MALAYSIAN JOURNAL OF MICROSCOPY

Journal homepage: https://malaysianjournalofmicroscopy.org

RESEARCH ARTICLE

ELUCIDATING THE miR-155-5p AND miR-181a-2-3p AS CIRCULATING MICRORNA BIOMARKERS OF THE INFLAMMATORY REGULATION IN THE PREMATURE ATHEROSCLEROSIS

Christianus Rustin[1], Muhammad Irfan Sazali Kamaruddin[1], Lilik Herawati[2], Azrina Zainal Abidin[3], Zulhabri Othman[3,4,*]

[1]School of Graduate Studies, Management and Science University, 40100 Shah Alam, Selangor, Malaysia.
[2]Faculty of Medicine, Universitas Airlangga, 60131 Surabaya, Indonesia.
[3] Faculty of Health and Life Sciences, Management and Science University, 40100 Shah Alam, Selangor, Malaysia.
[4] Faculty of Medicine, Manipal University College Malaysia, 75150 Melaka, Malaysia.

Abstract. MicroRNAs, short fragments of non-coding RNA, play a crucial role in gene regulation found within atherosclerotic plaque complex via inflammatory pathways, indicating their role as a biomarker for atherosclerosis. We hypothesize that miR-155-5p and miR-181a-2-3p are overexpressed in High-Fat Diet (HFD=5) compared to Normal Diet (ND=5) in Sprague Dawley (SD) rats. MicroRNAs quantification was confirmed by Real-Time PCR using SYBR Green within two timelines. The lipid profiling was measured using mission 3-in-1 cholesterol meter, and histology analysis of the aortas was performed using Haematoxylin & Eosin (H&E) method. Lipid profiling analysis revealed as significant higher ($p<0.05$) of low-density lipoprotein (LDL) (20.3±6.12) mg/dL, triglycerides (TG) (134.65±7.41) mg/dL and very low-density lipoprotein (VLDL) (21.54±8.23) mg/dL in HFD at week 12. Whilst the high-density lipoprotein (HDL) and total cholesterol (TC) were recorded to be significantly higher ($p<0.05$) in ND groups with 68.62±6.54 mg/dL and 54.09±8.40 mg/dL respectively. The microRNA analysis showed a significant difference ($p=0.04$; $t=7.09$) in the microRNA expression between HFD and ND groups. The average Ct value of rno-miR-181a-2-3p and rno-miR-155-5p in HFD group at week 12 were 27.08±10.84 and 25.96±11.27 respectively. The rno-miR-181a-2-3p showed higher average fold changes expression level compared to rno-miR-155-5p. Moreover, an obvious thickening of tunica intima of the aorta due to inflammation activities in HFD (152.50±7.52) µm compared to ND (148.41±1.94) µm. In summary, microRNAs were significantly expressed in HFD compared to ND and supported by the lipids level and aortas inflammation.

Keywords: Atherosclerosis, biomarkers, inflammatory, microRNA, miR-155-5p, miR-181a-2-3p.

Article Info

Received 22 September 2024
Accepted 26 November 2024
Published 6 December 2024

***Corresponding author: zulhabri.othman@manipal.edu.my**

ISSN: 1823-7010, eISSN: 2600-7444

1. INTRODUCTION

Atherosclerosis is an inflammatory disease due to abnormal changes in the arteries' pathophysiology that lead to heart remodelling such as myocardial infarction (MI) and other associated health complications [1]. Meanwhile, in Malaysia, cardiovascular diseases remain as the leading cause of mortality among the population, and statistically the number of cases appeared to be increasing a period [2]. Hypercaloric diets contain High-Fat Diet (HFD) enhanced synthesis of energy, however, an unbalance fatty acid level led to the accumulation of high Low-Density Lipoprotein (LDL) and triglycerides in the liver, suggesting that lipids enriched diets most probably initiate the occurrence and development of atherosclerosis [3]. An excessive LDL caused atherosclerosis via inflammatory pathway involving the recruitment of immune cells and activation of cytokines due to damage endothelial cell wall of coronary artery. Furthermore, an excessive uptake of LDL, triggers the recruitment of immune cells on the damage endothelial cells (ECs) resulting in the formation of atherosclerotic plaques,thus affecting the pathophysiology of the coronary artery and causing various heart-related diseases [4]. Moreover, the inflammatory pathway is facilitated by microRNAs either in an up-regulated or down-regulated manner corresponding to the expression of genes involved in atherosclerosis [5].

