular proliferation within the arterial walls, potentially resulting in disability and mortality [1-4]. Primary clinical manifestations of atherosclerosis include cardiovascular diseases (CVD), such as

coronary artery disease, peripheral artery disease, deep vein thrombosis (DVT), and cerebrovascular conditions like stroke [1,5-7].

Global Burden of Disease (GBD) reports that deaths due to cardiovascular disease (CVD) rose from 12.4 million in 1990 to 19.8 million globally in 2022 [5]. The World Health Organization

(WHO) reported that CVD-related deaths totaled 17.8 million in 2019 and are expected to increase to 23 million by 2030, driven by the rising prevalence among younger populations [4,8]. Atherosclerosis progresses through a complex process involving endothelial dysfunction, oxidized lipoprotein accumulation, macrophage infiltration, and atherosclerotic plaque formation [2,6].

Although many studies have been conducted to understand the mechanisms of atherosclerosis, more representative experimental models are needed to evaluate new therapies and further understand the disease pathogenesis. Animal models have been widely used to mimic atherosclerosis progression in humans. Common models include Apolipoprotein E / ApoE and Low-Density Lipoprotein Receptor/LDLr mice, which tend to develop atherosclerosis when fed a high-fat diet and cholesterol. Furthermore, models induced through the administration of high-fat diets or injections are frequently used to simulate chronic inflammatory and hyperlipidemia conditions that contribute to the development of atherosclerosis [9].

The success of this model is evaluated through histopathological analysis, in particular by calculating the number of foam cells and measuring the thickness of the arterial intima tunica. Foam cells are lipid-filled macrophages due to oxidized LDL phagocytosis (ox-LDL), and are a typical early marker of atherosclerotic lesion formation. An increase in the number of foam cells reflects an active and chronic inflammatory process. However, the thickness of the intima tunica indicates vascular remodeling and plaque development. Thickening of the intima layer indicates the progression of atherosclerosis lesions and endothelial dysfunction. These two parameters provide quantitative and morphological evidence relevant to atherosclerosis. Also, assessment of inflammatory biomarkers, including TNF- α and reactive oxygen species (ROS), is vital. Reactive oxygen species (ROS) is crucial in the inflammatory process by triggering activation of NF- κ B, which subsequently enhances the production of tumor necrosis factor-alpha (TNF- α), interleukin – 6 (IL-6) and C-reactive protein (CRP) [10].

The margarine diet model is clinically relevant as it mimics key human atherosclerosis risk factors, including high-fat diet consumption, hyperlipidemia, and inflammation. Also, this model is used to test the efficacy of novel therapeutic agents, such as lipid-lowering drugs, and anti-inflammatory, and nanoparticle-based

therapies. Although various models have been developed, each approach has its advantages and limitations. Therefore, this study aimed to develop and evaluate an atherosclerotic model in Wistar rats using margarine diet administration. This model is expected to provide a better picture of the histopathological changes and inflammatory biomarkers involved in atherosclerosis, as well as contribute to the development of more effective prevention and therapeutic strategies.

EXPERIMENTAL

Study design

This *in vivo* study employed a randomized post-test design to establish an atherosclerotic model in male Wistar rats (*Rattus norvegicus*) by administering a margarine-based diet.

Animals

A total of 12 adult male Wistar rats (*Rattus norvegicus*; aged 8 – 12 weeks) weighing 180 – 220 g were randomized into study and control groups (n = 6 in each group). The animals were housed in cages to acclimatize for 1 week under standard conditions (12-hour light/dark cycle, temperature of 22 $\pm$ 2 °C, and humidity level of 50 – 60 %) Control group received COMFEED AD II standard diet for 4 weeks, while study group received high-fat diet (margarine-based) to induce atherosclerosis over the same period. Margarine used contained 36 % saturated fat and < 1 % trans-fat per label specification. Feed intake was monitored daily and averaged 15 g/rat/day. Wistar rats were chosen for their well-characterized response to a high-fat diet and translational relevance to human lipid metabolism. Rats that died or developed diseases other than atherosclerosis were excluded. The study received ethical approval from The Research Ethics Committee of the Faculty of Medicine, University of Muhammadiyah Malang (approval no. E.5.a/181/KEPK/-UMM/VI/2024), and was conducted in compliance with the internationally accepted guide for the Care and Use of Laboratory Animals [11].

