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ORIGINAL ARTICLE

miR-342-5p Expression Levels in Coronary Artery Disease Patients and its Association with Inflammatory Cytokines

Reza Ahmadi [1], Esfandiar Heidarian [2], Reza Fadaei [3], Nariman Moradi [1], Mojtaba Malek [4], Soudabeh Fallah [1]

[1] Department of Biochemistry, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2] Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[3] Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[4] Research Center for Prevention of Cardiovascular Disease, Institute of Endocrinology & Metabolism, Iran University of Medical Sciences, Tehran, Iran

SUMMARY

Background: **Atherosclerosis is a progressive inflammatory disease and is the main underlying mechanism of coronary artery disease (CAD). Immune system cells and cytokines play pivotal roles in the development of atherosclerosis. Several studies have shown the role of microRNA in the inflammatory processes of atherosclerosis, and miR-342-5p has been shown to be involved in macrophage activation during atherosclerosis and cytokine secretion. But until now, there has been no data regarding the association of miR-342-5p with CAD and inflammatory cytokines.**

Methods: **This case control study was conducted on 82 CAD patients and 80 controls. Peripheral blood mononuclear cell (PBMC) miR-342-5p expression and gene expression of IL-6 and TNF-α were evaluated using real time-PCR. Also, the serum levels of IL-6 and TNF-α were measured using ELISA kits.**

Results: **The results demonstrated a higher expression of miR-342-5p in CAD patients compared to controls ($p < 0.001$). Moreover, logistic regression revealed an increased risk of CAD according to the expression of miR-342-5p after adjusting for CAD risk factors (OR [CI] = 6.1 [1.0 - 37.2], $p = 0.048$). Also, serum IL-6 and TNF-α showed higher levels in CAD patients ($p = 0.003$ and $p = 0.004$, respectively). Furthermore, there were positive correlations of miR-342-5p with gene expressions and serum levels of IL-6 and TNF-α.**

Conclusions: **The present study demonstrated higher levels of miR-342-5p in CAD patients and showed positive correlation with inflammatory cytokines. This result is in accordance with a previous study, and suggested a regulatory role for miR-342-5p in atherosclerosis and cytokine secretion, although more studies are required in this direction.**
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Correspondence:
Soudabeh Fallah
Department of Biochemistry
Faculty of Medicine
Iran University of Medical Sciences
Hemmat Highway
Tehran
Iran
P.O. Box: 141555983
Email: fallah.s@iums.ac.ir

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atherosclerosis, inflammation, TNF-α, IL-6, miR-342-5p, coronary artery disease

INTRODUCTION

Coronary artery disease (CAD) is one of the most common forms of cardiovascular disease (CVD), the leading cause of death globally, and is characterized by atherosclerosis in coronary arteries [1-3]. Lifestyle, environmental factors and genetic predisposition are directly in-

volved in CAD pathogenesis [4]. Indeed, the heritability of CAD has been estimated to be between 40 and 60% [5]. Importantly, the majority of signals for CAD identified by the genome-wide association study (GWAS) approach resided in the noncoding regions of the genome [4].
MicroRNAs (miRNAs, miR) are a non-coding single stranded RNA (containing about 22 nucleotides) that regulate the expression of a wide variety of target genes through binding to the 3' UTR of the mRNAs [6]. Also, several studies have shown the dysregulation of microRNA expression in the context of cardiovascular disease. Some microRNA have been suggested as diagnostic tools for cardiovascular diseases [7].
It has been reported that they are involved in many aspects of atherosclerosis progression including endothelial dysfunction, infiltration of inflammatory cells, low-density lipoprotein oxidation, and CAD-associated dyslipidemia [8]. Studies have shown the important role of microRNAs in the inflammatory aspect of atherosclerosis, for instance miR-155 plays a role in the inflammatory process and atherosclerosis [9]. Furthermore, a study showed that miR-342-5p promotes the production of inflammatory mediators and it has been suggested that it plays a role in atherosclerosis and was found to be one of the highly upregulated microRNA in early macrophage-rich lesions [10]. Studies have shown that miR-342-5p played a role in tumorigenesis and angiogenesis [11,12], but there is no data on the expression of miR-342-5p in CAD patients and its association with inflammatory cytokines and metabolic variables. Therefore, the present study aimed to evaluate the expression of miR-342-5p in CAD patients, controls and its association with inflammatory cytokines.