The circulating microRNAs have emerged as a new class of disease biomarkers to inform clinical diagnosis better and provide new avenues for personalised therapies [6]. The expression levels of some microRNAs changed significantly in different stages of atherosclerosis pathogenesis; therefore, microRNAs may become new diagnostic biomarkers for atherosclerosis [7]. Su et al [8] have suggested that miR-181a-3p is an antiatherogenic microRNA which regulates the inflammation process that may represent a novel therapeutic approach to managing atherosclerosis.

This study hopes to enhance more knowledge of the previous discovery of microRNAs. Thus, the findings of this research will potentially fulfil the criteria required for further advanced research in atherosclerosis by utilizing microRNAs as a promising therapeutic target for cardiovascular diseases [1]. Apart from that, the findings from the experimental animal models will provide an insight into the pre-clinical studies concerning the unbalanced diets intakes and the occurrence of heart diseases. Subsequently, it will help tremendously in the efforts to minimize such medical conditions. Finally, this study anticipated to facilitate the existing and upcoming ideas among researchers regarding the association between diets in the events of atherosclerosis in corresponding toward inflammatory microRNA expression [9]. The discovery of microRNAs has been significantly relevant and created positive impacts, including better prognosis for atherosclerosis and other cardiac-related diseases [10], however, there still the need to fill the research gap in microRNA as the potential therapeutic target for atherosclerosis. Hence, we aimed to elucidate the expression of circulating microRNAs found in SD rat's blood. We anticipated that all the selected inflammatory microRNAs were expressed in our study model, followed by abnormal lipid fluctuation and inflammation on the coronary arteries of SD rats induced HFD.

2. MATERIALS AND METHODS

2.1 Study Design

Ten male Sprague Dawley (SD) rats were divided into two groups used in this experiment. The treatment group was given high-fat diet (HFD) and control group was given normal diet (ND) for twelve weeks. SD rats were maintained in a controlled environment which 22–24 °C and 12 hours light/dark cycles and housed in polypropylene cages two rats per cage [5]. HFD consists of a 60% fat mixture and 40% standard rat pellets whilst ND is made up of pure pellets. Diet formulation was based on the diet dilutions method [4].

2.2 Lipid Profiling

Lipid profiling including the total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) was conducted using a cholesterol mission meter according to manufacturer protocol (ACON Laboratories, Inc) and the lipids measurement was performed according to Fredward's equation [9].

2.3 MicroRNA Analysis

Blood collection: A large volume of blood (4 ml) was collected. Blood samples were obtained from the marginal tail of SD rats using a 26G needle and syringe [11].

Primer Design: The primer sequence was derived from NCBI and synthesized by a manufacturer company (Integrated DNA Technologies, USA). Table 1 shows the primer sequences for mirR-155-5p and miR181-2-3p.

Table 1: Primers assay for microRNA biomarkers

MicroRNAs	***Primers Details***
miR-155-5p	Primer seq: 5'-tatatggccccagcatgcga-3'; 3'-cacttgtggcccaggtatgc-5'
	Probe & Dye: SYBR Green
	Assay: rno-miR-155-5p miRCURY LNA miRNA PCR Assay (YP02119303)
miR-181a-2-3p	Primer seq: 5'-tccatgtactgtagtgcttcgac-3'; 3'-tcagaccactccgccaatgc-5'
	Probe & Assay: SYBR Green
	Assay: rno-miR-181a-2-3p miRCURY LNA miRNA PCR Assay (YP02108605)

Total RNA extraction: Circulating small RNA was extracted from blood of SD rats induced with HFD and ND. Extraction of the total RNA was performed using the NucleoSpin® RNA Blood according to the manufacturer protocol (Qiagen, Germany). The 200 µL blood were added with 200 µL lysis buffer and 20 µL proteinase K and incubated at room temperature for 15 minutes. The lysate then transfer to spin column for further steps of binding DNA, desalt silica membrane and washing with buffer. The RNA was eluted with 60 µL RNase-free water.

