Original Article

Role of Plasma Soluble Lectin Like Oxidized Low-Density Lipoprotein Receptor-1 in Severity of CAD Patients and Relationship with Microrna-98

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Background: Coronary artery diseases are the most important cause of premature death and it is predominantly related to atherosclerosis. Soluble lectin like oxidized low-density lipoprotein receptor-1 (sLOX-1) and microRNAs are closely associated with atherosclerotic coronary heart diseases.
Aims: The current study investigated the relationship of plasma sLOX-1and the severity of coronary artery disease patients (CAD) and association with microRNA-98.
Study Design: Case control study.
Material and Methods: Angiographically documented 38 single coronary lesions, 75 double coronary artery disease, 62 multi-vessel coronary artery disease patients, 62 healthy control subjects, and 24h hypoxic (1% O2) HUVEC cells were included in this study. Circulating sLOX-1concentrations were determined through enzyme-linked immunosorbent assays and microRNA-98 expressions were measured by the quantitative real- time polymerase chain reaction.
Results: The expressions of plasma sLOX-1 levels were progressively and significantly higher in single, double, and multi-vessel CAD patients than healthy control subjects ($p<0.001$). Circulating sLOX-1 concentrations in multi-vessel, double vessel and single vessel CAD female subjects had evidently elevated than male subjects ($p<0.001$). Plasma sLOX-1 values were remarkably increased in female different age groups CAD patients as compared with the same male age subjects ($p<0.001$). Single vessel diseased (AUC 0.879), double vessel diseased (AUC 0.928) and multi-vessel diseased (AUC 0.943) CAD patients have been clearly differentiated from healthy participants with high sensitivity and specificity.
The expression of microRNA-98 noticeably down-regulated in single, double and multi-vessel occluded CAD patients and hypoxic exposed HUVEC than controls ($p<0.001$). Significantly elevated LOX-1 and caspase-3 activity and remarkably decreased cellular viability in hypoxic injured HUVEC. On the contrary, mimic of microRNA-98 markedly reduced caspase-3 and LOX-1 levels and highly increased cellular viability.
Conclusion: Elevated circulating plasma sLOX-1 levels have a potential impact to identify the severity of coronary artery disease and a strong correlation with aging as well as the female gender. Reduced plasma miR-98 level possible considers a risk factor for CAD, and agomiR-98 prevents atherosclerosis and cellular injury through targeting LOX-1.
Key words: Coronary artery lesions, Plasma sLOX-1, Female gender, MiR-98, HUVECs

Globally coronary atherosclerotic heart diseases are the major epidemic health problem and the predominant cause of increased morbidity and mortality. Early identification with proper management of stable coronary artery disease may significantly reduce further coronary atherosclerotic complications including unstable coronary artery disease, myocardial infarction, and markedly reduced cardiac mortality rate (1). The clinical presentations of stable coronary artery disease especially younger, geriatric people, diabetes mellitus, and neuropathies patients are atypical even sometimes they don't have any clinical symptoms. A large number of stable CAD patients may have normal electrocardiogram (ECG) findings and even exercise tolerance test (ETT) reports were also not significant. Cardiac

CT angiogram and especially invasive coronary angiogram may help to confirm the diagnosis of stable CAD patients but required special arrangements and it may produce simple to severe complications and also not suitable for all the patients. Therefore, blood-based biomarkers are necessary to diagnosis as well as the grading of stable coronary artery disease patients (1, 2).

Coronary atherosclerosis is a chronic multi-factorial inflammatory process within coronary arteries. Lectin like oxidized low-density lipoprotein receptor-1 (LOX-1) has critical roles for the development of atherosclerosis through binding, internalization, and degradation with oxidized low-density lipoprotein cholesterol (ox-LDL) in the arterial wall (3). The human LOX-1(OLR-1) is a 50 kDa transmembrane glycoprotein located in the 12 chromosome as a C-type lectin gene cluster. Initially, it was detected in bovine aortic endothelial cells subsequently identified from human coronary artery endothelial cells, cardiomyocytes, macrophages, fibroblasts, platelets, and smooth muscle cells. The extracellular neck area of the LOX-1 protein usually cleaved through an enzymatic reaction (metalloproteinase, ADAM10) and generated soluble LOX-1 (sLOX-1) which circulated in different body fluids including blood, and triggers to release many inflammatory cytokines and progress to atherosclerosis. This circulatory sLOX-1 has potential value to diagnosis and prognosis of many cardiovascular diseases (4).

An early stage of the atherosclerosis process mainly initiated by endothelial dysfunction and LOX-1-ox-LDL primarily involved in vascular injury by several inflammatory mechanisms. Activate LOX-1 significantly increased ROS production, endothelial cellular apoptosis, caspase-3, caspase-9 and inhibiting of anti-apoptotic BCL-2 protein (5). Moreover, PCSK9 and LOX-1 concentrations were elevated in human endothelial cells and vascular smooth muscle cells and they have significantly linked with ox-LDL and LPS. All these leads to arterial wall damage, a hallmark of atherosclerosis (6). Several clinical studies have been demonstrated that circulating sLOX-1 levels were significantly higher in essential hypertension, type-2 diabetes mellitus, metabolic syndrome, heart failure, ischemic heart disease patients, and ischemia-reperfusion injury (7, 8, 4). Recently, Valter's research group demonstrated circulating sLOX-1 concentrations remarkably increased in stable coronary artery disease and have a considerable impact on the diagnosis of stable CAD patients (9). It has also been reported that elevated circulating sLOX-1 levels were positively associated with lesions of stable coronary artery disease, acute coronary syndrome patients and traditional CAD risk factors (10, 11).