MATERIALS AND METHODS

Study population

The study population consisted of 162 Iranian individuals who underwent coronary angiography at the Hazrate Rasool Hospital, Iran University of Medical Sciences, Iran. The age and gender matched 82 CAD patients and 80 non-CAD (controls) were enrolled between April 2016 to February 2017.
All patients underwent coronary angiography to evaluate CAD. Patients with a history or evidence of diabetes, cancer, renal impairment or liver disease, or any other chronic illnesses were excluded. The diagnosis of CAD was based on typical coronary angiography. All angiograms were evaluated by a cardiologist, and, in this regard, CAD was defined as the presence of stenosis > 50% in at least one major coronary artery [13]. Moreover, the controls had normal coronary arteries (stenosis less than 5%). The medical history, drug consumption, and demographic data of the study population were collected on a questionnaire. The blood pressure, weight, and height were also recorded. Ethics approval was obtained from the research ethics committee of Iran University of Medical Sciences, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki (IR.IUMS.REC 1395.9221184201).

Biochemical parameter measurements

Blood samples were collected after 12 hours of fasting. The cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and fasting blood glucose levels were measured using aliquots of serum samples stored at -80°C until testing, using the standard enzymatic methods. In addition, TNF-α and IL-6 serum levels were evaluated using the human cytokine ELISA kits (International GmbH, IBL) according to the manufacturer's instructions.

PBMCs isolation and RNA extraction

PBMCs were isolated from 10 mL EDTA-containing whole blood tubes using Ficoll-Hypaque density-gradient centrifugation as previously described [14,15]. Subsequently, total RNA was extracted from the PBMC lysates using the miRNeasy mini kit (QIAGEN, USA) according to manufacturer's instructions. The concentration and purity of extracted RNA were evaluated by the NanoDrop spectrophotometer.

Quantitation of IL-6 and TNF-α mRNA

First-strand cDNA was synthesized from 300 ng of total RNA using cDNA synthesis kit (Takara, Japan). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara, Japan) and specific primers for IL-6 and TNF-α and beta-actin (QIAGEN, USA) in duplicate, according to the standard program on ABI-StepOne (Applied Biosystems, USA). The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to beta-actin.

First strand cDNA synthesis and miRNAs expression profiling

First-strand cDNA was synthesized from 300 ng of total RNA using miScript II RT Kit (QIAGEN, Germany). Quantitative Real Time-PCR was done using miScript SYBR® Green PCR Ki (QIAGEN, Germany) and specific primers for miR-342-5p and U6 (QIAGEN, USA). PCR amplification was carried out by use of ABI-StepOne (Applied Biosystems USA). The expression level of miRNA was determined using $2^{-\Delta\Delta Ct}$ and normalized to U6snRNA.

Statistical analysis

All statistical analyses were performed by SPSS Statistical Software Package (version 18.0). Normality was evaluated by the Shapiro-Wilk test. Categorical data were shown as frequencies and continuous data were shown as mean ± standard error of mean (SEM). Depending on the normality of the data, Student's *t*-test and Mann Whitney *U* test were carried out to compare continuous data between groups. Moreover, Pearson's correlation analysis was carried out to determine the correlation of miRNA expressions with biochemical pa-

rameters. Also, analysis of covariance (ANCOVA) was carried out to remove the possible effect of covariance on the expression of microRNA. Furthermore, binary logistic regression was performed to assess the risk of CAD, according to the expression of microRNA. p-values less than 0.05 were considered to be statistically significant.

RESULTS

Basic characteristics of the study population

The clinical and biochemical features of a total of 162 participants are presented in Table 1. As shown, CAD patients showed higher levels of BMI ($p = 0.014$) compared to non-CAD subjects. There were no statistically significant differences between CAD cases and normal subjects in terms of gender ($p = 0.438$), age ($p = 0.091$), FBS ($p = 0.263$), SBP ($p = 0.919$), DBP ($p = 0.649$), total cholesterol ($p = 0.331$), LDL-cholesterol ($p = 0.803$), and TG ($p = 0.065$) levels. Moreover, the controls had significantly higher levels for HDL-cholesterol ($p < 0.001$) than the CAD patients.