Reverse transcription: cDNA synthesis performed using the miRCURY® LNA® RT Kit (Qiagen, Germany) according to the manufacturer protocol in a thermal cycler at 42 ℃ for 60 minutes and 95 ℃ for 5 minutes. The reverse transcription master mix (10 µL) was prepared by adding the miRCURY RT reaction buffer (2 µL), RNase-free water (4.5 µL), miRCURY RT enzyme mix (1 µL), synthetic RNA spike (0.5 µL) and 2 µL of template RNA (5 ng/µL).

Quantitative Real-Time PCR (qRT-PCR): Performed using the SYBR® Green PCR Master Mix (Applied Biosystems, USA) on the One-Step RT-PCR system (Applied_Biosystems, 2016). A total of 50 µL reaction was prepared by adding the 2X SYBR Green PCR master mix (25 µL) with forward primers (50 nM), reversed primer (50 nM), cDNA template (10 ng) and water. The PCR was performed with denature at 95 ºC (15 seconds) and anneal/extension at 60 ºC (1 minute) for 40 cycles.

2.4 Histological Analysis

For the microscopic study, the aorta was isolated from the heart organ and cut into a small segment. The tissues were fixed in 10% formaldehyde and for 10 days at room temperature. Dehydration involved sample immersion in a series of ethanol concentration and xylene, embedded in paraffin wax using tissue block processor (Medite, Germany) and sectioning in a microtome (RM2235, Leica, Germany) with a blade adjusted to 5 µm in thickness and ribbon-like shape tissue sections were collected using glass microscopic slides and left to dry overnight at 40 ºC. Cardiac tissue staining was

performed using Haematoxylin and Eosin (H&E) to visualize inflammatory activities. The outer layer of the aorta was subjected to comparison microscopic (DM2700P, Leica, Germany) observation [12].

2.5 Data Normalization

The UniSp6 RNA spike-in was used as the exogenous control for the experimental genes; the primary purpose of the control genes is to provide the most possible accurate values of expression level and the reliability of the targeted genes or microRNAs expression by eliminating potential bias [13]. This involves the calculation of test-specific scaling factors to remove relative biases between tests.

2.6 Data Analysis

MiR-181a-2-3p, and miR-155 were analysed and interpreted via the amplification plot and melting curve using applied biosystems real-time PCR system software was used for the data interpretation [14].

2.7 Data Interpretation

All data were expressed as means and standard deviation (SD). To assess the difference between the mean among variables, a paired sample t-test was employed. The differences in miRNAs expression level between the treated group and control group were analyzed using delta-delta Ct Value (2^-(-ΔΔCT) according to Livak's method [15]. The methods allow for analysis of quantitative gene expression data using relative quantification. For further analysis, which is the correlation tests were performed only within the microRNA that has been expressed and with an obvious statistical difference. The analysis was conducted in SPSS software. A p-value of less than 0.05 ($p<0.05$) is considered statistical significance.

3. RESULTS AND DISCUSSION

3.1 Body Weight and Body Length

On average there was more than a 50% increase in weight in SD rats for every four consecutive weeks starting in week 4 among HFD (n=5) group, this corresponded to the increase in body length and the BMI of SD rats. Similarly, in ND (n=5) group, there were significant changes in body weight, body length, and BMI which began to take place in week 4. Although the overall changes among experimental rats from the HFD group were higher in comparison to the ND group. However, all changes were in increasing patterns throughout the duration of the experiment (Table 2).

Table 2: Baseline characteristics of SD rats for 12 weeks

	High-Fat Diet (HFD)			**Normal Diet (ND)**		
	Body Weight (g)	**Body Length (cm)**	**Body Mass Index (BMI)**	**Body Weight (g)**	**Body Length (cm)**	**Body Mass Index (BMI)**
Week 0	80.19±2.09	15.34±0.43	0.34±0.02	75.50±2.25	15.20±0.45	0.33±0.03
Week 4	177.40±0.38	19.40±0.55	0.47±0.02	127.20±2.87	17.20±0.87	0.43±0.07
Week 8	387.74±6.26	24.80±0.84	0.02±0.63	274.40±5.70	22.80±1.30	0.52±0.05
Week 12	472.73±2.26	24.98±0.32	0.76±0.04	361.22±4.99	23.60±0.55	0.65±0.09

