Assessment of Oxidative Stress Markers and Carotid Artery Intima-Media Thickness in Elderly Patients Without and with Coronary Artery Disease

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Abstract We aimed to assess whether measuring carotid intima-media thickness (CIMT) and oxidative stress markers such as protein carbonyls, malondialdehyde, nitrate and glutathione in plasma of elderly patients without and with coronary artery disease (CAD) identifies early risk for CAD. A total of 50 cases with cardiovascular risk factors over the age of 60 years without CAD, and 50 patients with angiographically documented CAD over the age of 60 years were included in the study. Control group consists of 200 healthy individuals without the risk factors. Demographic details were obtained from all the subjects and CIMT measured by high frequency ultrasound and oxidative stress markers such protein carbonyls, malondialdehyde and total glutathione were determined in plasma by spectrophotometric methods. The distribution of cardiovascular risk factors in without CAD and CAD cases were smokers (16 vs 56 %), hypertension (26 vs 64 %), diabetes (16 vs 56 %) and dyslipidemia (18 vs 58 %) and positive family history (4 vs 38 %). None of the control group had any cardiovascular risk factors. Among the CAD cases, 16 % had single vessel disease, 44 % had double vessel disease and 40 % had triple vessel disease. The

CIMT was significantly increased in CAD cases as compared to cases without CAD and healthy controls. On the other hand, CIMT was significantly increased in cases without CAD as compared to healthy controls. CIMT also increased with the duration of diabetes in patients without CAD and severity of disease in CAD cases. The levels of oxidants like plasma malondialdehyde, protein carbonyls, were significantly elevated and antioxidant glutathione levels and nitrate levels were significantly reduced in cases with and without CAD as compared to healthy controls. Oxidative stress markers and CIMT was found to be significantly increased in patients with cardiovascular risk factors like diabetes, family history of CAD, dyslipidemia, hypertension and smoking when compared to patients without risk factors. In patients with diabetes, CIMT increased as duration of disease increases and also in poorly controlled diabetes. In CAD group, when number of vessel involvement (severity of coronary disease) increases, the CIMT also increases confirming that CIMT is a quantifiable risk factor for CAD.

Keywords Coronary artery disease · Carotid intima-media thickness · Atherosclerosis · Oxidative · Stress

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Introduction

Reports on coronary artery disease (CAD) in Indians from different parts of the World have shown that Asian Indians have 3–4 times higher risk of CAD than white American, 6 times than Chinese, 20 times than Japanese [1]. Increase in age, family history of CAD, genetic constitution [2], hypertension, cigarette smoking, hyperlipidemia, diabetes, obesity, sedentary lifestyle, stress, heredity and race [3], etc. contribute to the etiology of CAD. Among these risk

factors specifically life-style risk factors are modifiable and hence are of interest in preventing CAD through life-style modification. Age is a significant risk factor in which it plays a role in susceptibility to CAD. CAD subjects with the age group of <60 years were found to show risk in causing CAD [4]. According to present trends in the United States, half of healthy 40-year-old males will develop CAD in the future, and one in three healthy 40-year-old women [5]. In both sexes, the risk of CAD increased markedly with age. Cigarette smoke is the single most potent risk factor for atherosclerosis [6]. Hypertension is a major modifiable risk factor cardiovascular disease, cardiovascular events and deaths. High blood pressure, especially high systolic blood pressure is the best predictor of heart disease [7]. The studies in experimental hypertension and hypertension in humans have demonstrated increased generation of reactive oxygen species (ROS) [8, 9]. ROS and oxLDL may play a critical role in the pathophysiology of hypertension. The involvement of reactive oxygen intermediates in essential hypertension is also suggested by the increased level of lipid peroxides and decreased concentrations of antioxidant vitamin E in plasma of essential hypertensive patients [10].

The importance of oxidative stress in the development of atherosclerosis is now widely accepted. Studies have shown that increased ROS production is a major cause of endothelial dysfunction (ED) in both experimental and clinical atherosclerosis [11, 12]. ED leads to a rapid decrease in NO production or bioavailability, partly due to inactivation of NO by superoxide. Several studies have reported that ROS are generated more in patients with diabetes, hyperlipidemia, smokers and other chronic inflammatory conditions [13]. Patients with neurodegenerative illnesses [14] diabetes and hypercholesterolemia [15] and children with juvenile chronic arthritis [16] were found to have elevated levels of total protein carbonyls, suggesting the potentiality of carbonylated proteins serving as a biomarkers for early diagnosis of these diseases.