Furthermore, microRNAs are highly conserved, endogenous, single-stranded non-coding RNAs that generally regulated gene expression at the post-transcriptional level through the 3′-untranslated region (3′-UTR). It has been well established that miR-217, miR-92, and miR-126 significantly regulated the atherosclerosis process (12). The expressions of circulatory miRNAs (miR-21, miR-149, miR-378, and miR-765) were remarkably altered in coronary artery disease patients (13, 14). Besides, miR-590, miR-155, and miR-let-7g-3p critically regulated LOX-1-ox-LDL mediated lipid metabolism and prevent human umbilical vein endothelial cellular (HUVECs) damage during hypoxic injury through modifying their target proteins(4,15). Recently demonstrated microRNA-98 significantly regulated foam cell formation and lipid accumulation through targeting LOX-1 in mice model, besides, it's modulated various inflammatory cytokines production such as IL-6, IL-8, IL-10, and TNF-α in human peripheral blood mononuclear cells (PBMCs)(16,17). However, the severity of coronary disease patients and its correlation with circulating sLOX-1 and microRNA-98 have not yet been fully investigated. Therefore, this study demonstrated the clinical impact of plasma sLOX-1 level in single, double, and multi-vessel diseased stable CAD patients and linked with gender and aging. Additionally, the role of microRNA-98 in CAD patients and its effect on LOX-1 expressions during hypoxic injured HUVECs also examined.

Materials Methods

Human study subjects

This study recruited angiographically confirmed 38 single vessel lesions, 75 double vessel lesions, 62 multi-vessels lesions coronary artery disease patients, and 62 healthy participants from January 2016 to December 2018. Three interventional cardiologists had been performed the invasive coronary angiogram through radial approach. Coronary arteries occluded at 50% or more than 50% were considered as coronary artery disease patients. Only one major (LAD, LCX, RCA) coronary artery blocked defined as a single vessel lesion, double vessel considered when blocked two major coronary arteries, and left main coronary artery also included as a double vessel diseased, two or more than two major coronary arteries including their diagonal or other branches stenosis have categorized as a multi-vessel diseased CAD patient. Previously implanted coronary artery stent, history of AMI and stroke, malignancy, chronic obstructive pulmonary disease (COPD), and bronchial asthma patients were excluded from this study. Free from cardiovascular diseases, no evidence of lipid disorders, chronic inflammatory conditions, chronic hepatic and renal disorders were included as a healthy volunteer. Written informed consent had taken from all the participants and their baseline, clinical, and medication data were collected following the instructions of the Declaration of Helsinki from the medical record files. The patient's 4 ml blood samples were obtained from an antecubital vein after 72 hours of coronary angiography. In accordance with company instructions plasma sLOX-1

levels were measured from all the human samples by Enzyme-linked immunosorbent assays (Bio-Rad Co, USA). Laboratory parameters including total cholesterol (T-CHOL), low-density lipoprotein (LDL), triglycerides (TG), high-density lipoprotein (HDL), creatinine, high sensitive C-reactive protein (hs-CRP) were demonstrated using standard laboratory methods by an automatic biochemical analyzer machine (Hitach75, Tokyo, Japan).

Cell culture and transfections

Human umbilical vein endothelial cells (HUVECs) were obtained (Chinese academy of medical sciences, Shanghai, China) and harvested in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Waltham, MA, USA) along with 20% fetal calf serum (FBS; HyClone, Logan, UT, USA) in 6 well plates at a density of 5×10^5 cells /well with 95% humidified atmosphere at 37°C in 5% CO2. Hypoxic HUVECs were generated by 18h incubated in 1% O2, 95% N2, and 5% CO2 by using a hypoxic modulator (Model 3131, Forma Scientific, Marietta, OH, USA) at 37°C. HUVECs were transfected with has-mimic-miR-98(50 nmol/L) for over-expression and has-mimic-negative control (NC)-miR-98(50 nmol/L) (RiboBio, Guangzhou, China) for serving as an inner control with using Lipofectamine 2000 transfection agent by following the company's instructions (Invitrogen, Carlsbad, USA), more details in our previous study(15).

RNA extraction and measurement of miR-98 levels by RT-qPCR

RNA was extracted from human plasma, normoxic, hypoxic, and transfected cultured HUVECs by using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's guidelines. The expression of miR-98 was measured by the 7300 Quantitative Real-Time PCR system (Applied Biosystems, Foster city, CA, USA) using Takara SYBR Green Master Mix reagents (Dalian, China). MicroRNA-98, endogenous microRNA-156a primers structured were obtained from RiboBio, and LOX-1, β-actin(inner control) primers were designed by the Shanghai Sangon Biotech (Shanghai, China), the methods were more explained in our past study(15).

Measurement of caspase-3 activity and cellular viability

Normal, hypoxic and transfected HUVECs caspase-3 activity was demonstrated by Beyotime assay kits (Shanghai, China) and cellular viability was measured by CCK-8 kits (Beyotime, Shanghai, China) following the company's instructions. Briefly, 10 μL of cell lysates were added with 90 μL of work solution containing caspase-3 substrate (Ac-DEVD-pNA), and subsequently incubated at 37 °C for 2hrs. HUVECs 96-well microplates were used and each plate absorbance was measured at 405nm for the caspase-3 activity and at 450nm for the cellular viability by the SpectraMax reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Normality distributions of the numeric variables data were analyzed by the Shapiro-Wilk test. Continuous variables data were measured as mean with standard deviation and categorical variables were expressed as numbers and percentages. The differences between two groups were conducted with Student's t-test for normally distributed data, Mann-Whitney U test was implemented for abnormally distributed data, and for multiple groups one-way analysis of variance method used followed by Tukey post hoc. Categorical variables were determined through the chi-square test. A medium effect size was 0.05, an α error of 0.05, power of the study 90%, and required a total sample size was192. Sensitivity and specificity were explored by the receiver operator characteristic (ROC) curve. All p values were 2 sided and $P < 0.05$ accepted as statistically significant. Windows SPSS 20 software was used for analyzing all the data (SPSS Inc, Chicago, IL, USA).

