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HIGH NEUTROPHILS/ LYMPHOCYTE RATIO AND MPO LEVELS ASSOCIATED WITH LOW EXPRESSION OF MIRNA-146A AND APOLIPOPROTEIN E IN TYPE2 DIABETIC PATIENTS WITH ATHEROSCLEROSIS

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Abstract
Background: Atherosclerosis is the chronic vascular inflammatory disease. Recent studies have showed that microRNA (miR)- 146a may serve to resolve the inflammation in atherosclerosis. However, the mechanisms by which miR- 146a exhibits its ant-inflammatory effects still unclear. Objective: To investigated the role of miR- 146a in type 2 DM with atherosclerosis and type 2DM only and its association with Apo E and other inflammatory markers .Methods: 100 participants were enrolled in this study: 40 Type 2 DM with Atherosclerosis(T2DM with ATHR), 30 type 2 DM group and 30 control group. Complete blood count parameters were assayed within tubes containing K2EDTA using hematology analyzer. Levels of ApoE, MPO and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). Levels of circulating miR-146a was measured by quantitative PCR. The data were analyzed by using SPPS and a P< 0.05 is considered a statistically significant. Results: T2DM with ATHER group showed an increase in N/L ratio (P< 0.05) compared with control. MOP and Apo E and levels were observed to increase significantly in T2DM with ATHER group, while levels of IL 6 showed a significant increase in T2DM with ATHER and T2DM groups compared to the control. Decreased levels of miR- 146a expression was indicated in T2DM with ATHER group compared with other groups. Conclusion: The results suggest lower levels of miR-146a expression and Apo E levels negatively regulate inflammatory response in T2DM with ATHER which enhance the inflammation and atherosclerosis

Keywords: atherosclerosis, microRNAs, apolipoproteins E, neutrophis/lymphoctyes ratio

INTRODUCTION

Cardiovascular disease (CVD), is considered is a major cause of death worldwide, numerous genetic and namely epigenetic mechanisms that have been recently indicated to play a role in the pathogenesis of CVD [1]. Atherosclerosis is a fundamental pathological change of CVD. It is an important macrovascular complication and many risk factors have been contributed to its pathogenesis, including, family history, hypertension, dyslipidemia, diabetes mellitus, obesity and smoking (DM) [2].

Type two diabetes mellitus (T2DM) is the most common form of DM characterized by hyperglycemia and insulin resistance, and identified to be a risk factor for the atherosclerosis pathogenesis [3]. The development of CVD associated with increase the morbidity and mortality in patients with T2DM [4].

Atherosclerosis is a chronic inflammatory condition that is developed and progressed via interactions of cellular elements of the artery wall and the inflammatory mediators [5].

inflammation in CVD is associated with excessive influx of inflammatory cells, neutrophils and macrophage resulting in an increase of neutrophil-to-lymphocyte ratio (NLR) [6]. Neutrophils accumulation causes extensive damage to cardiac tissue via release of ROS, enzyme that are stored in granules such as myeloperoxidase (MPO), and inflammatory cytokines thereby the inflammatory response is prolonged [7].

IL-6 is cytokine that has been shown pro-inflammatory effects in tissue injury [8] and also contributed to the

stimulation of the inflammatory response during atherosclerosis [9].

Apolipoprotein E (apo E) is well recognized as lipoprotein that has an essential role in the maintain of lipid homeostasis [10]. ApoE is known to have ability to exert anti-inflammatory effects and to suppress atherosclerosis [11]. Different signaling and molecular regulatory pathways are involved in the initiation and progression of atherosclerotic plaques, recent research has provided new molecular insight about the role of microRNA in these pathways [12] In addition to its role in the clearance of atherognic lipoproteins remnant from plasma, apoE is identified to regulate cellular signaling under controlling of microRNA in immune cells and vascular wall[13].

miRNAs are small non-coding RNA molecule about 21-25 nucleotides in length that function in the post-transcriptional of gene expression [14]. miRNAs have vital roles in the different biological processes including proliferation, apoptosis, and response to stress [15]. Anti-inflammatory miR-146a has been previously described to have effects on the immune response by regulation the proinflammatory signaling pathways [16,17] and also the inflammatory response during coronary heart disease [18].

miR-146a has been suggested to decrease the atherogenesis, this is because of suppression of nuclear factor-kappa B (NF-kB) signaling and endothelial cell activation in patients with CVD [19,20].

