Assessment of Endothelial Dysfunction in Health and Disease; Using Various Parameters

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Abstract Conditions like hypertension, atherosclerosis and diabetes are known to be the result of endothelial dysfunction which could begin early in the life of an individual. The markers of endothelial dysfunction studied in the present work are plasma hemoglobin, serum high sensitivity C-reactive protein (hsCRP) and plasma nitrite and nitrate. We studied the onset of endothelial dysfunction with increase in age as well as in disease condition like newly detected hypertensives and of hypertensive diabetic patients. The mean plasma hemoglobin and hsCRP values were found to increase with age. hsCRP and plasma hemoglobin levels were significantly higher in patient groups as compared to aged-matched controls. On the other hand nitrate and nitrite was significantly higher in patients who suffered from both diabetes and hypertension together and nitrate values were significantly lower in patients who suffered from hypertensive alone. Plasma hemoglobin and hsCRP are associated with endothelial dysfunction and increases both with age as well as in disease condition.

Keywords Plasma hemoglobin · hsCRP · Endothelial dysfunction · NO · Hypertension · Diabetes mellitus

Introduction

Endothelial dysfunction is defined as a change towards injurious processes and it contributes to vasospasm, vasoconstriction, excessive thrombosis and abnormal vascular proliferation [1]. Dysfunction of the endothelium is proposed to be the key event in the pathogenesis of many conditions like hypertension and atherosclerosis. Over the past decade, a large number of clinical studies have assessed endothelial function in health and disease [2].

The endothelium is a single layer of endothelial cells that lines the inner surface of all blood vessels including arteries and veins (as well as the innermost lining of the heart and lymphatics); it lies between the lumen and the vascular smooth muscle. This monolayer of cells plays a major role in modulating vascular tone by synthesizing and metabolizing vasoactive substances, including endothelium-derived relaxing factor (EDRF) now characterized as nitric oxide (NO), a powerful vasodilator [3].

The interaction of NO with hemoglobin has drawn increasing interest. The ability of hemoglobin to react with NO produced by the endothelium is limited by compartmentalization of hemoglobin inside the erythrocyte [4]. During intravascular hemolysis, this separation and the diffusional barriers are disrupted, resulting in efficient NO scavenging and endothelial dysfunction [5, 6]. Intravascular hemoglobin limits the amount of endothelial-derived NO available for vasodilation. Hemolysis has recently been suggested to contribute to endothelial dysfunction based on a mechanism of NO scavenging by cell-free hemoglobin [7]. Plasma hemoglobin has the ability to scavenge NO while heme possesses multiple proinflammatory and pro-oxidant properties [7].

The endothelium plays an active role during the process of inflammation and platelet adhesion. C-reactive protein

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(CRP) is one of the acute phase proteins. Laboratory and experimental evidence indicate that atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process [8]. Thus researchers have hypothesized that inflammatory markers such as high sensitivity C-reactive protein (hsCRP) may provide an adjunctive method for global assessment of cardiovascular risk [9]. In support of this hypothesis, several large-scale prospective epidemiological studies have shown that plasma levels of hsCRP are a strong independent predictor of risk of future myocardial infarction, stroke, peripheral arterial disease, and vascular death among individuals without known cardiovascular disease [10, 11].

Traditional risk factors such as arterial hypertension, dyslipidemia, hyperglycemia, smoking, age and obesity may initiate endothelial damage [12]. In 1992, endothelial dysfunction was also demonstrated in asymptomatic children and young adults with risk factors for atherosclerosis, such as hypercholesterolemia and cigarette smoking [13].

Conditions like hypertension, atherosclerosis and diabetes are most prevalent among Indian population. Atherosclerosis, a vascular disease, begins very early in life, the progression of the disease is slow and its secondary manifestations usually do not occur until after the fifth decade in life [14]. It is important to study the fundamental aspects of vascular function and its alteration during common disease conditions like hypertension and diabetes mellitus.

In the present study, an attempt is made to find out whether endothelial dysfunction develops with increasing age and hence we have included subjects of younger age group and have studied the changes that take place with age as well as in disease state. Endothelial dysfunction may be present in asymptomatic individuals who could later go on to develop hypertension, diabetes mellitus and heart related problems which are one of the major causes of morbidity and mortality in today's world.