3.2 Body Mass Index (BMI)

Body Mass Index (BMI) is one of the most important parameters widely used for the evaluation of health conditions such as overweight, diabetes, and obesity. The BMI is a universal parameter to measure health vital stats commonly practiced by clinicians within the healthcare setting. In our study, the first group of SD rats was fed with HFD, showing a significant increase in body weight, body length, and BMI from week 0 to week 12. It was anticipated that the HFD would cause SD rats to develop obesity. Whilst the ND group showed a slight change, suggesting that the risks of becoming obese are lower when consuming non-calorie foods. Previous reports have shown that the occurrence of obesity is due to the accumulation of adipose tissue inside the body system [16], which is associated with unhealthy diets. In addition, a study on the effect of HFD in SD rats suggested hypercaloric foods led to an increase in weight due to the increase in body fat mass corresponding toward higher energy consumption [17].

3.3 Lipid Profiling

We observed a higher level of LDL, VLDL, and TG in HFD compared to ND groups, whereas the HDL and TC were seen higher in ND groups. Statistically, there was a significant difference (t=0.7572, p=0.0016) in lipids level between the HFD and ND groups. Results have shown an increase in the average level of LDL (20.3±6.118) mg/dL in HFD groups between week 0 and week 12. Similarly, the average level of LDL (20.3±6.118) mg/dL, TG (134.65±7.405) mg/dL and VLDL (21.54±8.23) mg/dL were significantly increased in HFD groups. Whereas the level of HDL (68.62±6.54) mg/dL and TC (54.09±13.40) mg/dL were reported to be higher in ND groups (Figure 1).

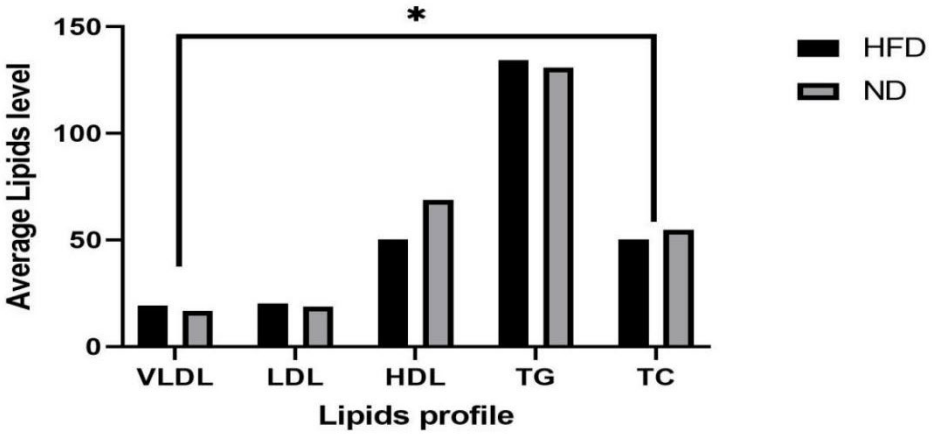

Figure 1: The average level of lipids at week 12 between HFD and ND groups, (*=*P<0.05*)

Based on the lipid profiling results, the overall pattern of LDL increased from week 0 to week 12, This finding revealed that HFD contains a higher level of LDL compared to ND. Whilst TG appeared to be higher and was in an increasing pattern in HFD, a constant level was observed in ND. The HDL levels in both HFD and ND were approximately similar from week 0 to week 12. TC in each group was around the same level throughout the course of dietary. One of the important elements causing atherosclerosis is LDL, most cases observed in HFD contain high fatty acid. The unbalanced level of fatty acid led to the activation of transcription factors associated with lipids regulation [5].

3.4 Amplification of rno-miR-155-5p and rno-miR-181a-2-3p in Week Six

The RT-PCR analysis revealed a significant difference and strong correlation between microRNAs amplification in HFD and ND, (r=0.995, t=13.54, df=1 and p=0.047). In the HFD groups, the mean Ct value for microRNA expression scored were (29.44±13.28) ΔRn/cycle and (31.46±5.21)

ΔRn/cycle for rno-miR-181a-2-3p and rno-miR-155-5p respectively. Whereas in the ND groups, higher Ct value was reported for microRNA rno-miR-155-5p (37.71±3.03) ΔRn/cycle and rno-miR-181a-2-3p (36.65±2.43) ΔRn/cycle. The results revealed the higher expression of both microRNAs in the HFD groups compared to ND groups. Furthermore, the overall score for fold change expression level in HFD was higher compared to ND. Meanwhile, our statistical analysis shown that, fold change expression level was higher for rno-miR-155-5p compared to rno-miR-181a-2-3p in SD rats induced with HFD and ND (Figure 2).