It was demonstrated that apoE downregulated the inflammatory process activated by NF-κB signaling pathway and atherosclerosis by enhancing the expression of miR-146a in mouse models [21], therefor the expression of miR-146a can provide a new strategy of therapy for inflammatory vascular diseases

The current study aimed to determine whether the circulating miR-146a levels in T2DM with atherosclerosis might associate with Apo E levels and accumulate of inflammatory cell, therapy contributing to release inflammatory mediators to determine its potential diagnostic value for atherosclerosis

MATERIALS AND METHODS

Patients

The study consisted of patients with T2DM for more than five years along with atherosclerosis (n=40 , 23 male, 17 female; mean age 51.7± 13.2 years), patients with T2DM without atherosclerosis (n=30, 18 male, 12 female ; mean age 59.23± 18.4years) and healthy control (n=30, male= 12 ,female= 18, mean age 52.4±22). All patients were recruited consecutively over a period of six months (November 2018 to April 2019) from diabetic unit of Al-Diwaniyah teaching hospital, Diwaniyah, Iraq. All experimental protocols were approved by the Ethical Committee of the medical college of university of Al-Qadisiyah. Informed consent was given to all individuals who participated in the study to fill it.

Patients with significant systemic disease including autoimmune chronic inflammatory conditions, previous coronary heart disease, heart failure and pregnant were excluded. Healthy controls were adjudged healthy based on medical history, physical examination and laboratory investigations.

METHODS

Blood sample (5 ml) was taken from each participant, and collected into two tubes (1 ml blood in K_2EDTA tube for hematological study and 4 mls in gel tube). Gel tube of blood was centrifuged at 4000 rpm for 10-15 min at 4 °C to separate the serum which was distributed into two parts , one part kept at (-80 ˚C) for miRNA146a analysis, while the other was kept at -20 ˚C for biochemical analysis such as fasting blood glucose, triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-c) and high density lipoprotein cholesterol HDL-c, which were determined spectrophotometry. Serum levels of Apo E, IL-6 and MPO were measured by ELISA.

Expression of miRNA146a was screened by qPCR. microRNA was isolated from serum (200 µ l) by Serum/Plasma microRNA purification kit (Bioworld, USA) and reverse-transcribed using Poly Polymerase (A) Tailing miRNA cDNA synthesis kit (abm, Canada). The resulting cDNA was mixed with miRNA-specific forward, reverse universal primers and cDNA BrightGreen master mix (Abm, Canada). U6 was used as the endogenous control. The relative level of miRNA was calculated by using the comparative threshold cycle (Ct) and ($2^{-\Delta\Delta Ct}$), the results indicated the fold change of expression

STATISTICAL ANALYSIS

Data were expressed as mean and standard errors (mean±SEM). SPSS software 23 was used for statistical analysis. one-way ANOVA followed by the multiple range tests (Tukey) was used to compare the difference between groups. $P < 0.05$ is considered statistically significant throughout.

RESULTS

A total of 100 individual were participated in this study. Patients' characteristics and the clinical parameters data are shown in Table 1. Regarding age, gender, and BMI no significant differences were detected between patients' groups and control ($p > 0.05$). Compared with the T2DM patients and the control, the fasting blood sugar, SBP, TG ,TC and LDL-c levels have to be higher ($P< 0.05$), as well as lower HDL-c levels, these results demonstrated that atherosclerosis is initiated and developed by risk factors such as obesity, dyslipidemia and hypertension.

As excepted, a significance difference in NLR was observed in patients with T2DM+ATHR group compared to control (Table.2) which related to change in neutrophil count. T2DM+ATHR group had higher neutrophil count than type 2DM and control groups. In contrast. lymphocytes count changed but not significantly between groups (Table. 2). MPO is a marker of neutrophil activation, therefore serum MPO levels were significantly elevated in T2DM+ATHR group than in control (Table.2). Serum IL-6 levels were significantly increased in patients with T2DM+ATHR and T2DM groups compared to control group (P value < 0.05, Fig 1), while serum level of Apo E is significantly decreased in patients with T2DM+ATHR compared to other groups (P value < 0.05, Fig 2.). qPCR results showed a significant decrease in miRNA-146a expression levels in patients with T2DM+ATHR group

compared to control group (P < 0.05, Fig.3), however its levels were decreased but non significantly in type 2DM group.

DISCUSSION

CVDs-caused by atherosclerosis are contributor to increase mortality in patients with T2DM. Atherosclerosis is a chronic inflammatory vascular disease characterized by unresolved inflammatory responses initiated by the accumulation of oxLDL [22] [20]. In the present study, levels of TG, TC and LDL-c were significantly increased in T2DM+ATHR, while HDL-c levels were relatively lower compared to control group (Table.1). Consistent with these results, studies have been previously recorded that dyslipidemia is higher in T2DM patients compared to healthy subjects [23,24] [21,22] The mechanism explaining dyslipidemia in the pathogenesis of T2DM is β-cell dysfunction and increased fatty acid influx secondary to insulin resistance.

The current study showed a significant increase in NLR in patient with T2DM+ATHR compared with other groups, these results consistent with other studies which suggested NLR as inflammatory markers in coronary vascular disease [25,26] Atherosclerosis is characterized by prolonged inflammatory response at all levels, in which inflammatory cells (WBC) infiltrate to site of damage, therefore NLR could be considered as indicator for systemic inflammation.