The measurement of carotid intima-media thickness (CIMT) is already established as surrogate marker of early cardiovascular disease and sub-clinical atherosclerosis in patients with conventional risk factors [17, 18]. An increase in CIMT in CAD patients and cardiovascular risk factors like diabetes has already been well established. The assessment of CIMT in elderly patients with cardiovascular risk factors may predict CAD risk in those individuals. The non-invasive measurement of CIMT by ultrasonography is regarded as one of the most reliable markers for atherosclerotic vascular changes. Thus, measuring intimal medial thickness (IMT) and oxidative stress markers in individuals with cardiovascular risk factors may predict early CAD events in elderly. In our recent study, we

demonstrated that IMT was significantly increased in T2D patients, as compared with non-diabetic healthy controls [19]. There was an insignificant increase IMT with the increase in the age of T2D patients was also observed. The influence of age was found in patients with various cardiovascular risk factors, including diabetes [20]. In a recent study, it was demonstrated that oxidative stress markers are independently associated with the increase in CIMT even after adjusting the conventional risk factors in men [21]. Hence the present study was carried out to investigate the role of oxidative stress markers and CIMT in elderly patients with cardiovascular risk factors without CAD and also in elderly patients with CAD.

Methods and Materials

Study Subjects

A total of 300 subjects from Nizam's Institute of Medical Sciences, Hyderabad, India were recruited in the study. Among all the subjects, 50 were elderly patients (>60 years) who are angiographically documented CAD cases, 50 are elderly patients (>60 years) without CAD with any of the cardiovascular risk factors and 200 healthy controls. Subjects with carotid narrowing having symptomatic cerebrovascular disease, inflammatory bowel disease, rheumatoid arthritis, neurological disease and patients on Statins therapy for ≥ 3 months were excluded from the study. This study was approved by the Institutional Ethical committee of Nizam's Institute of Medical Sciences and informed consent was obtained from all the subjects and demographic characteristics were recorded and shown in Table 1.

Sample Collection and Storage

5 ml of blood sample was collected in EDTA tube from all the subjects followed by centrifugation at 3000 rpm for 5 min and the plasma was collected and stored at -80°C till the date of analysis.

Estimation of Biochemical Parameters

Plasma malondialdehyde was determined as thiobarbituric acid reactive substances (TBARS) [22], Protein carbonyl content present in plasma was quantified with 2, 4-dinitrophenylhydrazine (DNPH) [23], Plasma nitrite, a measure of nitric oxide (NO) levels was determined by using the Griess reagent [24] and reduced glutathione levels was determined by Ellman's method [25].

Table 1 Demographic details of study subjects

Characteristics	Healthy controls	Patients without CAD	Patients with CAD
Age (years)	50 ± 18.91	64.70 ± 4.56	65.32 ± 5.92
Males	106	29	30
Females	94	21	20
BMI (mean ± SD)	23.60 ± 1.91	24.19 ± 2.94	26.309 ± 3.67
HbA1c (%) (mean ± SD)	5.51 ± 0.18	7.0 ± 1.18*	9.0 ± 1.75*
Total cholesterol (mean ± SD)	172.25 ± 10.61	178.50 ± 26.97	217.45 ± 35.75*
Triglycerides (mean ± SD)	137.96 ± 20.10	166.30 ± 31.22*	178.79 ± 30.38*
LDLC (mean ± SD)	102.24 ± 8.87	94.50 ± 12.82	115.24 ± 15.65*
HDLC (mean ± SD)	45.79 ± 8.49	40.80 ± 6.64	38.55 ± 2.32*
Diabetes (n)	None	8 (16 %)	28 (56 %)
Hypertensives (n)	None	13 (26 %)	32 (54 %)
Smokers (n)	None	8 (16 %)	28 (56 %)
Postive family history (n)	None	2 (4 %)	19 (38 %)
Dyslipidemic pts (n)	None	9 (18 %)	29 (58 %)
IMT (mm)	0.71 ± 0.04	0.80 ± 0.13*	1.14 ± 0.17*

n number of subjects, * *P* < 0.001 versus control

CIMT Measurement

Carotid Intima-media thickness is measured using B-mode USG scan using 5–10 MHz probe and when required colour Doppler scan is used. Carotid artery scanning was performed with a high resolution Sonos ACCUSON X300 (SIEMENS) with a duplex B-mode scanner and a curvilinear phased array transducer of 2–5 MHz frequency. Scanning was undertaken by a Radiologist who was unaware of the clinical status of the subjects. Intima-media thickness was taken as the distance between the leading edge of the first echogenic line of the far wall of the carotid artery (lumen intima interface) and the leading edge of the second echogenic line (media-adventitia interface). Measurements of IMT were made at end-diastole (peak of the R wave) at three segments on each side: the distal 1 cm of common carotid artery just before the bifurcation, the carotid bifurcation, and the proximal 1 cm of internal carotid artery and then average of all three taken into consideration, both right and left sides are measured like this method [26]. Measurements were taken only on longitudinal scans and not on transverse scans.