Analysis of miR-342-5p expression

Analysis of miR-342-5p expression in PBMCs revealed higher expression of miR-342-5p in CAD patients compared to controls ($p < 0.001$) (Figure 1A). In addition, ANCOVA was performed to remove the effect of age, gender, and BMI on the expression of miR-342-5p, and after adjustment the difference remained significant ($p = 0.001$). Also, binary logistic regression demonstrated a significant relationship between miR-342-5p and the presence of CAD (OR [CI] = 8.8 [2.2 - 35.7], $p = 0.002$), and after adjusting for classical CAD risk factor, it was found that miR-342-5p is significantly associated with the risk of CAD presence (OR [CI] = 6.1 [1.0 - 37.2], $p = 0.048$) (Table 2). Also, the ROC curve was plotted to assess the ability of miR-342-5p expression for the diagnosis of CAD. The result of this study demonstrated that the area under curve was 0.702 (CI = 0.620 - 0.783, $p < 0.001$) (Figure 2).

Comparison of cytokine levels between CAD patients and controls

The results revealed that TNF-α ($p = 0.004$) and IL-6 ($p = 0.003$) gene expression in PBMCs was significantly higher in CAD patients compared to controls (Figure 1B and Figure 1C). Furthermore, TNF-α demonstrated higher serum levels in CAD patients (12.69 ± 1.12 pg/mL) compared to controls (8.78 ± 0.52 pg/mL) ($p = 0.002$) (Figure 2A). Likewise, the IL-6 serum levels were found to be higher in CAD patients (6.45 ± 0.31 pg/mL) compared to controls (5.44 ± 0.31 pg/mL) ($p = 0.023$) (Figure 2B).

Association of miR-342-5p with molecular and biochemical parameters

The correlation of miR-342-5p and molecular, biochemical, and anthropometrical parameters were determined using Pearson's correlation analysis. The results are presented in Table 3. Correlation analysis in controls showed inverse correlation of miR-342-5p with DBP and HDL-C. Furthermore, miR-342-5p was found to be positively correlated with LDL-C, TNF-α, and IL-6 gene expressions as well as TNF-α and IL-6 serum levels. Also, the analysis of correlations in CAD patients showed inverse correlation between miR-342-5p and HDL-C. Moreover, miR-342-5p was found to be positively correlated with TNF-α and IL-6 gene expressions as well as TNF-α and IL-6 serum concentration.

DISCUSSION

Inflammation plays a substantial role in the development of atherosclerosis and regulation of inflammatory signaling and cytokines secretion that could be a potential therapeutic target for atherosclerosis and CAD [16]. Several studies have shown the association of microRNA with the feature of atherosclerosis. Some of them have been suggested as potential biomarkers for CAD, especially myocardial infarction [17]. Although, several microRNA have been studied in the context of CAD and atherosclerosis [18,19], only one study has evaluated the role of miR-342-5p in the pathogenesis of atherosclerosis in animal model and cell line [10]. The present study evaluated miR-342-5p as a new regulatory factor for macrophage activation in atherosclerosis [20]. The present findings demonstrated that the PBMC expressions of miR-342-5p was higher in CAD patients than in control subjects. This result was independent of age, gender and BMI. Also, miR-342-5p showed a relationship with CAD, independent from the classical risk factors for CAD. Two studies evaluated the role of miR-342-5p in cancer tumorigenesis and angiogenesis [11, 12]. One study has reported a role for miR-342-5p in the development of atherosclerosis in an animal model [10]. However, to the best of our knowledge, the present study is the first *in vivo* report regarding the association of miR-342-5p with CAD.

Yuanyuan et al. reported miR-342-5p as highly upregulated in macrophage-rich lesions. It also suggested that miR-342-5p plays a role in macrophage activity during atherosclerosis [10]. In line with this result, the findings of the present study demonstrated a higher expression of miR-342-5p in the PBMCs of CAD patients. PBMCs include mononuclear cells of the immune system that are involved in the development of atherosclerosis. Monocytes, as a part of PBMCs, infiltrate into the arterial wall during atherosclerosis and differentiate to macrophages which uptake Ox-LDL. The upregulation of miR-342-5p might increase the inflammatory response during atherosclerosis, although more studies are needed to prove this concept.