Results

Baseline and clinical information of the study subjects

Current study conducted with 38 single vessel, 75 double vessel, 62 multi-vessel occlusions coronary artery disease patients (CAD), and 62 healthy study subjects. The characteristics of the study participants were shown in Table 1. Body mass index (BMI), family history of CAD, high-sensitivity C-reactive protein (hs-CRP) were significantly changed between controls and single, double, also multi-vessel CAD patients ($P < 0.001$), but within single, double, and multi-vessel CAD patients were not statistically significant. T-CHOL and HDL concentrations in double and multi-vessel CAD patients were significantly higher than healthy subjects whereas, triglyceride (TG) and LDL levels were significant differences observed among healthy and multi-vessel CAD patients ($P < 0.001$).

----------Please insert---------- Table 1----------here----------

Plasma sLOX-1 concentrations in healthy and coronary artery occluded CAD patients

The plasma sLOX-1 expressions were significantly elevated by 6.6 fold in multi-vessel CAD (632±54)pg/mL, 4.4 fold in double vessel CAD (418±51)pg/mL, and 3.8 fold in single vessel CAD(367±40)pg/mL patients as compared with healthy participants(95±12)pg/mL ($p<0.001$). The pattern of sLOX-1 expressions within single, double and multi-vessel lesions CAD patients were highly different ($p<0.001$) (Figure-1A). Furthermore, single vessel, double vessel, and multi-vessel occluded female CAD patients had markedly higher plasma sLOX-1 levels than males, indicated circulating sLOX-1 concentrations have significantly linked with female CAD subjects (Figure-1B).

Besides, circulatory plasma sLOX-1 expressions in female healthy subjects were slightly higher than male healthy study groups but not significant.

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The relationship of plasma sLOX-1 expression with age variations in CAD lesions and control subjects

Plasma sLOX-1 levels were evidently increased in female (29-49yrs), (50-69yrs) and (70-80yrs) groups multi-vessel lesions CAD patients than same male age groups multi-vessel lesions CAD patients ($p<0.001$), while, within male subjects among different ages circulating sLOX-1 were not significant, it suggested multi-vessel diseased female CAD patients associated with sLOX-1 concentrations (Figure-2A). In double vessel diseased (50-69yrs) and (70-80yrs) female CAD groups were significantly higher plasma sLOX-1 expression than a double vessel occluded same age groups male CAD subjects($p<0.001$), both female and male double vessel CAD patients among (29-49yrs) groups were not significant (Figure-2C). The expression of circulating sLOX-1 concentrations in female (29-49yrs) and (50-69yrs) single vessel diseased CAD subjects were noticeably up-regulated than similar male age groups single vessel diseased CAD subjects ($p<0.001$) (Figure-2C). Moreover, sLOX-1 concentrations were slightly increased in both male and female healthy subjects among all the age groups (Figure-2D).

----------Please insert---------- Figure 2----------here----------

Impact of sLOX-1 in the severity of CAD patients

The clinical impact of plasma sLOX-1 to identify the degree of coronary artery stensosis was evaluated through the ROC curve. Circulating sLOX-1 expression in single vessel lesion coronary artery patients were strongly distinguished from healthy subjects with high specificity and sensitivity (Table 2) with an AUC of 0.879 (Figure 3A). In double vessel diseased CAD patients, sLOX-1 levels were evidently differentiated from healthy participants with remarkable-sensitivity and sensitivity, and the area under the curve (AUC) was 0.928 (Figure 3B). The ROC curve of circulating plasma sLOX-1 between controls and multi-vessel diseased CAD patients revealed a significant discrimination with a prominent AUC of 0.943 (Figure 3C). Moreover, ROC curves of plasma sLOX-1 were also highly significant and able to discriminate between single, double, and multiple vessel CAD subjects. These findings recommended that up-regulated plasma sLOX-1 has an essential impact to categorize the severity of coronary artery stensosis.

----------Please insert---------- Figure 3----------here----------

----------Please insert---------- Table 2----------here----------

Expression of microRNA-98 in Human plasma and HUVEC cells

Circulating human plasma miR-98 expressions were significantly and progressively lower in single, double, and multi-vessel lesions CAD patients than that in the healthy control groups($p<0.001$). Besides, plasma miR-98 concentrations in multi-vessel CAD patients were remarkably down-regulated as compared with double and single vessel diseased CAD subjects (Figure 4A). Moreover, miR-98 expressions were evidently reduced in 18h hypoxic incubated HUVEC cells than normal HUVEC cells ($p<0.001$(Figure 4B).

----------Please insert---------- Figure 4----------here----------

Effect of microRNA-98 in LOX-1, Caspase-3 and cellular viability in Hypoxic induced HUVECs

LOX-1 is the direct target gene of microRNA-98 identified from the target scan. The expressions of LOX-1 mRNA protein were significantly elevated in 18h hypoxic induced HUVECs as compared with normal HUVECs but LOX-1 expressions were amazingly reduced while hypoxic HUVECs transfected with mimic-miR-98($p<0.001$) (Figure 5A). The relative activities of capase-3 in 18h hypoxic injured HUVECs were noticeably higher compared with normoxic HUVECs. Conversely, caspase-3 activities in 18h hypoxic HUVECs were markedly down-regulated with over-expression of microRNA-98 ($p<0.001$), and negative control (NC) showed no obvious reaction with hypoxic groups (Figure 5B). Cellular viability markedly reduced in hypoxic conditions, but HUVECs viability distinctly increased with mimic-miR-98(Figure 5C).

