Progressivity of TNF-α production and miR-29b-3p expression during hypercholesterolemia

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Abstract: Hypercholesterolemia triggers atherosclerosis, characterized by releasing TNF-α and miR-29b-3p that may be applicable for biomarker or therapeutic targets. This study was aimed to analyze the correlation between TNF-α production and miR-29b-3p expression during hypercholesterolemia progression. Total 12-male-Sprague Dawley rats weighted of 170-180 gr were randomly divided into normal control group and hypercholesterolemia (HC) group, equally. The Sprague Dawley (SD) rats were given only standard feed for seven weeks, combined with placebo for normal group and 2 ml of cholestrol/200 grBM/day, orally for hypercholesterolemia group. The miR-29b-3p expression analysis was performed by qRT-PCR and TNF-α using ELISA, by followed manufacture procedures. The miR-29b-3, then analyzed using Kruskal-Wallis analysis, whereas TNF-α was analysed using t-test. During, initial week, TNF-α was produced 5.19±0.20 pg/ml in the normal group and significantly different with HC group there was 4.43±0.32 pg/ml, then it significantly increases up to 6.12±0.21 pg/ml in the normal group and 14.10±0.33 in the HC group. Increased of miR-29b-3p expression was occurred in the normal group by from 1.33±0.24-fold change in 1st week to 2.55±0.08 fold change in 8th week, then 1.66x104±1.06x104 to 1.94x104±1.19x104 fold change in HC group.

1. Introduction

Hypercholesterolemia triggers oxidative stress due to low-density lipoprotein-cholesterol (LDL-C) oxidation in tunica of blood vessels. This oxidation process activates monocytes to become pro-inflammatory [1], triggers inflammation, and release tumor necrosis factor-alpha (TNF-α) [1–3]. Oxidative stress is also predicted to trigger the release of miR-29b-3p, which is associated with upregulation of the nitric oxide synthase (Nos2) gene, thereby increasing the production of nitric oxide (NO) in normal endothelial cells [4]. However, miR-29b-3p was also reported to be positively correlated with ox-LDL [5]. The incidence of inflammation and IL-6 were significantly increased in patients with atherosclerosis than in normal people [6]. The miR-29b-3p is expressed simultaneously with the NF-kB and small mothers' activation against decapentaplegic-mitogen-activated protein kinase (SMAD-MAPK) signaling pathways which are active when there is an increase in ROS as a result of LDL-C oxidation in tunica [7].

The description of the findings above indicates that miR-29b-3p is a type of miRNA that directly correlates with inflammatory conditions, so it has the potential to be developed as a pathophysiological biomarker of oxidative stress. However, the progress of miR-29b-3p production and expression and its
relation to TNF-α as an inflammatory marker in hypercholesterolemic conditions have not been developed much. Based on this, this study was conducted to analyze the progress of TNF-α production and its relation to miR-29b-3p expression in hypercholesterolemic conditions. The results of this study are expected to explain the molecular processes of cells when consuming high amounts of cholesterol to detect and prevent cardiovascular disease.

2. Methods

As many as 12 male-Sprague Dawley rats with a body weight around 170-180 gr were randomly divided into normal control group and hypercholesterolemia (HC) group, equally. The cage's condition was set at 60% of humidity, 20-24 °C, 12-14 hours of light exposure, and nonstop ventilation. The SD rats were given only standard feed for seven weeks, combined with placebo for normal group and 2 ml of cholesterol/ 200 grBM/ day, orally for hypercholesterolemia group. The study was approved using experimental animals from the Health Research Ethics Commission, Faculty of Medicine, Universitas Gadjah Mada, registered number: KE/ FK/ 1003/ EC/ 2019.

Before blood collection, the rats were anesthetized using chloroform inhaled to avoid trauma, and miR-29b-3p troubleshot [8]. Blood was collected weekly from the first to the seventh week through sinus orbitalis using microhematocrit and put into a 2 ml sterile tube and left at room temperature for 30 minutes. The plasma was separated in a different tube as much as 200 µl for miR-29b-3p expression analysis. The remaining plasma was used for cytokine analysis. TNF-α production was measured using TNF-α ELISA Kit for Rat Cat No. KRC3011 (Invitrogen: Vienna, Austria). The analysis step was conducted by following the manufacturer's procedures.