Atherosclerosis induction

Control Group (n = 6) was administered a standard COMFEED AD II diet along with distilled water for four weeks. Study group (n = 6) was fed a high-fat diet consisting of COMFEED AD II mixed with margarine in a 9:1 ratio for 4 weeks. Each rat was estimated to consume 15 g of feed per day. Since each cage housed six

rats, the total daily feed required was 90 g (diet consisted of 10 g margarine (Blue Band) per 90 g feed (10 % margarine by weight). Baseline lipid profiles were assessed before induction to obtain normal levels.

Determination of bodyweight

The body weight of animals was measured weekly.

Lipid profiles

After 4 weeks, blood samples (2 mL) were obtained through cardiac puncture, centrifuged, and lipid profiles (total cholesterol, LDL, HDL, triglycerides) quantified using a spectrophotometer and blood chemistry analyzer (ICHEM-I UBIO 535).

Reactive oxygen species (ROS)

Oxidative stress was assessed using a serological assay (ROS1/ROS ELISA Kit; Sandwich ELISA; LS Bio, Catalog no. LS-F9759).

Foam cell formation, tunica intima thickness, and expression of TNF- α

Histological analysis of carotid artery sections was examined for foam cell formation and tunica intima thickness. After 4 weeks, the rats were euthanized under anesthesia, followed by exsanguination. Carotid arteries were harvested and preserved in 10 % formalin. Tissue samples were fixed in 10 % neutral-buffered formalin for 24 – 48 h at room temperature to ensure proper fixation and preservation of tissue morphology for histological analysis. Hematoxylin and Eosin (H&E) staining was conducted to evaluate foam cell formation and thickness of the tunica intima. Immunohistochemistry was conducted to evaluate TNF- α expression using TNF- α antibodies (Abcam-EPR 19147). Foam cell count, tunica intima thickness, and TNF- α

expression were measured using a Nikon Eclipse Ei light microscope.

Statistical analysis

Data was analyzed using Statistical Package for the Social Sciences (SPSS version 22.0, IBM, Armonk, NY, USA). Results were expressed as mean $\pm$ standard deviation (SD) and compared using unpaired t-test. Pearson correlation analysis was used to evaluate the relationship between LDL levels and TNF- α expression. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in body weight, and lipid profile

The Shapiro-Wilk test confirmed that the data followed a normal distribution ($p > 0.05$), while Levene's test indicated that the variance was homogeneous ($p > 0.05$), thus the unpaired t-test could be used for comparison. During the 4 weeks, study group showed significantly greater weight gain compared to control group ($p < 0.05$). Levels of total cholesterol, LDL, and triglycerides were significantly higher, while HDL levels were significantly lower in study group compared to control group ($p < 0.05$; Table 1).

Post indicated that the measurements (LDL, HDL, triglycerides, and cholesterol) were taken at the end of the experimental period. The term 'post' was used to distinguish these final values from any potential baseline or intermediate measurements, emphasizing that lipid profiles were collected after treatment completion.

Foam cell formation, tunica intima thickness, expression of TNF- α and ROS

Study group showed a non-significant increase in ROS levels, TNF- α expression, foam cell count and tunica intima thickness compared to control group ($p > 0.05$; Table 2).

Table 1: Changes in body weight, and lipid profile (mean $\pm$ SD, n = 6 in each group)

Variable	Group	Mean $\pm$ SD	P-value
Weight (g)	Control	202.93 $\pm$ 9.85	0.001
	Study	223.40 $\pm$ 4.16	
Chol post (mg/dL)	Control	51.74 $\pm$ 9.22	0.000
	Study	98.40 $\pm$ 9.84	
LDL post (mg/dL)	Control	47.37 $\pm$ 17.40	0.000
	Study	147.57 $\pm$ 13.23	
TG post (mg/dL)	Control	49.37 $\pm$ 17.40	0.000
	Study	147.57 $\pm$ 13.23	
HDL post mg/dL)	Control	44.00 $\pm$ 9.80	0.021
	Study	32.33 $\pm$ 3.76	

Table 2: Foam cells, tunica intima thickness, and ROS (mean $\pm$ SD, n = 6 in each group)

Variable	Group	Mean $\pm$ SD	P-value
Foam cell (cells per microscopic field)	Control	24.33 $\pm$ 1.74	0.064
	Study	20.20 $\pm$ 4.22	
Thickness TI (μ m)	Control	40.90 $\pm$ 9.24	0.470
	Study	44.37 $\pm$ 7.41	
TNF- α (pg/mL)	Control	20.92 $\pm$ 3.69	0.308
	Study	31.47 $\pm$ 3.39	
ROS (U/mL)	Control	194.50 $\pm$ 25.92	0.173
	Study	242.95 $\pm$ 72.78	

Histopathology and immunohistochemistry of carotid arteries

Tunica intima

Study group showed thickened tunica intima compared to control group ($p < 0.05$; Figure 1).