----------Please insert---------- Figure 5----------here----------

Discussion

Coronary artery disease is usually occurred due to formation of atherosclerotic plaque within coronary artery. Increased plasma sLOX-1 levels considered as an early predictor of endothelial injury and atherosclerotic plaque [7]. Present study found that plasma sLOX-1 levels were significantly higher expressions in multi-vessel CAD patients followed by double vessel, single vessel as compared with healthy controls. Moreover, circulating sLOX-1 expressions within single, double and multi-vessel diseased CAD patients were also highly significant. Besides, sLOX-1 concentrations in single, double and multi-vessel CAD female subjects were noticeably elevated than male subjects. These results suggested plasma sLOX-1 concentrations have significant role to differentiate simple and complex coronary artery disease patients, and its level may be potentially useful to determine the progression of CAD lesions. Zhao et al. demonstrated patients with complex CAD lesions had remarkable higher circulating serum sLOX-1 concentrations as compared with simple CAD lesions patients, which closely linked with present clinical

research (18). Research studies reported that sLOX-1 levels highly expressed in three-four vessel occluded CAD patients and also from proximal segment of LAD lesions than distal portion of LAD lesions, their results are directly supported present study [19, 20]. The sensitivity and specificity of CAD lesions have been investigated by the ROC curve. The single vessel, double vessel and multi-vessel diseased CAD patients were clearly differentiated from healthily subjects with AUC of 0.879, 0.928, and 0.943 respectively. The sensitivity and specificity values between single, double and multi-vessel were evidently higher and statistically significant. These findings indicated up-regulated plasma sLOX-1 may be used as a novel clinical parameter for determining the severity of the CAD lesions. These results also were in accordance with other clinical studies (21-22,8). Zhao et al. and Liu et al. clinical studies reported circulating serum sLOX-1 concentrations have significant linked with major cardiovascular events and it may be used as prognostic marker and risk stratification in stable CAD patients after percutaneous coronary intervention (23, 24).

Although age and gender is the major non modifiable risk factors for coronary artery diseases but there is lack of information regarding sLOX-1 and aging. The present study first time demonstrated sLOX-1 concentrations among different ages between male and female in healthy subjects, single, double and multi-vessel CAD patients. Circulating sLOX-1 expressions in different ages of female healthy subjects were fairly higher than male healthy subjects**.** Very interestingly, present research found that circulating sLOX-1 values were markedly up-regulated in female (29-49yrs), (50-69yrs) single vessel CAD lesions compared with similar ages male subjects, but sLOX-1 expression among (70-89yrs) female and male subjects have not been prominently elevated, may be due to less number of single vessel patients were found in this age group. In (50-69yrs), (70-89yrs) female double vessel CAD patients had remarkably higher plasma sLOX-1 than identical male age groups, whereas (29-49yrs) female and male subjects have not shown any oblivious different, probably double vessel CAD are usually not very common in this female age group patients. Circulating plasma sLOX-1 levels in (29-49yrs), (50-69yrs), (70-89yrs) female multi-vessel diseased CAD patients were remarkably up-regulated as compared with the same male age groups patients. These results strongly supported that sLOX-1 concentrations are evidently associated with progression of CAD patients and closely linked with female gender. Andreas et al. identified sLOX-1 expressions were significantly higher in the wall of epicardial coronary arteries in male (median age 61 yrs) 3 vessel coronary artery disease patients but they didn't explore expression pattern in female gender (25). Current study found that highest expression of hs-CRP levels were measured in multi-vessel CAD patients followed by double and single vessel diseased CAD patients and they were statistically significant. Its suggested hs-CRP have major role in the initiation and progression of CAD lesions. Nicole et al. investigated that LOX-1 positively correlated with CRP through ox-LDL and enhanced endothelial dysfunction during atherosclerosis process (26). In our previous study also demonstrated CRP levels were significantly increased in CAD patients as compared with healthy participants [14]. In addition, family history of CAD were significantly higher in single, double and multi-vessel diseased CAD patients, BMI measurement were distinctly higher levels in double and multi-vessel CAD, TC levels prominently elevated and HDL remarkably decreased in double and multi-vessel CAD, TG and LDL levels obviously increased in multi-vessel CAD patients than healthy subjects. These data recommended lipid abnormalities had intimately related with severity of CAD lesions.

Moreover, microRNAs are significantly involved in various cardiovascular diseases including coronary artery disease. Several circulating miRNAs have potential utility as diagnostic and prognostic biomarkers for ischemic heart disease patients [14]. A very important finding of the current study is that plasma miR-98 markedly decreased in single vessel followed by double and multi-vessel CAD patients compared with healthy participants. Furthermore, the expressions of microRNA-98 were significantly down-regulated in 18h hypoxic exposed HUVECs than normoxic cell. Recent clinical research demonstrated serum miR-98 expressions were evidently lower in bronchial asthma patients than healthy subjects (27). However, present research excluded COPD and bronchial asthma patients to reduce possible error. This study through targetscan, mirfinder, miranda bioinformatics databases and also from other studies confirmed that LOX-1 is the direct target protein of miR-98(16,28). Present study revealed LOX-1 expressions in 18h hypoxic injured HUVECs were markedly up-regulated than normoxic cells, while over-expression of miR-98 amazingly down-regulated LOX-1 expressions as close to normoxic cells, its recommended miR-98 significantly controlled LOX-1 expressions during hypoxic damage of HUVECs. Yao dai et al. reported miR-98 have essential role to control foam cell formation and prevent excess cholesterol accumulation in ApoE mice through modification of LOX-1 protein expressions (16). Chen et al. found over-expression of microRNA-98 remarkably increased HUVEC cellular viability through regulation of LOX-1 gene expression (28). Chuan Sun research group established agomiR-98 significantly reduced caspase-3 activity and increased cellular viability during ischemic injury (29).

This research found that caspase-3 activity levels were exceptionally increased in 18h hypoxic HUVECs than normal cells; on the contrary, caspase-3 activities were obviously decreased in mimic-miR-98 hypoxic HUVECs.