Isolation of miRNA was completed using RNA miRNeasy Serum/ Plasma Kit cat. No. 217184 (Qiagen: California, USA). The isolation phase of RNA begins with miRNeasy Serum/ Plasma Spike-In Control with C. elegans ce-miR-39-1 mimic miRNA as a housekeeping gene. The procedure was conducted following the manufacturer's procedures. After RNA isolation, cDNA was synthesized from the sample using miRCURY® LNA® RT Kit (reverse transcriptase-PCR) (Qiagen: California, USA), miRNA measurement stage that was carried out using quantitative real-time PCR (qRT-PCR) with MiRCURY LNA SYRR Green (Qiagen: California, USA), primary set (forward and reverse) has-miR-29b-3p, cDNA. The miR-29b-3p expression was calculated using Livak & Schmittgen (2001).

The TNF-α parameters were performed using a t-test with a 95% confidence level. While the miR-29b-3p expression data were analyzed using Kruskal-Wallis analysis with a 95% confidence level. Correlation between parameters was analyzed using Pearson Bivariate Correlation analysis, Statistical analysis was performed with 24-Mac Statistical Products, and Service Solution (SPSS) then presented using Microsoft Excel 2019.

3. Results and discussion

In this study, administration of cholesterol for seven weeks significantly increased TNF-α levels and miR-29b-3p expression. The increase was 17.92% in the normal group, while the HC group increased 218.28%. In addition, it also significantly increased miR-29b-3p in the first week to the seventh week from the first day of cholesterol administration (Table 1).

| Groups | Normal TNF-α (pg/ml) | miR-29b-3p (fold change) |
|--------|----------------------|--------------------------|
|        | 1st week *           | 7th week *               | 1st week | 7th week |
| Normal | 5.19±0.20 a          | 6.12±0.21 a              | -0.88±0.05 (down-regulated) | -0.79±0.08 (down regulated) |
| HC     | 4.43±0.32 a          | 14.10±0.33 b             | 1.66x10^{4}±1.06x10^{4} a (up-regulated) | 1.94x10^{4}±1.19x10^{4} a (up regulated) |

Note: the alphabet (a-d) after number show significant different (p < 0.05) between treatment groups. The star mark (*) representing significantly different in periodic measurement (1st and 7th weeks).
miR-29b-3p was categorized as up-regulated when the gene-expression score \( \geq 1.00 \) fold change and down-regulated when \(< 1.00 \) fold change.

TNF-\( \alpha \) production on a weekly basis shows an increasing curve, in contrast to miR-29b-3p, which moves fluctuate. A positive exponential increase occurred in the production of TNF-\( \alpha \), while in the expression of miR-29b-3p, there was a decrease in concentration until it rose again in the sixth week towards the seventh week. A weekly increase was observed significantly between the normal group and the treatment group. Continuously the most significant weekly increase occurred in the second week to the third week. This suggests that the intense consumption of cholesterol may lead to an increase in TNF-\( \alpha \) production (Figure 1-A).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** the TNF-\( \alpha \) titer (A) and miR-29b-3p expression (B) weekly.

The miR-29b-3p was increased since the first week, which suggests that cholesterol input may be related to miR-29b-3p expression. Up-regulation of miR-29b-3p expression in cholesterol-induced mice suggests that this miRNA expression may be associated with TNF-\( \alpha \) production. However, miR-29b-3p is also expressed in small amounts. This suggests that miR-29b-3p may also be involved in normal cell activity. The miR-29b-3p is increased alongside the production of NO in normal endothelial cells and aids in the dilation and constriction of blood vessels [4]. Furthermore, a decrease in miR-29b-3p correlates with a decrease in blood vessel dilatation in aortic aneurysms. [10]. In addition, miR-29b-3p is reported to have increased significantly in patients with atherosclerosis [6].