Statistical Analysis

All values are expressed as mean ± SD. The analysis of variance (ANOVA) was used to compare the results among the three groups followed by Student's *t* test and the level of significance was set as <0.05.

Results

As shown in Table 1, the age of healthy controls, patients without CAD and with CAD were 50 ± 18.9, 65.3 ± 5.9 and 64.7 ± 4.5 years respectively. In patients without CAD and CAD, the percentage distribution of diabetes (16 vs 56 %), hypertension (26 vs 64 %), smokers (16 vs 56 %), dyslipidemia (18 vs 58 %), positive family history (4 vs 38 %), respectively. There were no significance differences in BMI among all the three groups. Serum total cholesterol, LDL-cholesterol, triglycerides were significantly increased whereas HDL cholesterol levels were decreased in CAD cases as compared to patients without CAD and healthy controls (Table 1).

The CIMT was significantly increased in CAD cases as compared to patients without CAD and healthy controls (Table 1). Similarly, patients without CAD showed significant increase in CIMT as compared to healthy controls. The CIMT in CAD, patients without CAD and healthy controls were 1.14 ± 0.17, 0.80 ± 0.13 and 0.71 ± 0.04 mm respectively. The measurement of right common coronary artery (RT.CCA) IMT and left common coronary artery (LT.CCA) IMT showed significant increase in CAD cases as compared to patients without CAD (Table 2). As shown in Table 3, in patients with CAD, the CIMT significantly increases as the number of vessels involved. In patients without CAD, with age more than 70 years showed significant increase in CIMT compared to <70 years (Table 4).

The male patients with CAD showed significantly higher CIMT than females. There was no significant difference in CIMT among males and female patients without CAD (Table 5). As shown in Table 6, the data was segregated based on the duration of diabetes to <5 years, 6–10 years and >10 years, there was a significant increase in CIMT in patients without CAD cases with the increase of duration of diabetes. However, no such difference was observed in CAD cases.

In CAD cases with diabetes, hypertension, smoking, dyslipidemia, or family history showed significant increase in CIMT as compared to patients without CAD with these risk factors (Table 7). On the other hand, the data was compared between patients without CAD with these risk factors with healthy controls showed significant increase in CIMT.

Table 2 CIMT in patients with CAD and without CAD

Parameter	n	CIMT (mm)	<i>P</i> value
<i>RT.CCA IMT</i>			
CAD	50	1.23 ± 0.16	0.0001*
without-CAD cases	50	0.82 ± 0.13	
<i>LT.CCA IMT</i>			
CAD cases	50	1.3 ± 0.25	0.0001*
without-CAD cases	50	0.81 ± 0.15	
<i>AVG.IMT</i>			
CAD	50	1.14 ± 0.17	0.0001*
without-CAD cases	50	0.80 ± 0.13	

RT.CCA IMT right common coronary artery IMT, *LT.CCA IMT* left common coronary artery IMT, *AVG.IMT* average IMT

Table 3 CIMT in severity of CAD

No. of vessels involved	n	CIMT (mm)	P value
1	8	0.95 ± 0.105	<0.0001
2	22	1.08 ± 0.105	
3	20	1.27 ± 0.156	

Values are expressed as mean ± SD

Table 4 Relationship of CIMT with age in CAD

Group	Age group	n	CIMT (mm)	P value
Patients with CAD	60 to <70 years	39	1.12 ± 0.18	0.35
	≥70 years	11	1.19 ± 0.15	
Patients without CAD	60 to <70 years	40	0.775 ± 0.12	0.02
	≥70 years	10	0.905 ± 0.15	

All values of expressed mean ± SD

Further, we measured oxidative stress markers like, protein carbonyl, malondialdehyde and total glutathione and nitrite in CAD, without CAD and healthy controls and data is depicted in Fig. 1. Compared to healthy controls, there was significant increase in protein carbonyl, malondialdehyde and significant decrease in total glutathione and nitrite levels in CAD and in patients without CAD cases. Data also showed a significant increase in protein carbonyl, malondialdehyde and significant decrease in total glutathione and nitrite levels in CAD as compared to patients without CAD.