Also, it has been shown that miR-342-5p promotes the synthesis of inflammatory mediators such as NOS2 and CCL2 [10]. Moreover, the miR-342-5p inhibitor re-

Table 1. Anthropometric and laboratory measurement of study population.

Parameter	Controls (n = 80)	CAD patients (n = 82)	p
Gender (male/female)	41/39	35/47	0.438
Age (years)	57.86 ± 0.97	60.10 ± 0.89	0.091
Body mass index (kg/m^2)	26.14 ± 0.49	27.56 ± 0.47	0.014
Systolic blood pressure (mmHg)	129.8 ± 2.1	132.7 ± 3.3	0.919
Diastolic blood pressure (mmHg)	82.06 ± 1.40	82.13 ± 1.80	0.649
Fasting blood sugar (mg/dL)	92.54 ± 1.11	93.93 ± 1.22	0.263
LDL-Cholesterol (mg/dL)	75.73 ± 3.34	74.56 ± 2.82	0.803
HDL-Cholesterol (mg/dL)	44.93 ± 0.98	39.11 ± 1.02	< 0.001
Triglycerides (mg/dL)	131.2 ± 5.36	139.8 ± 4.57	0.065
Total Cholesterol (mg/dL)	134.7 ± 3.1	139.1 ± 3.2	0.331

Table 2. Odds ratios of CAD incidents according to miR-342 expression in PBMCs.

Model	B	S.E.	OR	95% C.I. for OR	p
1	2.172	0.716	8.8	2.1 - 35.7	0.002
2	1.815	0.919	6.1	1.0 - 37.2	0.048

Model 1 - Crude model.
Model 2 - Adjusted for age, gender, BMI, smoking, SBP, DBP, Chol, HDL-C, LDL-C, IL-6, TNF-α.

Table 3. Correlation analysis of miR-342 with anthropometric, laboratory, and molecular data.

	Controls		CAD	
	Correlation coefficient	Sig. (2-tailed)	Correlation coefficient	Sig. (2-tailed)
Age (years)	-0.144	0.204	-0.005	0.966
Systolic blood pressure(mmHg)	-0.218	0.052	-0.127	0.257
Diastolic blood pressure (mmHg)	-0.264	0.018	-0.058	0.604
Body mass index (kg/m^2)	-0.178	0.114	0.184	0.098
Fasting blood sugar (mg/dL)	-0.067	0.557	-0.111	0.320
Triglycerides (mg/dL)	-0.194	0.085	-0.070	0.533
Total Cholestrol (mg/dL)	0.194	0.085	0.003	0.979
LDL-Cholestrol (mg/dL)	0.357	0.001	0.109	0.331
HDL-Cholestrol (mg/dL)	-0.282	0.011	-0.233	0.035
TNF-Expression	0.257	0.021	0.220	0.047
IL6-Expression	0.0.260	0.020	0.416	0.000
TNF-α (pg/mL)	0.305	0.006	0.303	0.006
IL-6 (pg/mL)	0.233	0.038	0.503	0.000