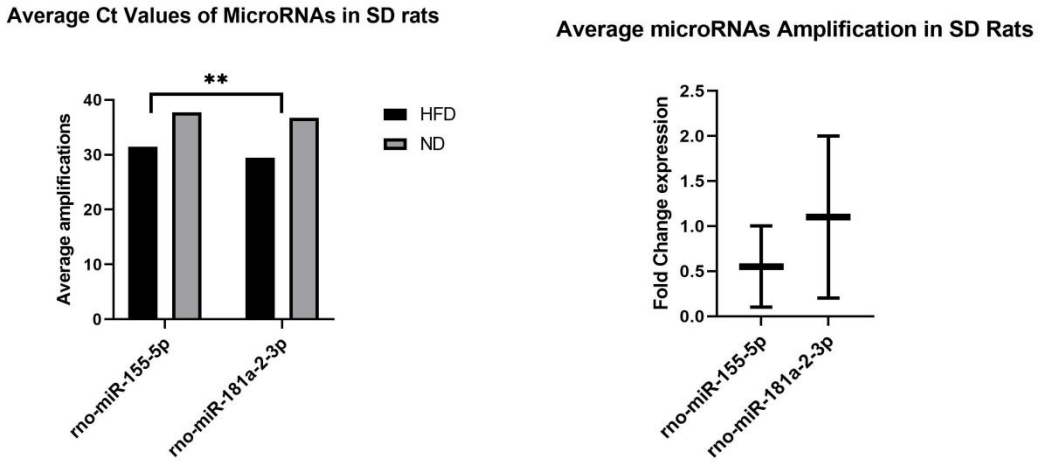

Figure 2: The average microRNAs amplifications and fold change expression level at week six between HFD and ND, (**=*P<0.05*)

3.5 Amplification of rno-miR-210-5p and rno-miR-181a-2-3p in Week Twelve

The RT-PCR revealed that, the lower average Ct value in HFD group was rno-miR-155-5p (25.96±11.27) ΔRn/cycle, followed by rno-miR-181a-2-3p (27.08±10.84) ΔRn/cycle. The amplifications pattern indicated HFD contributed toward the higher level of those circulating microRNA (Figure 3).

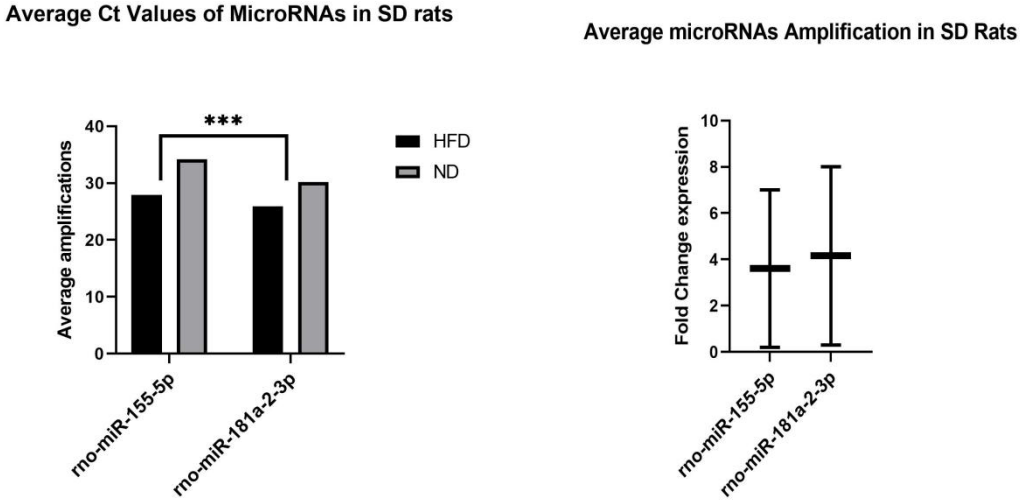

Figure 3: The average microRNAs amplifications and fold change expression level in week twelve between HFD and ND groups (***=*P<0.05*)

