ORIGINAL ARTICLE

Correlation of Serum Nitric Oxide, High Sensitivity C-reactive Protein and Lipid Parameters in Diabetics with and without Coronary Artery Disease

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Abstract:

Background: Coronary Artery Disease (CAD) and Diabetes Mellitus (DM) top the list among non-communicable diseases. Nitric Oxide (NO) preserves normal vascular physiology. Uncoupling of endothelial nitric oxide synthase enzyme occurs in the blood vessels of diabetics leading to endothelial dysfunction and excessive production of superoxide anion causing decreased bioavailability of NO. *Aim and Objectives:* To assess the serum NO levels, high-sensitive C-reactive Protein (hsCRP), lipid parameters and their association with CAD in diabetics. *Material and Methods:* The study comprises total 195 participants. There are three groups, each group consist of 65 participants. Three groups were diabetes with CAD, diabetes without CAD and control. NO assessed by modified Griess method. hsCRP by immunoturburdimetric method FBS and lipid parameters were analysed in fully automated analyzer. *Results:* There was a significant decrease in NO levels and significant increase in hsCRP levels in diabetes without CAD and diabetes with CAD patients compared to controls. NO showed negative correlation with Fasting Blood Sugar (FBS) and hsCRP in DM without CAD patients. NO showed negative correlation with DBP in DM with CAD patients. NO showed negative correlation with HbA1c in both the groups. *Conclusion:* The study concludes that estimation of NO and hsCRP along with lipid profile, help in early detection of endothelial dysfunction in diabetese patients. Reduced NO and increased hsCRP levels in diabetese patients may be strong indicator of coronary artery disease.

Keywords: Nitric oxide, high-sensitive C-reactive protein, Diabetes mellitus, Coronary artery disease

Introduction:

Cardiovascular Disease (CVD) is the leading cause of death and disability in developing countries [1]. In India three million deaths per year are due to cardiovascular diseases [2]. Coronary Artery Disease (CAD) and Diabetes Mellitus (DM), top the list among non-communicable diseases. Increasing incidence of morbidity and mortality due to cardiovascular complications including CAD has been observed in DM. The risk of CAD is two to four times higher in diabetic subjects. CAD strikes Indians at younger age that is in their productive age group. DM and CAD share several common risk factors like age, dyslipidemia, obesity, life style, environmental & genetic factors, hypertension and stress [3]. According to National Cholesterol Education Program (NCEP) guidelines, diabetes has been considered as cardiovascular risk equivalent [4]. Complications of DM include macrovascular i.e CAD, peripheral vascular disease, stroke and microvascular complications include retinopathy, nephropathy and neuropathy.

Among all these, endothelial dysfunction is more common [5]. Endothelium secretes many substances like Nitric Oxide (NO), endothelin-1. Impairment of endothelial function is the early feature of cardiovascular disease in DM [6].

NO is an endogenous gaseous molecule secreted by endothelium by a family of Nitric Oxide Synthase (NOS) enzymes. It is a key signaling messenger in the cardiovascular system [7]. It is having very short half life of few seconds, with a potent vasodilator and endothelial relaxing factor. NO produced in endothelium controls vascular tone and permeability, maintains vascular integrity by inhibiting the platelet aggregation, leukocyte endothelium adhesion and vascular smooth muscle proliferation [8]. Diabetes mellitus, atherosclerosis, hypertension, stroke and congestive heart failure have been linked to abnormalities in NO signaling [9, 10]. Adequate levels of NO preserve normal vascular physiology. Uncoupling of endothelial nitric oxide synthase enzyme occurs in the blood vessels of diabeteses leading to endothelial dysfunction and excessive production of superoxide anion causing decreased bioavailability of NO [11].

Pro-inflammatory markers like Tumor Necrosis Factor alpha (TNF-a), CRP and interleukin-6 are strongly associated with development of CAD. C-reactive protein (CRP) is a biomarker of inflammation due to acute-phase response. High-sensitive C-reactive protein (hsCRP) is considered as an important, powerful predictor of future cardiovascular disease [12]. Measurement of hsCRP helps to quantify low grade inflammation, in the absence of overt systemic inflammation and immunologic disorders. Asian Indians were shown to have elevated CRP levels, suggesting that pro-inflammatory factors may contribute to increased risk for diabetes and CAD [13].