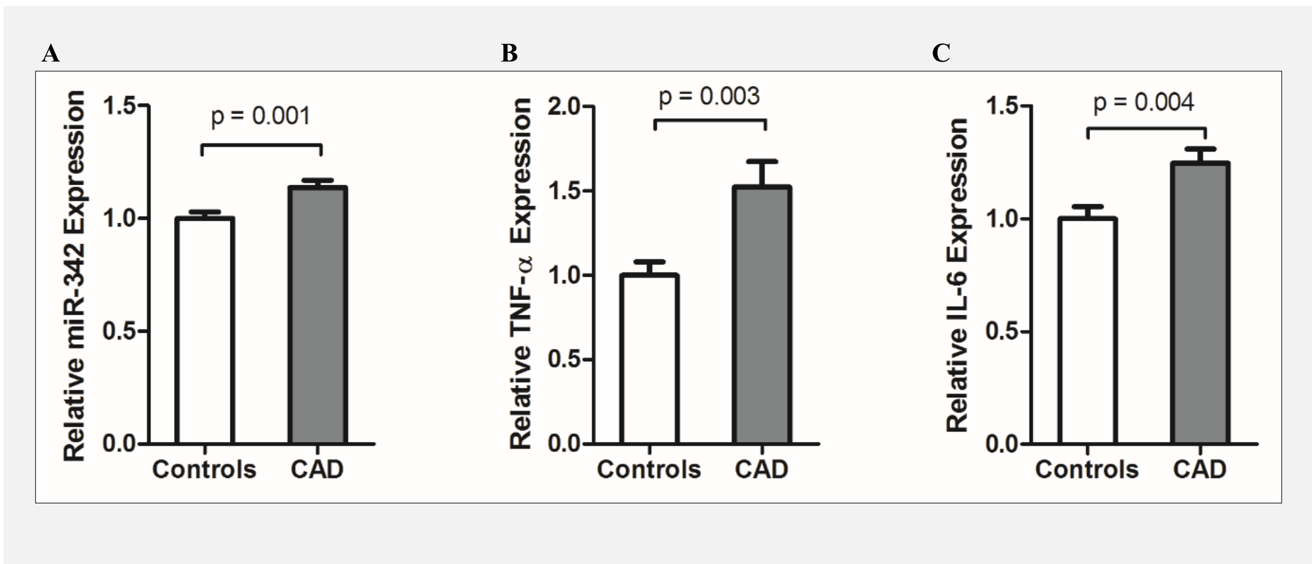

Figure 1. miR-342-5p expression and gene expressions of TNF-α and IL-6 in PBMCs.

A) miR-342-p5 showed higher expression in CAD group (p = 0.001). B) TNF-α gene expression in PBMCs was found to be higher in CAD patients (p = 0.003). C) IL-6 gene expression in PBMCs of CAD patients was higher than controls (p = 0.004).