MPO levels are increased significantly in patients with T2DM+ATHR compared to control (P< 0.05). Atherosclerosis associate with increasing circulatory leukocyte which primarily produced MPO. Neutrophils account and NLR increase in T2DM+ATHR group which released more MPO into blood.

Hyperglycemia can initiate the production of ROS like H2O2leading to an increase in MPO activity [27]. Also increased production of inflammatory cytokines during atherosclerosis for example TNF-alpha, IL-6 and IL-8 result in activation of neutrophils which might enhance release of MPO from neutrophils and increased it levels in blood [28]

The present results indicated that IL-6 levels were significantly increased in T2DM+ATHR and T2DM groups compared to control (Fig.1). IL-6 was identified as proinflammatory cytokines that play a key role in inflammatory diseases like atherosclerosis and DM [29]. Previously it has been shown that high levels of IL-6 associated with atherosclerotic burden [30,31]. Patients with T2DM are known to be at an elevated risk for atherosclerosis [32]. Hypercholesterolemia activated mechanisms like Oxidative stress elevate IL-6 in type 2 diabetes in association with insulin resistance [33] therefore high IL-6 level in type 2 diabetes is an independent predictor of cardiovascular events [34,35]. ApoE is recognized to have a protective effect against atherosclerosis. Serum Apo E levels were significantly decreased in T2DM+ATHR compared to other groups (Fig.2). Low levels of apoE in the serum have been known to stimulate atherosclerosis by inhibiting the efflux of cellular cholesterol, thus initiating the formation of foam cell in the blood vessel wall [36]]. Also, it was identified the apoE's anti-atherogenic properties such as suppress myelopoiesis and the activation of monocytes in hyperlipidemic mice [37] these protective properties due to the: capacity of cellular expression of Apo E lipid efflux [38] and by its role to improve the ability of plasma HDL-c for cholesterol efflux. Results of present study indicated that low level of Apo E is linked with upregulation of IL-6 which suggested that apoE modulates inflammatory and immune responses [39]. Circulating miRNAs have been described as diagnostic biomarkers and potential therapeutic targets for CVD, including atherosclerosis [40]. MiR-146a has been identified to suppress inflammatory signaling pathways [41,42]. Downregulation of miR-146a expression was observed in the T2DM with ATHER and T2DM groups compared to control (Fig.3). This may be contributed to the strong activation of NF-κB signaling in plaque

The underlying mechanisms of these results are the levels of NF-κB were elevated by miR-146a down expression in T2DM patients with ATHER, miR-146a has been identified to have a role in negative regulation of NF-κB under high glucose conditions [43]. Activation of NF-κB was recognized in inflammatory cell like monocytes and macrophages in patients with CVD [44]. Also experiments in myeloid cells using animal models have contributed the initiation and progression of atherosclerosis to the activation of NF-κB [45]. Our findings demonstrate that low levels of apoE might participate to decrease miR-146a levels and suppress cellular activation. This may be to the inflammatory response signaling through TLR receptors under hyperlipidemic conditions. The results suggest that decreasing the expression of miR-146a in T2DM is likely to activate atherogenesis by activation NF-κB signaling [46,47].

CONCLUSIONS

This study explored the involvement of miR-146a and Apo E in the regulation of vascular inflammation in the atherosclerosis, a chronic inflammatory disease. Results demonstrate that decreased levels of apoE expression initiates inflammation by down regulation of miR-146a expression that enhances inflammatory cytokine IL-6. With an increase in NLR and MPO levels, Transcriptional and post-transcriptional events affect cellular miRNA levels. The decrease MiR-146a expression may be attributed to NF-κB transcriptionally induction during inflammatory processes. The results highlight the fact that cellular regulation of miR-146a by apoE, and target it for potential use to resolve the inflammation and atherosclerosis. Further studies are needed to determine whether monitoring apoE-related inflammatory effects in blood by miR146a could provide a biomarker relevant to apoE-dependent atherosclerosis.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

Dr. Anwar J almzaiel and Dr. Nawal Kanteel were contributed to design the research, to the analysis of the results and to the writing of the manuscript. The authors

approved the final version for submission.

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Table 1. Comparison of baseline characteristics for the study groups.