There is paucity as well as controversial data were reported on NO levels in DM and CAD patients. Hence the present study was undertaken to assess the serum NO levels, hsCRP, lipid parameters and their association with CAD in diabetes.

Material and Methods:

Study design:

The research work was carried out in the department of Biochemistry, S Nijalingappa Medical College and Hanagal Sri Kumareshwara Hospital Research centre, Bagalkot, Karnataka. It was hospital based observational study. Study was conducted for two year 2015-16. The study protocol was approved by the Institutional Human Ethics Committee (SNMC/IECHSR/2014-15/A-18-1.1). Informed consent was taken from all patients and healthy controls at the beginning of the study.

Sample size:

Sample size was calculated by using the software, at 95% confidence interval and 80% power, it was calculated by taking into consideration of mean values from study by [14]. Study comprises total 195 participants, among them 65 were diabetes with coronary artery disease patients. CAD was documented on the basis of angiographically confirmed cases diagnosed by cardiologists i.e stenosis in major vessels, ECG findings, cardiac markers, previous medical records. DM was confirmed by WHO criteria (FBG >126 mg/dL or PPBG >200 mg/dL or HbA1c >6.5%). Sixty five were diabetese patients without CAD. Age and sex matched 65 healthy controls were taken who were free of any clinical manifestations i.e., diabetes, hypertension and cardiac disease. Smokers without any cardiac event were taken as controls.

Smokers with cardiac event were taken as cases. Patients with chronic liver and kidney diseases, nephrotic syndrome, thyroid disorders, pregnant women, cancer, patients on OCP's steroids and statins were excluded from the present study.

Methodology:

In all the patients, detailed history was taken, anthropometric characteristics like height, weight, blood pressure were noted and Body Mass Index (BMI) was calculated. Six mL of fasting venous blood sample was collected from all subjects by venipuncture. Two mL of EDTA sample used for estimation of HbA1c, determined by High Performance Liquid Chromatography (HPLC) principle, using Bio-Rad D-10 instrument. Remaining 4mL plain blood was allowed to clot and serum was separated. The blood was centrifuged at 3000 revolution per minute for 10 minutes. Serum was used for analysis of nitric oxide, hsCRP, lipid profile and Fasting Blood Glucose (FBG). Serum nitric oxide was estimated by Griess method. FBG by Glucose oxidase and peroxidase (GOD-POD) method, Total Cholesterol (TC) by cholesterol oxidase-peroxidase method, Triglycerides (TG) by glycerol phosphate oxidase-peroxidase method and High Density Lipoprotein-cholesterol (HDL-c) by direct method were assessed. Low Density Lipoprotein-cholesterol (LDL-c) was calculated by using Friedwald's formula. hsCRP by immunoturbidemetric method. All these were analysed in fully automated analyzer in Biosystem A-25 using commercially available kits.

Statistical analysis:

Quantitative data was expressed Mean ± SD. Comparison of three groups by Analysis of Variance (ANOVA) test and followed by *Post-hoc* multiple comparison tests. Correlation of NO with other parameters was done by Pearson correlation. The data was analyzed by using software SPSS version 17. $p < 0.05$ was considered as statistical significant.

Results:

Baseline characters of the study group are shown in table-1. There was no difference in the percentage of smokers and family history of CAD in different groups. The study groups were age and sex matched. There was significant increase ($p<0.001$) in blood pressure, BMI, fasting glucose and HbA1c in cases compared to controls shown in table-2. Diabeteses with CAD had significant increase ($p<0.001$) in systolic as well diastolic blood pressure compared to controls.

Table 1: Baseline Characters in the Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)
Male: Female	37:28	38:27	42:23
Smoking (%)	13 (20%)	12 (18.76%)	12 (18.76%)
Family history of CAD (%)	8 (12%)	9(13.84%)	13 (20%)
Duration of DM in year	-	6.8 ± 2.3	8.3 ± 3.2