Foam cell

Study group showed higher number of foam cells compared to control group ($p < 0.05$; Figure 2).

Tumor necrosis factor – alpha (TNF- α) expression

The study group showed increased TNF- α expression compared to control group (Figure 3).

Correlation between LDL and TNF- α levels

A strong positive correlation ($r = 0.73$, $p < 0.01$) was found between LDL and TNF- α levels, confirming that increased LDL is closely related to increased inflammation which is a trigger for atherosclerosis (Figure 4).

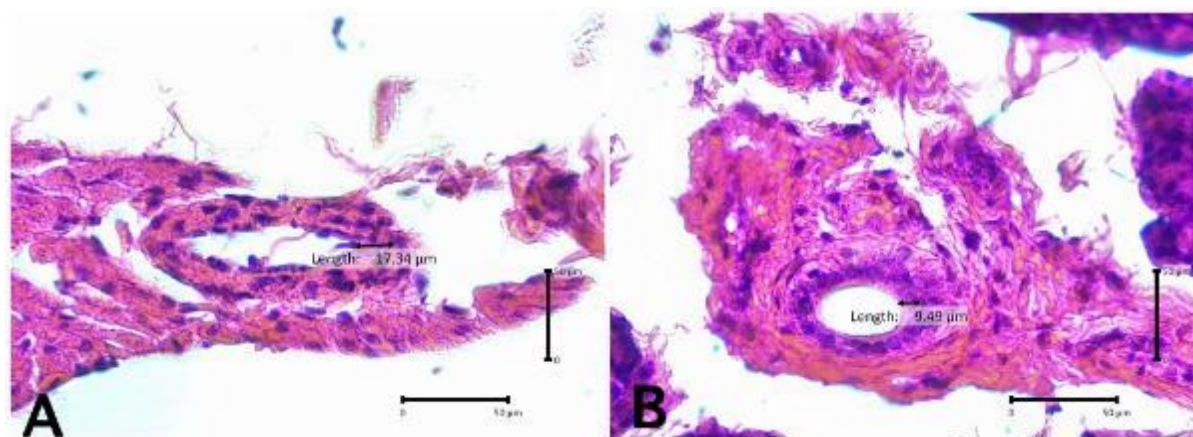


Figure 1: Histo-architecture of the tunica intima. A: Study group, B: Control group