Discussion

Consistent with the literature, in the current study, the cardiovascular risk factors like diabetes, hypertension, dyslipidemia, smoking, positive family history are strongly associated with CAD. The percentage of individuals with these risk factors is significantly higher in CAD than patients without CAD cases. This indicates that patients without CAD with these risk factors may be at future risk for developing CAD. CIMT is associated with a number of factors, including age, sex, hypertension, smoking, lipid profile and BMI [27]. The CIMT >0.9–1.0 mm is strong suggestive of atherosclerosis and generally referred as an intermediate phenotype for early atherosclerosis [28].

The relation of CIMT to CAD in many studies like in the ARYA study [27], the ARIC study done by Howard et al. [29], Gupta et al. [30] from India showed that mean IMT was high in CAD patients. In the current study, the CIMT was significantly increased in CAD and in patients without CAD as compared to healthy controls. And the

Table 5 Relationship of CIMT with Gender in CAD

Group	Sex	N	CIMT (mm)	P value
Patient with CAD	Male	30	1.19 ± 0.1918	0.0047
	Female	20	1.06 ± 0.1171	
Patient without CAD	Male	29	0.80 ± 0.1458	0.6129
	Female	21	0.79 ± 0.1338	

Values are expressed as mean ± SD

Table 6 Relation of CIMT with duration of Diabetes in CAD

Group	Duration of diabetes (years)	n	CIMT (mm)	<i>P</i> value
Patients with CAD	≤5	7	1.07 ± 0.10	0.1485
	6–10	7	1.15 ± 0.2	
	>10	15	1.25 ± 0.17	
Patients without CAD	≤5	6	0.86 ± 0.068	0.0322
	6–10	1	0.9	
	>10	1	1.15	

Values are expressed as mean ± SD

Table 7 CIMT with cardiovascular risk factors

Parameter	CIMT (mm) in patients without CAD	CIMT (mm) in patients with CAD	<i>P</i> value
Diabetes	0.90 ± 0.11 (8)	1.19 ± 0.19 (28)	0.0001
HTN	0.89 ± 0.11 (13)	1.17 ± 0.15 (32)	0.0001
Smoking	0.88 ± 0.14 (8)	1.19 ± 0.20 (28)	0.0001
Family history	1 (2)	1.16 ± 0.20 (19)	0.0001
Dyslipidemia	0.93 ± 0.11 (9)	1.16 ± 0.21 (29)	0.0001
Total group	1.14 ± 0.17	0.80 ± 0.13	0.0001

Values in parenthesis are number of subjects; values are expressed mean ± SD

IMT was more in 80 % of CAD patients with disease severity. Our study also shows significant association of IMT with the CAD.

The mean age of the patients was 65 years. Around 80 % of patients in both CAD cases and patients without CAD groups were <70 years age. The mean IMT increases as age increases which has a positive correlation and is statistically significant. In Cardiovascular Health Study [31], studied in 5201 patients concluded that prevalence and severity of carotid atherosclerosis continued to increase with age even in late decades of life. In another study in type 2 diabetic patients and found that mean CIMT was positively correlated with age [32]. Our present study is very much comparable to the above studies.

Fig. 1 Plasma levels of **a** protein carbonyl, **b** MDA, **c** glutathione, **d** nitrite levels in healthy controls, patients without CAD and with CAD. Values are expressed mean ± SD. *P* < 0.001 versus control

In the current study, in CAD with diabetes, hypertension, smoking, dyslipidemia showed increased CIMT as compared with patients without CAD with these risk factors. On the other hand, without CAD cases with these risk factors showed increased CIMT as compared to healthy controls. This suggests that patients without CAD with these risk factors may be at increased risk for developing of CAD. In a recent study, it was demonstrated that higher mean CIMT in CAD than patients without CAD and significantly correlated with the prevalence of any coronary plaque and obstructive coronary plaque disease [33]. In one study by Agarwal et al. [34], observed the duration of diabetes as a predictor of CIMT which was statistically proved in the study ($P < 0.002$). In another study, Chennai Urban Population Study (CUPS) done in 2000 demonstrated that diabetic subjects have higher intima media thickness [35]. Our results corroborate with the earlier studies as diabetes showed a positive correlation with intima thickness that is statistically significant in CAD and without CAD groups with uncontrolled diabetes as evidenced by raised glycated haemoglobin in both the groups. The results of the study also indicate that duration of diabetes increases, there was an increase in CIMT. An increase in CIMT and carotid plaques were detected in young CAD cases from Nepal [36].