CAD = Coronary Artery Disease, DM = Diabetes Mellitus

Table 2: Anthropometric and Biochemical Parameters in Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)	F	p- value
Age in Year	056.12 ± 14.62	056.00 ± 15.60	061.09 ± 14.72	2.87	0.06
SBP (mm of Hg)	112.00 ± 10.00	124.00 ± 10.00*	136.00 ± 10.00*#	70.939	0.00
DBP (mm of Hg)	078.00 ± 06.00	084.00 ± 08.00	094.00 ± 06.00*#	68.128	0.00
BMI (Kg/m^2)	023.73 ± 01.25	026.47 ± 02.18*	026.37 ± 03.43*	10.27	0.00
FBG (mg/dL)	081.49 ± 16.08	158.20 ± 44.03*	162.47 ± 48.93*	88.10	0.00
HbA1c (%)	005.10 ± 0.50	008.21 ± 01.57*	008.90 ± 01.75*	131.66	0.00

DM = Diabetes mellitus, CAD = Coronary artery disease, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, BMI = Body mass index, FBG = Fasting blood glucose, HbA1c = Glycosylated hemoglobin
** Comparison with control, # comparison between DM without CAD and DM with CAD, P < 0.05 statistical significance*

Table 3: Comparison of Lipid Parameters, hsCRP and NO Levels in Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)	F	p-value
TC (mg/dL)	153.60 ± 26.81	163.41 ± 48.23	154.08 ± 37.10	1.35	0.262
TG (mg/dL)	097.52 ± 23.70	117.54 ± 46.75*	141.23 ± 83.25*#	9.59	**0.001**
HDL-C (mg/dL)	039.68 ± 07.40	036.20 ± 8.35*	032.16 ± 05.78*#	15.36	**0.001**
LDL-C (mg/dL)	094.41 ± 24.08	103.69 ± 47.38	093.29 ± 34.16	1.59	0.21
VLDL-C (mg/dL)	019.50 ± 04.74	023.51 ± 09.35	028.62 ± 19.06*#	8.61	**0.001**
hsCRP (mg/L)	001.10 ± 0.24	003.67 ± 01.02*	004.80 ± 1.04*#	301.04	**0.001**
NO (mmol/L)	045.01 ± 10.2	036.2 ± 12.47*	023.48 ± 7.65*#	61.48	**0.001**

*DM = Diabetes mellitus, CAD = Coronary artery disease, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, VLDL-C = Very low density cholesterol, hsCRP = High sensitive C-reactive protein, NO = Nitric oxide, * comparison with control # comparison between DM without CAD and DM with CAD P < 0.05 statistical significance*

Summary of lipid parameters, hsCRP and NO levels in three groups is shown in table-3. There was significant increase ($p<0.001$) in TG, VLDL and hsCRP and significant decrease ($p<0.001$) in HDL and NO levels in diabetese without CAD patients and diabetes with CAD group than compared to controls. Even though TC and LDL increased in cases, the difference was not statistically significant. Inflammatory marker hsCRP was 4.8 ± 1.04 mg/L in DM with CAD patients, 3.6 ± 1.02 mg/L in DM without CAD patients and 1.1 ± 0.2 mg/L in controls. NO in DM with CAD group was drastically reduced 23.48 ± 7.66 mmol/L, compared to 36.92 ± 12.47 mmol/L in DM without CAD and in controls 45.01 ± 10.1 mmol/L.

Comparison between DM without CAD and DM with CAD group

As expected, both diabetes groups had higher ($p<0.001$) BMI, fasting glucose and HbA1c as compared to controls. There was no significant difference in BMI, FBG and HbA1c between two diabetes groups. DM with CAD patients had a longer duration of diabetes than compared to diabetes without CAD. TG, VLDL, hsCRP were significantly increased ($p<0.001$), HDL and NO was significantly decreased ($p<0.001$) in DM with CAD group compared to DM without CAD group. Correlation of NO with different parameters is depicted in table-4. NO shows significant negative correlation ($p<0.05$) with FBG, HbA1c and hsCRP

Table 4: Correlation of NO with Other Parameters in DM without CAD and DM with CAD Patients

Parameters	DM without CAD		DM with CAD	
	r-value	p-value	r-value	p-value
SBP (mm of Hg)	0.12	0.83	0.009	0.94
DBP (mm of Hg)	- 0.13	0.27	-0.295	0.01*
FBG (mg/dL)	- 0.247	0.05*	-0.226	0.07
HbA1c (%)	- 0.728	0.00*	-0.439	0.00*
TC (mg/dL)	0.018	0.87	-0.188	0.13
TG (mg/dL)	0.15	0.23	-0.128	0.31
HDL-C (mg/dL)	0.149	0.23	0.081	0.52
LDL-C (mg/dL)	-0.037	0.76	-0.134	0.28
hsCRP (mg/L)	-0.467	0.00*	- 0.235	0.08

*DM = Diabetes mellitus, CAD = Coronary artery disease, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, FBG = Fasting blood glucose, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TGs = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, NO = Nitric oxide, * Comparison with control, P < 0.05 statistical significance*

in DM without CAD patients. In DM with CAD group NO shows significant negative correlation (P<0.05) with DBP and HbA1c. Even though there was negative correlation between NO with FBG and hsCRP in DM with CAD group, it was not statistically significant. NO had no correlation with lipid parameters in both the groups.