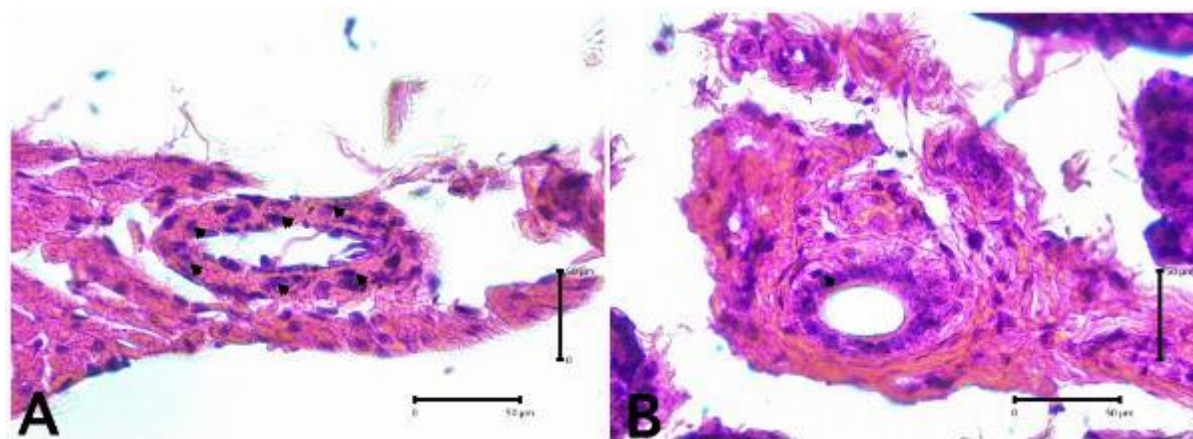


Figure 2: Histo-architecture of the Foam cell. A: Study group, B: Control group

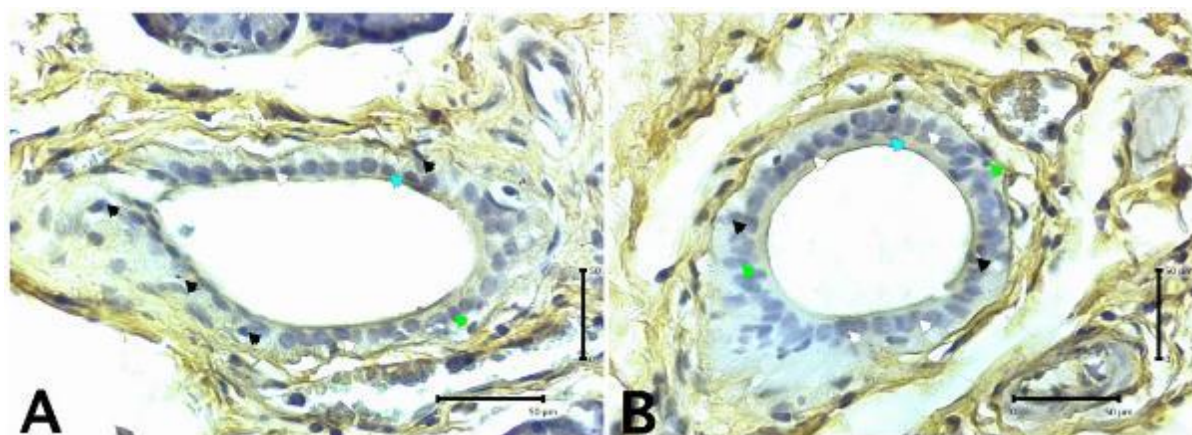


Figure 3: Histo-architecture of TNF- α expression foam cell. A: Study group, B: Control group

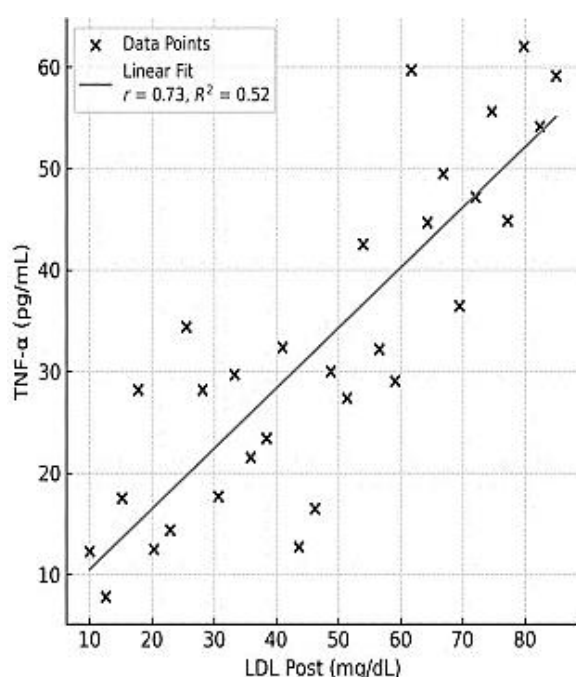


Figure 4: Correlation of LDL and TNF α -levels

DISCUSSION

The atherosclerosis model used in this study effectively replicates the physiological and histopathological characteristics of atherosclerosis. The induction of hyperlipidemia through a margarine diet led to increased body weight, dyslipidemia, and inflammation in study group compared to control group. These results are in line with previous research which showed that a high-fat diet triggered lipid accumulation in the arteries and increased vascular inflammation [13]. Margarine contains trans-unsaturated fatty acids, which negatively affect lipid profiles by lowering HDL while increasing LDL cholesterol and triglyceride (TG) [14,15].

Fatty acids naturally occur as esters in triglycerides and undergo hydrolysis to produce either saturated or unsaturated fatty acids, depending on the presence of double carbon bonds. Unsaturated fatty acids, which contain double bonds, exist in two structural forms, namely *cis* and *trans* isomers. Natural unsaturated fatty acids are predominantly *cis* fatty acids, but *trans* isomers constitute only a small proportion. However, processing methods such as hydrogenation and high-temperature heating significantly increase *trans* fatty acids (TFA) content in foods, particularly margarine [15].

Previous studies show that the presence of TFA in food is strongly associated with adverse health outcomes, particularly coronary heart disease [16]. Negative effects of TFA surpass those of saturated fatty acids and cholesterol [17]. Epidemiological studies have recognized *trans* fatty acids (TFA) as a major risk factor for coronary heart disease, as it raises LDL levels (similar to saturated fatty acids) and reduces HDL, a function not observed with saturated fats. Additionally, TFA promotes the formation of atherogenic lipoproteins, such as lipoprotein (a), thus increasing its negative impact beyond that of saturated fats or high cholesterol intake [14,15].