Parameter	T2DM+ATHR Mean ±SEM	T2DM Mean ±SEM	Control Mean ±SEM	P-value
Age (year)	51.7± 3.2	54.23± 4.5	52.4±2.2	P >0.05
BMI	26.2 ± 3.8	24± 3.1	26.5± 4.2	P >0.05
Sytosilic BP (mmHg)	145.02±16.03 *	138.02±14.15 *	126.55±13.22	P< 0.05
Diastolic BP (mmHg)	83.4±5.6	82.3±2.9	80.2±6.2	P>0.05
Total cholesterol mg/dl)	224.63 ± 43.13*	198.03 ± 59.48*	116.16 ± 24.31	P< 0.01
Triglycerides (mg/dl)	268.3 ±81.31*	207.9 ± 64.55*	125.5 ±31.24	P< 0.01
HDL-c (mg/dl)	42.9±1.2*	43.5±1.8*	47.6±2.3	P< 0.05
LDL-c (mg/dl)	151.46±6.3*	147.95±10.03*	130.13±10.3	P< 0.01
FBG (mg/dl)	255.3 ±71.60*	223.12±81.25*	96.76 ± 10.28	P< 0.01

* indicates significant differences compared to the control

Table 2. Neutrophils counts, lymphocytes count, NLR and MPO levels in the study groups

Parameter	T2DM+ATHR Mean ±SEM	T2DM Mean ±SEM	Control Mean ±SEM	P-value
Neutrophils counts (1000/ µl)	7.9±0.24*	5.22±0.54	3.2±0.12	P <0.05
Lymphocytes counts (1000/ µl)	1.59±0.34	1.72±0.15	1.45±0.21	P >0.05
NLR	5.62±0.22*	3.4±0.12	2.5±0.14	P< 0.05
MPO (ng/ml)	82.41±15.8*	60. 3±11.41	52.31±13.8	P< 0.05

* indicates significant differences compared to the control

Table 3. correlation between miRNA-146a and other parameters in the study groups

Parameter	T2DM+ATHR Mean ±SEM	T2DM Mean ±SEM	Control Mean ±SEM	P-value
ApolipoproteinE	7.9±0.24*	5.22±0.54	3.2±0.12	P <0.05
miRNA-146a	1.59±0.34	1.72±0.15	1.45±0.21	P >0.05
NLR	5.62±0.22*	3.4±0.12	2.5±0.14	P< 0.05
MPO (ng/ml)	82.41±15.8*	60. 3±11.41	52.31±13.8	P< 0.05

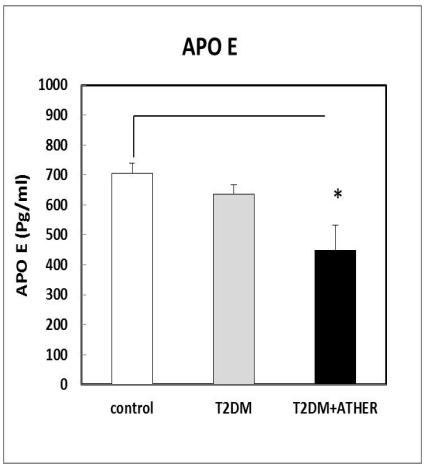
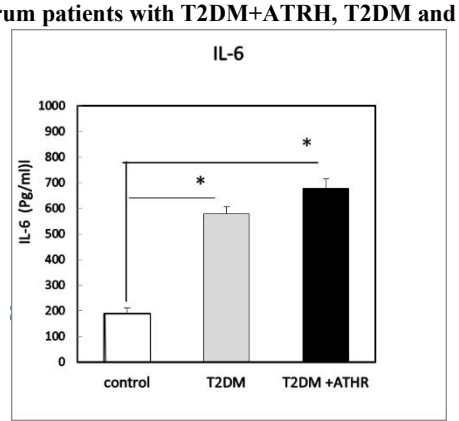


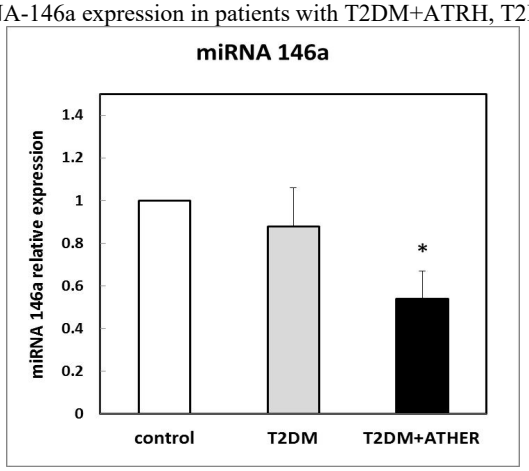
Figure 1. Interlukine-6 levels in serum patients with T2DM+ATRH, T2DM and control groups. Data are expressed as



means ± SEM, *indicates significant differences compared to the control, (P < 0.05)

Figure 2. Apo E levels in serum patients with T2DM+ATRH, T2DM and control groups. Data are expressed as means ± SEM, *indicates significant differences compared to the control, (P < 0.05)

Figure 3. Serum fold change miRNA-146a expression in patients with T2DM+ATRH, T2DM and control groups. Data are



expressed as means ± SEM, *indicates significant differences compared to the control, (P < 0.05)