Moreover, statistically, the overall fold change expression level showed higher score for rno-miR-155-5p in comparison to rno-miR-181a-2-3p in SD rats induced with HFD and ND (Table 3). Additionally, according to the t-test, there was a significant difference and strong correlation in microRNAs amplification in SD rats between groups (r=0.981, t=7.085, df=1, p=0.04). Overall, the expression patterns for rno-miR-155-5p and rno-miR-181a-23p were over-expressed among SD rats induced with HFD groups compared to ND groups.

Table 3: Differential expression of microRNAs between HFD and ND groups (Week 12)

MicroRNA	HFD ΔCt (Mean ±SD)	ND ΔCt (Mean ±SD)	NTC ΔCt (Mean ±SD)	Average Fold Changes	
				HFD	ND
rno-miR-181a-2-3p	27.08±10.84	32.16±3.12	0	8	0.3
rno-miR-155-5p	25.96±11.27	32.23±10.47	0	7	0.2

3.6 Inflammatory microRNAs Biomarker Expression

Several inflammatory microRNA biomarkers were known to be expressed in atherosclerosis via the inflammatory signaling pathway [5]. Our study revealed that the two known inflammatory microRNAs were successfully expressed in both HFD and ND groups. According to the RT-PCR result, rno-miR-155-5p and rno-miR-181a-2-3p were successfully expressed in both the treated group (HFD) as well as in the control group (ND). MiR-155 is known for its cellular function in promoting atherosclerosis through the downregulation of Bcl6 in macrophages [10]. Previous evidence showed the expression pattern of miR-155 was in increasing pattern on the aortic wall in a mouse with diet-induced atherosclerosis after ten months, they conducted further analysis and demonstrated the expression of proinflammatory molecules during macrophage activation is closely related to the presence of miR-155. In the meantime, the downregulation of miR-155 was shown to elevate the expression of proinflammatory agents, including IFN-ϒ and IL-4 [10,18]. Furthermore, the oxidation of LDL molecules is also associated with the expression of miR-155.

The mechanism of miR-155 in the expression of cytokine was evaluated using the luciferase assay in which the complementary binding of miR-155 to the mRNA of Bcl6. However, the inhibition of proinflammatory such as the NF-Kb reduced the expression of Bcl6. In addition, the role of Bcl6 in the sense of protection against atherogenesis was shown through the expression pattern of the gene. An increase in the Bcl6 expression contributes to the increasing rate of plaque formation [19]. Whilst, in our study the expression of miR-155 was observed after the first month in SD rats induced with HFD. Study by Zhu et. al. [20] suggested that miR-155 is a part of a negative feedback loop, which down-modulates inflammatory cytokine production and decreases atherosclerosis progression. It may also be involved in the posttranscriptional regulation of the inflammatory response and MAPK pathway by targeting mitogen-activated protein kinase kinase kinase 10 [20].

Meanwhile, the mechanism between miR-181 and the oxidized LDL (ox-LDL) in triggering the activation of dendritic cells (DCs) is one of the essential elements in inflammatory activities corresponding to atherosclerosis occurrence. The initial outcome suggested that there was an increase in the level of inflammatory response and up-regulated miR-181a expression in the animal model fed with a high-fat diet and was found to be hyperlipidaemia [21]. Therefore, the expression of miR-181 in our study suggested the importance of that biomarker as an indicator of the occurrence of atherosclerosis. Our RT-PCR analysis revealed the overexpression of miR-181 in rats induced with HFD suggesting that the microRNA was actively involved in the mechanism of atherosclerotic plaque formation due to the apoptosis of endothelial cells [10].

3.7 Histological Analysis of Tunica Intima of Aorta

The histology analysis revealed a significant ($p<0.05$) thickening of the tunica media layer with an average mean of 152.50±7.52 µm in HFD group (A), compared to ND group (B) with slight thickening of tunica media with an average mean of 148. 41±1.94 µm (Figure 4). The photomicrographs as in HFD group suggested the thickening of the outer layer of the aorta wall, hence narrowing the lumen.