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The percentage of TNF-\( \alpha \) titer (A) and miR-29b-3p expression (B) increase weekly.

The increase in cholesterol input triggers a buildup of LDL-C in tunica and increases radical oxygen species (ROS), resulting in various cell signaling pathways. The presence of ROS influences the following mechanisms, 1) triggers macrophage activation to produce TNF-\( \alpha \) as an inflammatory
response [11]; 2) phosphorylating Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) as a TNFR1 adapter molecule that initiates signaling. TRADD activates TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein kinases (RIP) [12] which continuously activates the protein IκB kinase (IKK); 3) phosphorylates the IκBα complex, and releases NF-κB. This mediates transcription including TNF-α and Interferon (IFN) -γ [12-13].

The presence of TNF-α acts autocrinally, which then increases the inflammatory response by secreting IL-1β, IFN-γ, and IL-6. In inflammatory atherosclerosis conditions, the increased production of TNF-α also affects the secretory cells and triggers more production of TNF-α and other cytokines [14]. In addition, INF-γ, produced in the NF-κB pathway, binds to endothelial cell surface receptors and triggers the activation of Janus kinases-signal transducer and activator of transcription proteins (JAK-STATs). INF - γ and surface receptors complex, makes dimerization receptor. The JAKs then phosphorylate on tyrosine residues (activation loops), JAKs phosphorylated tyrosine in the receptor, causing the STATs to dissociate from the receptor [15]. The phosphorylation makes STATs bind to the Src-homology 2 (SH2) domains receptor. These activated STATs form hetero- or homodimers. The SH2 domain of each STAT binds the phosphorylated tyrosine of the opposite STAT, and the dimer then translocates to the nucleus to induce gene transcription [16]. The STATs protein may also be phosphorylated directly by receptor tyrosine kinases.

The miR-29b-3p is directly involved in the aorta’s calcification by dysregulation of the matrix metalloproteinase-2 (MMP-2) gene. The regulation involves gelatinase, which has a significant role in matrix degradation and vascular remodeling [10]. The regulation of MMP2 expression and activity is shown in the migration of vascular smooth muscle cells (VSMC), which mediates the degradation of the lamina elastica resulting in the production of soluble elastin peptide and the stimulation of the TGF-β1 signaling pathway from the extracellular matrix (ECM) of blood vessel walls [10]. MMP2 is the direct target gene for miR-29b-3p, with a negative correlation between them. Overexpression of miR-29b-3p in VSMC mice resulted in decreased expression of MMP2 at protein levels and resulted in decreased arterial calcification and VSMC [17].

The certainty of the effect of the miR-29b-3p expression on the endothelial wall is shown by suppression of MMP2. However, when referring to NF-κB pathway cell signaling, the presence of miR-29b-3p has increased in inflammatory conditions. This is because NF-κB will be activated in oxidant stress caused by LDL-C oxidation [18]. In other words, these miRNAs may increase along with inflammation in atherosclerosis and act as anti-fibrosis [19]. However, further research needs to be done to see a direct correlation between the presence of miR-29b-3p and the expression of pro-inflammatory cytokines such as TNF-α and interleukins (IL) such as IL-10 and IL6. The facts found in this study including 1) an increase in miR-29b-3p has a positive correlation with an increase in the rate of inflammation which is indicated by an increase in TNF-α production; 2) the initiation of inflammation may have started immediately after the induction of cholesterol, where an increase in the amount of cholesterol that enters the body results in a buildup in the intima area.

4. Conclusion
Simultaneous administration of cholesterol has been shown to cause an increase in TNF-α levels, which may indicate an inflammatory response. The increase in TNF-α was greater as the intensity of cholesterol induction in the model mice was also followed by an increase in miR-29b-3p. This suggests a possible relationship between inflammation in hypercholesterolemic conditions and miR-29b-3p expression. Further research is needed to determine the role of miR-29b-3p involvement in the TNF-α-controlled inflammatory mechanism. Research could be developed to investigate which mRNA of specific proteins has decreased due to the miR-29b-3p silencing program.

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