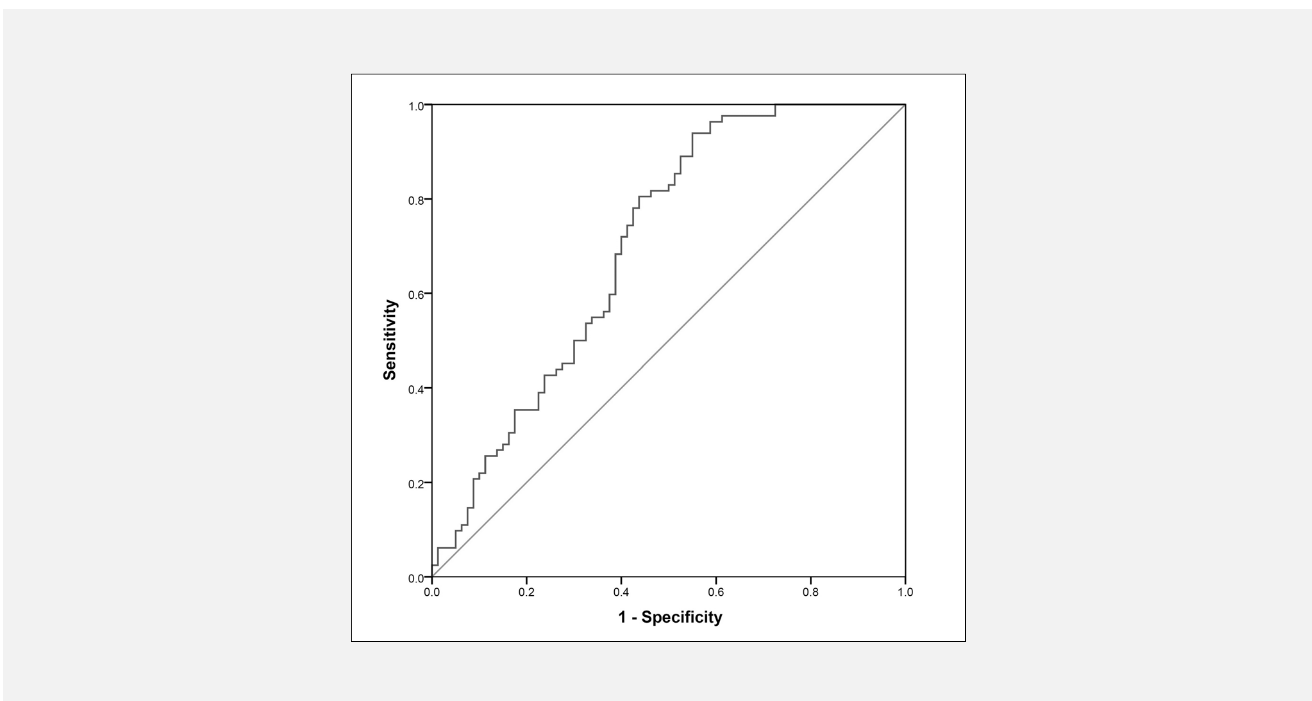

Figure 2. ROC curve for diagnosis of CAD.

duced IL-6 and TNF-α expression [10]. In line with this result, the positive correlation of miR-342-5p expression with IL-6 and TNF-α gene expressions and serum levels was detected. The result of the present study is an *in vivo* evidence for the association of miR-342-5p with inflammation that could be a possible underlying mechanism for the association of miR-342-5p with CAD. miR-342-5p was found to be positively associated with

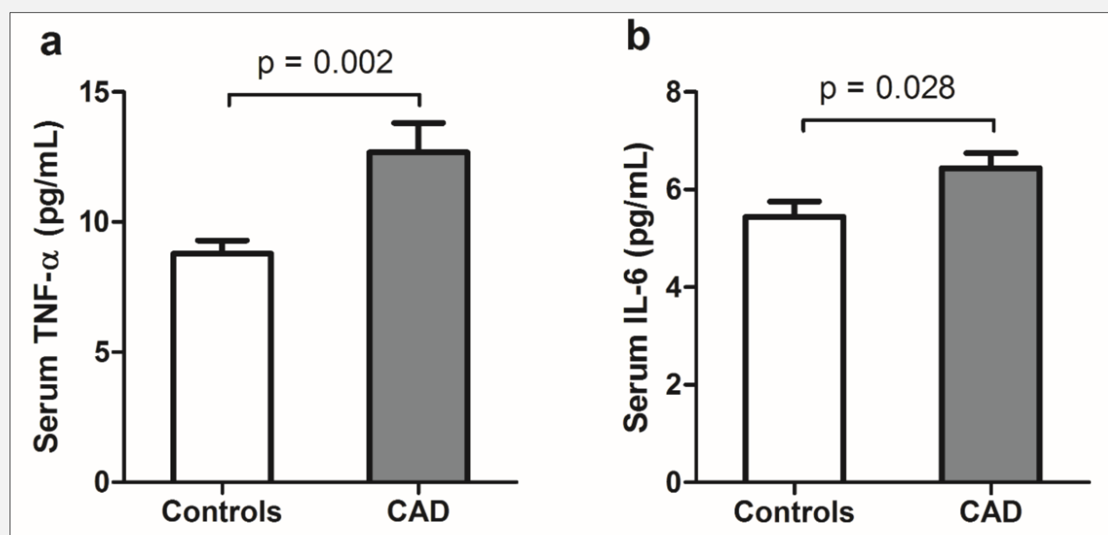

Figure 3. Serum concentration of cytokines.

A) Serum levels of TNF-α showed higher levels in CAD group (p = 0.002). B) IL-6 serum concentration was found to be higher in CAD group (p = 0.028).

LDL-C and negatively associated with HDL-C. A previous study showed that highly oxidized LDL upregulated the expression of miR-342-5p [10]. This result suggested a possible relationship between miR-342-5p and lipoprotein metabolism; however, further studies are needed to detect a possible underlying mechanism. Taken together, the results obtained serve as *in vivo* evidence for the relationship of miR-342-5p with CAD and inflammatory cytokines and is in line with a previous study by Yuanyuan et al. that suggested a role for miR-342-5p in atherosclerosis by promoting the expression of L-6 and TNF-α [10].

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Declaration of Interest:
The authors declare no conflict of interest

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