Furthermore, cellular viability significantly lower in hypoxic induced HUVECs groups than normal groups, whereas, cellular survivability remarkably enhanced in hypoxic HUVECs treated with agomiR-98. These results indicated mimic-miR-98 protect cellular injury in hypoxic conditions, may propose a therapeutic strategy for coronary atherosclerotic disease. To reduce angiographic dye and catheter effect on vascular injury as well as circulatory expression of sLOX-1 and miR-98, blood samples had taken after 72hours of coronary angiography from all the confirmed CAD patients.

Some limitations of the current study need to be addressed. Importantly, the data collected from only one provincial tertiary care hospital and the sample sizes among different groups were relatively small. Reporter gene assay have not been performed. Further multicenter with larger study samples are required to establish the circulating sLOX-1 as risk stratification for detecting the severity of coronary heart disease patient. Moreover, the relationship between sLOX-1 and mRNA-98 to the lipid levels and also linked with inflammatory markers such as IL-6, TNF, hs-CRP, need to more study to discover their underline molecular mechanism.

Conclusion

Circulating sLOX-1 can be use a potential predictor to recognize the severity of CAD patients. Reduced plasma microRNA-98 levels may consider a risk factor for CAD patients. Over-expression of miR-98 suppressed caspase-3 activity and improved cellular viability through targeting LOX-1, providing a possible therapeutic strategy against atherosclerotic CAD patients.

Disclosures

None

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TABLE 1. Clinical parameters of the study groups

Characteristic	Healthy subjects	Single vessel CAD	Double vessel CAD	Multi-vessel CAD	P_1	P_2	P_3
Sample size	62	38	75	62			
Age, yrs	54.7±10.8	61.93±11.5	63.95±11.9	66±12.7	0.256	0.197	0.143
Gender, (male/female)	33/29	22/16	41/34	33/29	0.688	0.867	1.000
BMI, kg/m^2	22.7±2.8	23.9±4.6	25.7±5.1	25.9±8.7	0.128	0.000	0.000
CAD risk factors: %							
Tobacco smoker	61(37)	69(26)	71(53)	76(47)	0.403	0.208	0.083

Hypercholesterolemia	0	83(31)	86(64)	88(54)	-	-	-
Type-2 Diabetes mellitus	0	21(8)	30(22)	33(20)	-	-	-
Family history of CAD	8(5)	57(21)	61(45)	64(39)	0.000	0.000	0.000
Hypertension	0	71(27)	75(56)	81(50)	-	-	-
Investigation data:							
High-sensitivity C-reactive protein, mg/L	1.92±0.35	14.2±6.17	18.5±9.41	22.7±11.86	0.000	0.000	0.000
Creatinine(μmol/L)	82.5±17.41	86.4±28.13	89.2±35.19	89.81±37.6	0.773	0.426	0.215
T-CHOL(mmol/L)	3.59±0.04	5.36±3.26	6.97±1.85	8.28±7.13	0.094	0.000	0.000
TG (mmol/L)	1.53±0.62	2.05±0.49	2.86±0.64	3.95±0.82	0.118	0.072	0.000
Low-DL(mmol/L)	2.57±0.81	3.95±1.25	4.22±1.07	5.16±1.88	0.217	0.094	0.000
High-DL(mmol/L)	1.33±0.56	0.92±0.17	0.74±0.52	0.23±0.69	0.310	0.000	0.000
Left ventricular EF %	61.25±11.17	59.33±8.31	54.87±6.27	49.87±4.53	0.479	0.281	0.064
Medications: *%*							
Anti-platelet agents	0	76(29)	89(67)	92(57)	-	-	-
Beta blockers	0	81(31)	85(64)	87(54)	-	-	-
Nitrates groups	0	64(24)	73(55)	77(48)	-	-	-
Calcium blockers	0	7(3)	10(7)	14(9)	-	-	-
Statins drugs	0	68(26)	71(53)	74(46)	-	-	-
P1: Healthy and single vessel CAD, P2: Control with double vessel CAD, P3: Healthy subjects versus multi-vessel occlusions CAD							

TABLE 2. The receiver operator characteristic curve analysis (ROC) of plasma sLOX-1 ratios in the prediction of CAD patients

Plasma sLOX-1	AUC	Cut-off point	Sensitivity	Specificity	CI (95%)	Positive value	Negative value	*P* value
Single vessel CAD vs control subjects	0.879	1.79	0.89%	0.90%	0.815 to 0.942	38	62	p<0.001
Double vessel CAD vs healthy subjects	0.928	1.97	0.99%	0.98%	0.878 to 0.978	75	62	p<0.001
Multi-vessel CAD vs Controls	0.943	1.89	0.96%	0.94%	0.896 to 0.989	62	62	p<0.001
The AUC curve, cut-off point, sensitivity, specificity, positive and negative value, confidence interval (95%), and P values of sLOX-1 were shown in Table 2.								