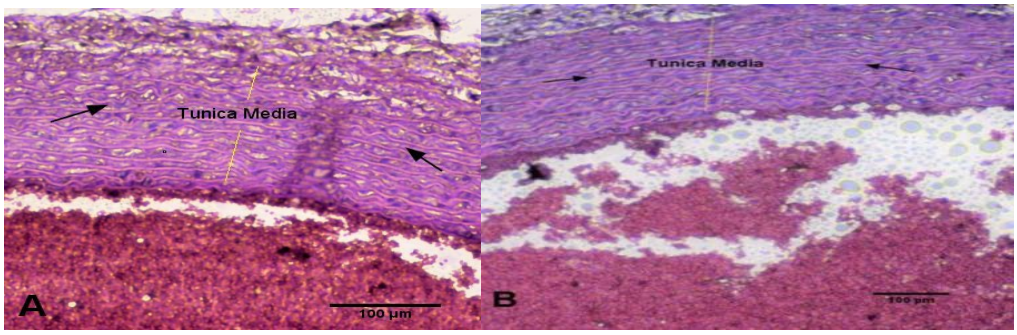

Figure 4: Photomicrographs of coronary arteries of SD rats induced HFD (A) and ND (B) showing the thickening of tunica media layer (arrows), (H&E, X100 magnification)

A hypercaloric diet containing a high level of LDL which accumulated and became too concentrated on endothelial cell walls led to the activation of immune cells and another associated signaling molecule on site greatly influencing the formation of atherosclerotic plaque. Our findings showed that the histological analysis, HFD caused the lesion formation on the aorta wall, therefore, increasing the risks of SD rats developing cardiovascular disease, which shortens the life span of SD rats. Contrary, previous research proved that HFD did not influence the rate of atherogenesis and its association with the aforementioned disease, hence, HFD was not affecting the survival time and the abnormalities in rat's aorta [22]. The components of the diets given to these rats might contain either pro-inflammatory or anti-inflammatory agents that influenced the systemic inflammatory state. One of the main contributors to the occurrence of inflammation is the type of diet. For instance, a hypercaloric diet containing high-level high-density lipoprotein which accumulated and became too concentrated on endothelial cell walls led to the activation of immune cells, another associated signaling molecule on-site. Thus, it will greatly influence the formation of atherosclerotic plaque which subsequently creates chronic cardiovascular diseases.

Plaque rupture results in a very serious medical condition since the cardiac muscle will be affected and lead to MI [5]. The tunica media layer is made up of vascular smooth muscle cells (VSMCs) and enriched with an elastic element such as fibers and collagen designed to resist pressure. In addition, immune cells such as dendritic cells are present on the intima layer which played a significant role in the proatherogenic stimuli, hence facilitated the proliferation of VSMCs which in turn yields a high concentration of modified extracellular layer that contributed to the occurrence of intimal thickening [23]. According to the histological analysis, HFD and ND showed morphological changes, specifically the thickness of the tunica media layer of the SD rat's aorta. However, there was an obvious lesion observed from the HFD group compared to the ND group. The overall observation led to the conclusion that HFD contributed more toward the abnormal thickness in tunica media layer of the coronary artery wall of SD rats.

4. CONCLUSIONS

Hypercaloric and normal diets influenced the expression pattern of the inflammatory microRNA biomarkers. Both rno-miR-181a-2-3p and rno-miR-155-5p were over expressed in SD rats. This indicated the up-regulation of rno-miR-181a-2-3p and rno-miR-155-5p in SD rats induced with

HFD. Overall, HFD showed significant expression of the microRNAs in HFD compared to ND. Furthermore, HFD caused an increase in BMI and lipids levels, and the occurrence of inflammation activities caused morphological changes in the tunica intima of coronary arteries. The microRNA is a potential disease-specific biomarker that might play an important role for the early detection of cardiovascular disease. Further less the microRNA still requires further research to embark on clinical study, especially the standardized protocol, technology and analysis.

Acknowledgements

The authors would like to thank the Ministry of Higher Education (FRGS/1/2018/SKK08/MSU/02/2) and Management and Science University (SG-042-012020-SGS) for funding this study.

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards. Ethical approval (MSU-RMC-02/ FR01) for this research was obtained from the Research Ethics Committee of Management and Science University.

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