Lipid profile analysis revealed a significant increase in total cholesterol, LDL, and triglyceride levels, along with a decrease in HDL, indicating dyslipidemia. Dyslipidemia, a lipid metabolism disorder characterized by imbalances in plasma lipid fractions, is a major risk factor for atherosclerosis [18]. The key lipid components involved in this process are total cholesterol, triglycerides, LDL, and HDL. During atherosclerosis progression, dyslipidemia promotes lipid accumulation in the bloodstream, which leads to structural and functional

endothelial changes [14,19]. Dyslipidemia also contributes to endothelial dysfunction, which results to inflammation and an immune response that encodes pathogens called macrophages. These macrophages engulf oxidized LDL (ox-LDL, formed when LDL undergoes oxidation by free radicals) for capturing by scavenger receptors, such as LOX-1, SRA, and CD36. LOX-1, an oxidized low-density lipoprotein (ox-LDL) receptor on the endothelium, is upregulated by TNF- α . Oxidized LDL is internalized by the cells following binding to LOX-1, leading to the generation of ROS which contributes to endothelial damage. The intracellular ROS subsequently activates protein kinases and NF- κ B transcription factors [11,20].

Low-density lipoprotein (LDL) which is the largest lipoprotein in plasma, constitutes approximately 70 % of plasma cholesterol with a high activity against oxidative stress. Oxidized LDL is a major contributor to atherosclerosis progression, as it stimulates ROS production, endothelial dysfunction, and foam cell formation, which initiates atherosclerosis. The atherogenic effect of LDL is obtained from the oxidized LDL component. Oxidized LDL causes endothelial activation and changes in its characteristics [21]. Once macrophages engulf oxLDL, they differentiate into foam cells and release pro-inflammatory cytokines, including TNF- α , IL-1, IL-6, and IFN- γ . These cytokines cause inflammation in endothelial cells which results in tissue necrosis.

Inhibiting NF- κ B activation is a possible target for preventing atherosclerosis. Targeting the NF- κ B transcription pathway is a potential strategy for reducing inflammation in chronic conditions such as atherosclerosis [22]. One of the mechanisms that inhibit NF- κ B activation is the use of antioxidants [10]. High levels of inflammatory biomarkers such as TNF- α in the study group indicated the link between inflammation and atherosclerosis progression [10]. A strong correlation between LDL levels and TNF- α ($r > 0.73$, $p < 0.01$) indicated that elevated LDL contributes to plaque formation and triggers an inflammatory response, which in turn accelerates endothelial dysfunction and increases the risk of cardiovascular complications. These findings revealed that atherosclerosis results not only from lipid accumulation in the arteries but also from inflammatory mechanisms that worsen vascular conditions. Therefore, therapies that target inflammation, such as through NF- κ B inhibition, may be an effective approach in addition to hyperlipidemic therapies such as statins. In addition, lifestyle changes, including a low-fat diet and exercise, remain the main

preventive measures to reduce the risk of atherosclerosis.

Limitations of the study

Some limitations of this study include the relatively small sample size and the limited duration of the study. Furthermore, genetic and environmental factors that contribute to atherosclerosis were not thoroughly analyzed. Further studies with larger sample sizes, longer dietary induction periods, and more detailed analyses are needed to validate these findings.

CONCLUSION

Margarine diet increases LDL, triglycerides, and total cholesterol levels while lowering HDL. These changes are accompanied by elevated ROS and inflammatory biomarkers such as TNF- α , which contribute to the progression of atherosclerosis. Furthermore, this study shows a strong correlation between LDL levels and inflammatory biomarkers, confirming the role of dyslipidemia and inflammation in atherosclerosis pathogenesis. Therefore, therapeutic interventions that target lipid level reduction and inflammatory modulation may be an effective strategy in the prevention and treatment of atherosclerosis.

DECLARATIONS

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Conflicts of Interest

No conflict of interest is associated with this work.

Contributions of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mochamad Bahrudin: Conceptualization, Methodology, Software, Data curation, Writing-Original draft preparation. Asra Al Fauzi, Paulus Sugianto: Supervision, Validation, Writing-Reviewing and Editing. All authors have read and approved the manuscript for publication.

Ethical approval

The animal study protocol was approved by the Ethics Committee of the University of Muhammadiyah Malang (Number E.5.a/181/KEPK-UMM/VI/2024, approved on 28 June 2024).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Use of Artificial Intelligence

The authors declare no use of Artificial Intelligence

Use of research reporting tools

The authors declare no use of research reporting tools.

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