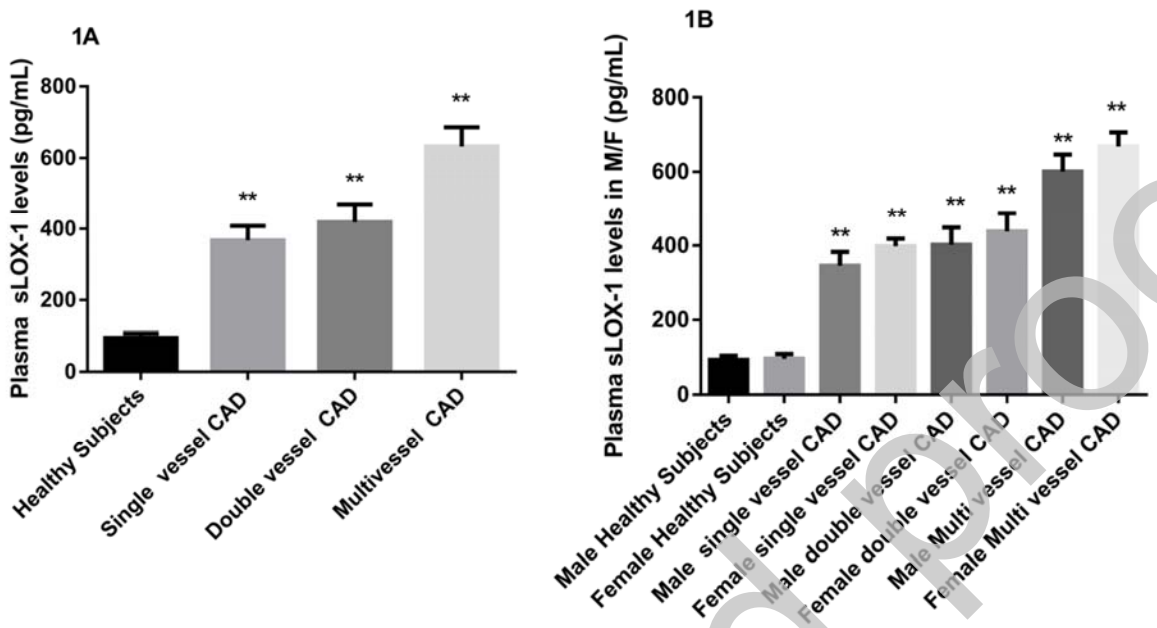

FIG. 1. A. The expression of circulating soluble LOX-1 between healthy versus single, double and multi-vessel CAD subjects. Figure 1B: The relative expression of plasma sLOX-1 in male and female gender among different CAD lesions subjects and also in healthy participants. Plasma sLOX-1 was evaluated by ELISA technique and quantitative data were expressed as mean ± SD from three independent experiments. ** ($p<0.001$)

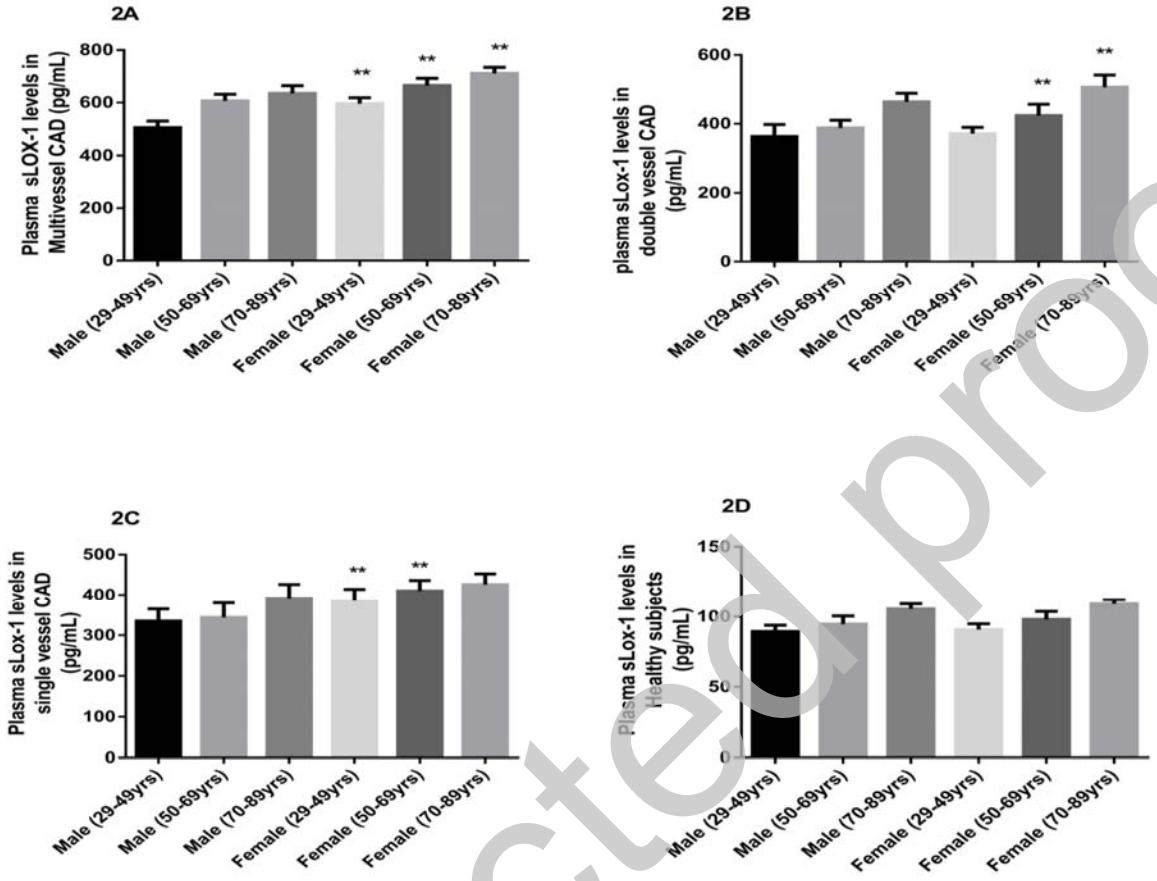

FIG. 2. A. Circulating sLOX-1 levels were determined by ELISA method. Comparison of plasma sLOX-1 concentrations in multi-vessel lesions CAD patients in (29-49yrs, 50-6yrs, 70-89yrs) age groups in both genders; ** ($p<0.001$). Figure 2B: Expression of plasma sLOX-1 in double vessel occluded CAD patients among different age's male and female subjects. Female (50-69yrs, 70-89yrs) CAD subjects compared with male (50-69yrs, 70-89yrs) CAD; ** ($p<0.001$). Figure 2C: Soluble blood LOX-1concentrations in single vessel CAD subjects at different ages male and female. Female (29-49yrs, 50-6yrs) CAD groups as compared with male (29-49yrs, 50-6yrs) CAD groups; ** ($p<0.001$). Figure 2D: Plasma sLOX-1 expressions in healthy male and female subjects among different age groups; $p>0.05$.