In the present study, 56 % of study group are smokers with increased CIMT than non-smoker. Cardiovascular study in young Finns study done by Raitakeri et al. [37] and study by Kablak et al. [38] showed similar relation of smoking with CIMT as observed in the current study. Yamasaki et al. [39], Anath Oren et al. [27] in the ARYA study, Cardiovascular Risk in Young Finns study done by Raitakari et al. [37] and the ARIC study done by Howard et al. [29] demonstrated that LDL-C, total cholesterol was positively associated with significant increase in CIMT. Our study also had similar results, the total cholesterol, triglycerides and LDL cholesterol values were significantly high in CAD group whereas HDL cholesterol levels were low. The mean IMT is significantly more in patients with dyslipidemia in the both the groups and is statistically significant.

Our previous studies and others have shown that oxidative stress is involved in diabetes, hyperlipidemia, and smoking are the potential risk factors for CAD [40–42]. Oxidative DNA damage is directly or indirectly involved in the pathophysiology of CAD. Conventional risk factors of CAD such as diabetes, hypertension, hyperlipidemia, and smoking are associated with oxidative stress [40, 43]. Many of these risk factors, including hyperlipidemia, hypertension, diabetes and smoking are associated with the overproduction of ROS or increased oxidative stress [40]. ROS, under the physiological conditions act as signalling molecules that regulate the

vascular smooth muscle cell contraction and relaxation and growth [44]. Increased lipid peroxidation (MDA), protein modification (protein carbonyls), and decreased anti-oxidant (glutathione) levels were observed in CAD and patients without CAD cases. The levels of MDA and protein carbonyls were significantly higher in CAD cases as compared to without CAD cases. In a recent study by Yoon et al. [21] showed that oxidative stress markers like MDA, 8-hydroxy-2'-deoxyquanosine (8-OHdG) and 8-iso-prostaglandin F₂α (Isoprostane) are independently associated with the increase in CIMT in men even after adjusting the cardiovascular risk factors like age, alcohol consumption, low density lipoprotein, BMI, smoking history, and metabolic syndrome. This indicates the role oxidative stress in the increase in CIMT. Nitric oxide, a product of eNOS, has vasodilatory, anti-platelet and anti-proliferative, permeability-decreasing and anti-inflammatory properties [45]. In the current study, NO levels were decreased in elderly patients without CAD and CAD. Decreased NO bioavailability in the vasculature is a key feature of all the classical risk factors for atherosclerosis, and it can be the result of NO's decreased synthesis and increased peroxynitrite levels that oxidize BH₄ to BH₂. Several studies have demonstrated that the bioavailability of NO is decreased in hyperhomocysteinemia [46], which might be attributable to decreased NO production or to alternative mechanisms such oxidative stress or nitrosylation [46, 47].

In a previous study, a correlation of decreased GSH with IMT in patients with atherosclerosis was demonstrated [48]. In the current study, we observed decreased levels of GSH in CAD and patients without CAD cases as compared to healthy controls. GSH normally plays the role of an intracellular radical scavenger and is the substrate for many xenobiotic elimination reactions. In earlier studies a marked decrease in GSH were reported in diabetic patients [49, 50]. In hyperglycemia, glucose is preferentially used in polyol pathway that consumes NADPH necessary for GSH regeneration by the glutathione reductase, therefore, hyperglycemia may indirectly cause GSH depletion causing increase in oxidative stress [51]. The limitation of the present study is its sample size. Future studies on different ethnic groups and populations with large sample size warrant further investigation.

Conclusions

In patients without and with CAD, the higher levels of oxidative stress markers were observed. Similarly, CIMT was also increased in patients without CAD and with CAD. The intensity of the oxidative stress markers and CIMT was significantly higher in patients with CAD as compared

to patients without CAD. This data clearly indicates that in elderly patients with cardiovascular risk factors will likely to have higher risk for developing CAD. This study once again confirms that oxidative stress is associated with increase in CIMT and also utility of measuring CIMT in prevention of future CAD in patients with cardiovascular risk factors.

Compliance with Ethical Standards

Conflict of interest All authors have no conflict of interest exist.

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