Table-5 shows the mean values of serum NO levels in DM without CAD patients in different biochemical parameters. Poorly controlled diabetes has decreased NO levels compared to good glycemic controlled diabetics. As the inflammation increases NO levels are reduced which was statistically significant (p<0.01) in DM without CAD patients

Serum NO levels was significantly decreased (p<0.05) with increase in HbA1c levels in DM with CAD patients shown in Table-6. NO levels also decreased with increase in TC levels in DM with CAD patients. There was not much difference in NO levels with change in BMI, TG, HDL and LDL levels.

Table 5: Serum NO Levels in DM without CAD Patients in Different Biochemical Parameters

Parameters		N	Mean ± SD	p-value
BMI (Kg/m^2)	<25	19	35.3 ± 12.7	0.5
	>25	46	37.5 ± 12.4	
HbA1c (%)	<7.5	29	46.3 ± 8.7	**0.00***
	>7.5	36	29.3 ±9.4	
TC (mg/dL)	<200	55	36.4 ±11.8	0.4
	>200	10	39.8 ±15.72	
TG (mg/dL)	<150	56	36.9 ±11.9	0.9
	>150	9	36.5 ±15.9	
HDL-C (mg/dL)	<40	43	35.6 ± 9.34	0.2
	>40	22	39.3 ± 8.56	
LDL-C (mg/dL)	<100	36	36.9 ± 12.06	0.9
	>100	29	36.8 ± 13.18	
hsCRP (mg/L)	<3	16	43.6 ± 11.72	**0.01***
	>3	49	34.7 ± 12.03	

*BMI = Body mass index, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, * Comparison with control, P < 0.05 statistical significance*

Table 6: Serum NO Levels in DM with CAD Patients in Different Biochemical Parameters

Parameters		N	Mean ± SD	p-value
BMI (Kg/m^2)	<25	21	22.6 ± 5.7	0.6
	>25	44	23.8 ± 8.4	
HbA$_1$c (%)	<7.5	18	26.4 ± 7.2	**0.05***
	>7.5	47	22.3 ± 7.5	
TC (mg/dL)	<200	60	24.2 ± 7.5	**0.01***
	>200	5	15.2 ± 2.3	
TG (mg/dL)	<150	52	23.9 ± 7.1	0.3
	>150	13	21.7 ± 9.8	
HDL-C (mg/dL)	<40	55	23.3 ± 7.8	0.8
	>40	10	24.03 ± 6.9	
LDL-C (mg/dL)	<100	43	23.8 ± 7.4	0.5
	>100	22	22.7 ± 8.13	
hsCRP (mg/L)	<3	1	18.3	0.5
	>3	64	23.5 ± 7.69	

*BMI = Body mass index, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, * Comparison with control, P < 0.05 statistical significance*

Discussion:

Endothelial dysfunction and reduced production or bioavailability of NO is an important factor in the pathogenesis of diabetic vascular complications. In our study, we observed decrease NO levels in both DM without CAD and DM with CAD patients. Reduced NO levels in diabetes can be explained as due to, hyperglycemic stimulation leading to formation of Advanced Glycation End (AGE) products which enhances polyol pathway and activates protein kinase C. This leads to oxidative stress and formation of reactive oxygen species. These ROS rapidly quench NO, leading to formation of peroxynitrite which is a cytotoxic oxidant. Reduced NO availability leads to the development of atherosclerosis [15]. Peroxynitrite degrades the Tetrahydrobiopterin (BH4) which is one of the cofactor of eNOS leading to uncoupling of eNOS [16]. Peroxynitrite is one of the mediators of oxidation of LDL and in turn accelerates the pathogenesis of atherosclerosis [17]. A study by Rodriguez–Mannas *et al*., observed that AGEs are important modulators of NO activity and relevant

to the impairment of endothelial function in poorly controlled diabetes patients [18].