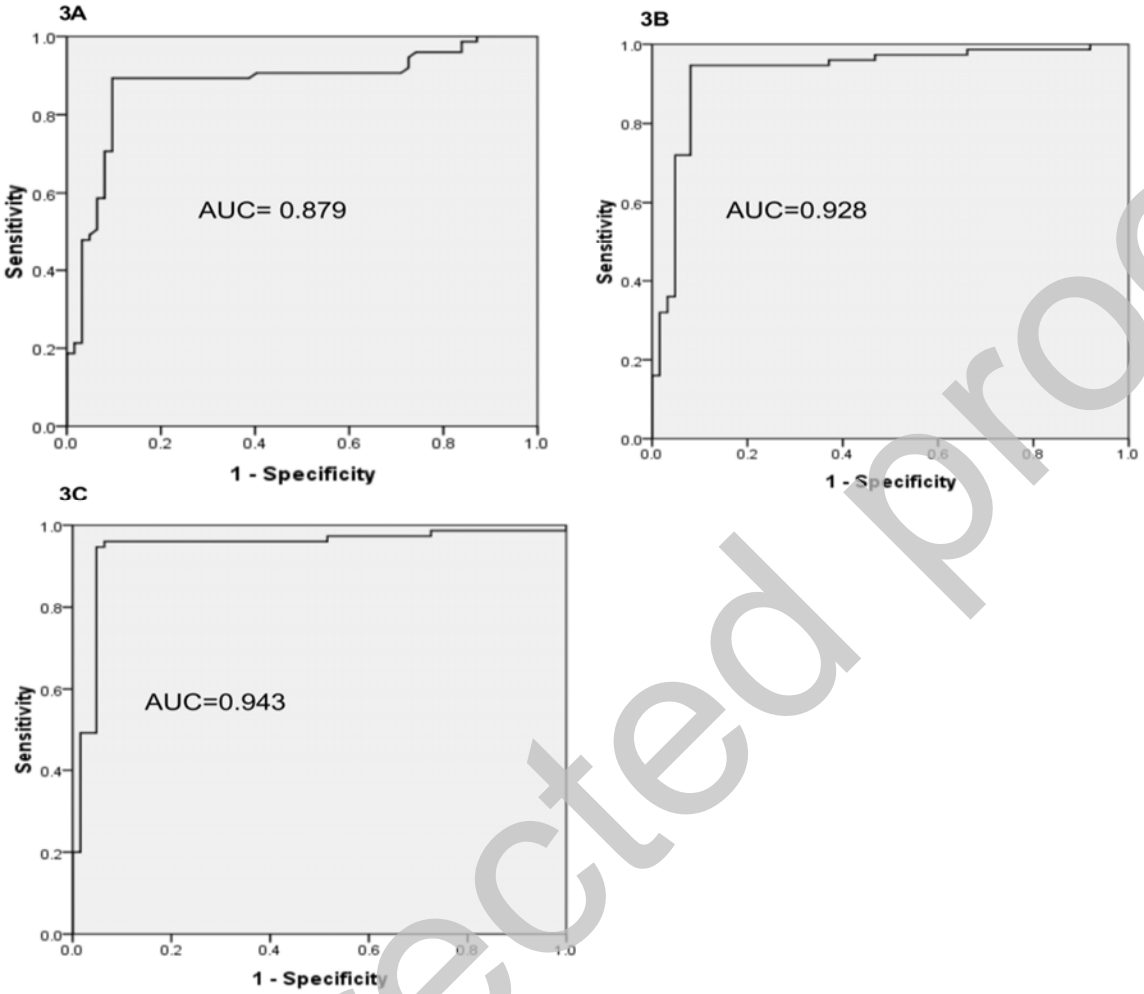

FIG. 3. ROC curve analysis was applied for discrimination of severity of CAD lesions in single, double and multi-vessel CAD patients. Figure 3A: Single vessel CAD and healthy participants (AUC 0.879). Figure 3B: Comparison between patients with double vessel CAD and healthy volunteers (AUC 0.928). Figure 3C: Control subjects with multi-vessel CAD patients (AUC 0.943).

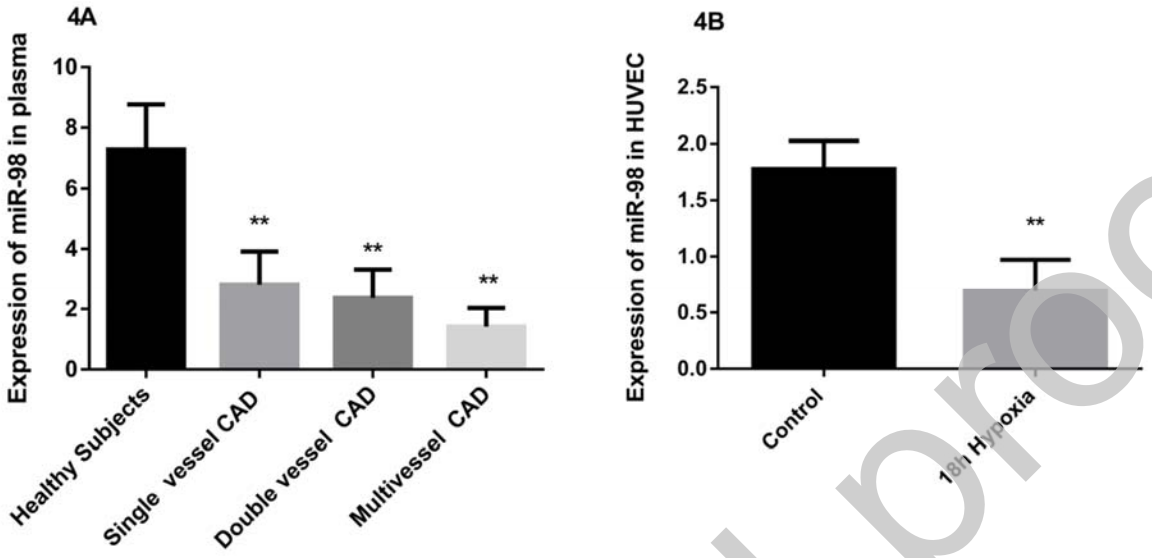

FIG. 4. A: The relative expressions of microRNA-98 in plasma were measured by Real-time PCR between healthy and multi-vessel, double, single vessel CAD subjects. Figure 4B: MicroRNA-98 expression in normoxic HUVECs and 18h hypoxic exposed HUVECs; ** ($p<0.001$).

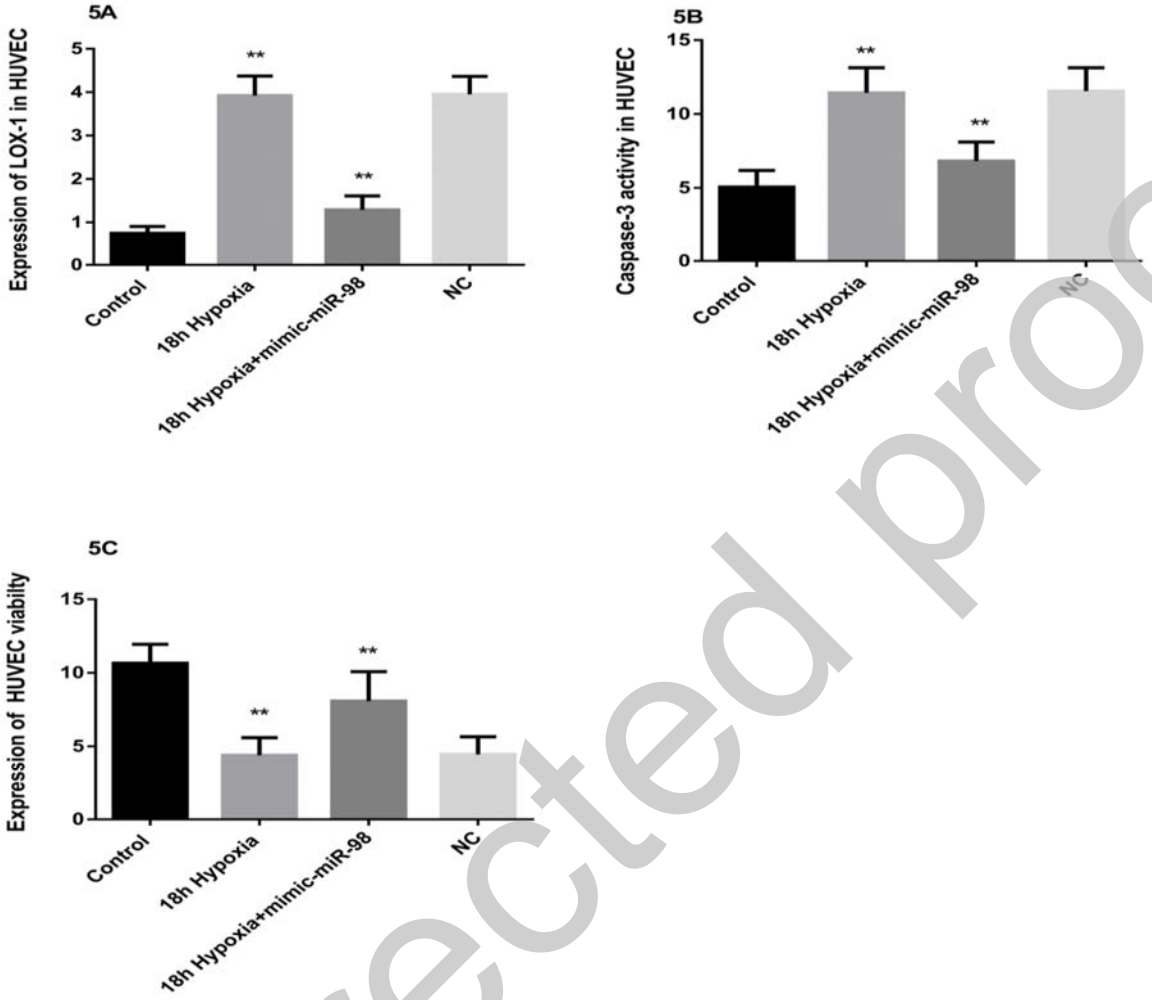

FIG. 5. A. Expressions of LOX-1 were detected in normal, 18h hypoxic (1% O2), 18h hypoxic plus tarnsfected with-mimic-98 and negative control (NC) HUVEC cells by qRT-PCR. Control HUVECs and 18h hypoxic HUVECs; **p<0.001. 18h hypoxic groups compared with 18hypoxic + mimic-98 HUVECs; ** p<0.001. 18h hypoxic incubated HUVECs versus NC; p>0.05. Figure 5B: Caspase-3 activity in agomiR-98 transfected hypoxic HUVEC cells and hypoxic cell; ** p<0.001. Normoxic cells compared with18h hypoxic HUVECs; **p<0.001. 18h hypoxic induced HUVECs and NC; p>0.05. Caspase-3 activities were measured by the SpectraMax reader. Figure 5C: Cellular viability between 18h hypoxic and miR-98 treated hypoxic HUVECs; ** p<0.001. 18h hypoxic exposed HUVECs and normoxic condition HUVECs;**p<0.001. Negative control HUVECs compared with 18h hypoxic injured HUVECs; p>0.05.The relative expressions of cellular viabilities were calculated through qRT-PCR.