According to Manju *et al.*, significant decrease in the serum NO values was observed in diabetese patients compared to controls [19]. They also observed that diabetes mellitus affects the vascular endothelium, vascular tone by affecting NO levels, as the severity of diabetes increases there will be increase in BP, due to decrease in serum NO levels. A study in Italy by Tessari *et al*. concluded that NO production from arginine decreased in type 2 diabetes and nephropathy [20]. A study at north-eastern part of India by Ghosh *et al*., sum up that NO was lower in diabetes patients which was statistically significant. Due to wide variations in NO levels, they proposed the need for the standardization of NO estimation, by conducting multi centric study [21]. Reduced NO levels in diabetes may be due to inactivation of NOS. Diabetes accelerates kidney dysfunction; it may prevent the elimination of NOS competitive inhibitors like Asymmetric Dimethyl Arginine (ADMA) which is and thus limits the NO production [22].

Few studies also showed increase in serum NO level; this could be due to diabetic complications and the stage of the disease [23, 24]. Study by Adela *et al*, even though showed increased NO levels in DM, significant low levels of NO in diabetes who were more than five years compared to the subjects having diabetes history of less than five years. Their cell culture data confirmed that high glucose exposure enhanced NO production at early time point but reduced subsequently after exposure for longer duration [23]. This holds good for us, as all diabetic patients in our study group were above six years of duration. Weide *et al*., found lower levels of NO in diabetes patients with HbA1c > 9% and associated with development of vascular complications in type-2 diabetes mellitus [25].

In our study, we found decreased NO levels in DM with CAD patients. Reduced NO availability engages in initiation, progression and complications of atherosclerosis. Lack of NO bioavailability in the coronary and peripheral artery has been regarded to be prospective cardiovascular events [26].

The alteration in endothelial function is due to imbalance between endothelial vasoprotective factors like NO, endothelium-dependent hyperpolarization, oxidative stress state and generated vasoconstrictors [27]. Hyperlipidemia i.e oxidized-LDL brings endothelial dysfunction by uncopling the eNOS, which results in increase in superoxide anions (O_2^-) production. This superoxide spontaneously reacts with NO to form peroxynitrite anion ($ONOO^-$) which is highly reactive and cytotoxic induces lipid peroxidation and endothelial dysfunction [28].

In our study there was negative correlation between NO and Diastolic Blood Pressure (DBP) in DM with CAD patients. A study by Manju *et al*, on diabetes patients observed, there was negative correlation between NO with HbA1c and Mean Arterial Pressure (MAP). They also showed as the severity of diabetes increases there was increase in BP, due to marked reduction in NO levels [19].

In our study there was negative correlation between NO with HbA_1c in DM without CAD and DM with CAD patients, which is in accordance with other studies [19, 25, 29]. Study found that serum NO was significantly low in diabetes normotensive as well as diabetese hypertensive patients as compared to controls. A negative

correlation was found between serum NO with glucose and HbA1c [29]. Poorly controlled diabetics have decreased NO levels.

In our study there was significant increase in hsCRP in diabetics without CAD as well as diabetics with CAD patients compared to controls, which is in accordance with other studies [30-32]. A south Indian study on 150 participants showed CRP levels were higher among diabetes with and without CAD compared to non diabetic subjects [31]. Elevated hsCRP levels associated with diabetes and CVD, but had no correlation with disease duration or glucose control [32]. hsCRP act as inflammatory marker by activating complement pathway, induces adhesion molecule, enhances LDL uptake by macrophages and induces plasminogen activator inhibitor-1 [33], inturn enhances endothelial damage [34]. We observed negative correlation of hsCRP with NO in diabetes without CAD patients. This may be due to opsonizing property of the hsCRP and recruitment of monocytes into atheromatous plaque and also inducing endothelial dysfunction by suppressing basal and induced NO release [33].

The study concludes that estimation of NO and hsCRP along with lipid profile, help in early detection of endothelial dysfunction in diabetes patients. Reduced NO and increased hsCRP levels in diabetes patients may be a strong indicator of coronary artery disease. Estimation of NO and hsCRP levels may contribute to early detection of coronary artery disease and in turn may reduce the morbidity and mortality associated with it in diabetes.

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