The aim of the present study was, screening of asymptomatic individuals in population for risk markers of hypertension and atherosclerosis leading to vascular events, also investigation of subjects with recently detected hypertension and diabetes includes present work.

Materials and Methods

This Study was approved by the Scientific and Ethical Committee of Lokmanya Tilak Municipal Medical College and General Hospital (LTMMC & GH), Sion, Mumbai.

A total of 179 subjects were included in this study. 2 control groups, 30 Group I healthy controls (Age range: 18–24 years) and 56 Group II controls included apparently

healthy, asymptomatic individuals whose blood pressure values did not exceed 139/89 and whose fasting blood sugar (FBS) values were ≤ 110 mg% (Age range: 25–60 years). Our study included two patient groups 50 Group III newly/recently detected hypertensive subjects whose blood pressure values were $\geq 140/90$ without medication (Age range: 25–60 years) and 43 Group IV patients suffered from both hypertension and diabetes mellitus, having blood pressure values $\geq 140/90$ without medication and a FBS level of ≥ 110 mg% (Age range: 25–60 years). Patients who had uncontrolled diabetes or who had any complication caused due to extremely high sugar levels were not taken in this study. Patients of Group III and IV were from the Medicine OPD of Lokmanya Tilak Municipal Medical College and General Hospital. All 179 subjects were enrolled in the trial after being shown the patient information sheet and signing on the consent form, after which, the various biochemical parameters and markers of endothelial dysfunction on each individual was studied.

The height, weight as well as blood pressure of each individual were recorded. 12–14 h fasting venous blood samples were collected for the study of various parameters and taken in fluoride bulbs for estimation of FBS, in sodium citrate vial and in plain vial (without anticoagulant). Samples were used for the estimations of FBS, serum complete lipid profile [serum total cholesterol (TC), serum triglycerides (TG) and serum HDL-cholesterol] plasma hemoglobin, plasma nitrite, plasma nitrate, serum hsCRP.

Height and weight of each individual from each group was measured and body mass index (BMI) was calculated (kg/m^2) [15]. Blood pressure values of each individual were recorded in a resting sitting position by standard sphygmomanometry.

Methods

Fasting blood sugar was done by the glucose oxidase-peroxidase method (GOD-POD method) [16]. Serum complete lipid profile that is; TC by enzymatic method [13], TG also using enzymatic method, [17], high density lipoproteins (HDL) using the precipitation by phosphotungstate/manganese method [18]. Low density lipoproteins (LDL) and very low density lipoproteins (VLDL) were calculated using Friedewald's formula [19] from TC, HDL-C and TG values.

Plasma Hemoglobin was estimated by 3,3',5,5'-tetramethyl benzidine/peroxidase method. The technique described below is adapted from Crosby and Furth's [20] modification of Wu's original peroxidase method [21]. Tetramethyl benzidine has been recommended as a non-carcinogenic analogue of benzidine [22].

Determination of the NO radical itself is difficult because of its radical nature and very short half-life [23].

The NO radical is rapidly metabolized into the stable end products nitrite and nitrate [24]. Hence determination of nitrite and nitrate in plasma is most often used as a measure for the production of NO radical. Nitrate is converted to nitrite, and it was estimated using Griess Reaction [25]. This assay determines NO concentrations based on the conversion of nitrate to nitrite by a metal (copper-coated cadmium).

Serum hsCRP was estimated by immuno turbidometric assay method [26]. By constructing a standard curve from the absorbance of the standards, hsCRP concentration of sample can be determined.

Noninvasive assessment of the intima-media thickness (IMT) of the left and right carotid arteries is widely used to study atherosclerosis in populations at large [27]. There is a growing belief that carotid intima media thickness can be regarded as an indicator of generalized atherosclerosis [28]. In our study we assessed the common carotid artery intima-media thickness (CCA-IMT) in 61 subjects by B-mode ultrasonography. 18 subjects from Group I, 11 subjects from Group II, 24 hypertensive patients (Group III) and 8 patients from Group IV were assessed for CCA-IMT. Since there was no difference observed in IMT of the subjects from the various groups (Groups I–IV), this procedure was discontinued.

Statistical analysis was carried out using unpaired 't' test. *P* value less than 0.001 ($P < 0.001$) was considered as significant.

Results and Discussion

Results

Results are summarized in Tables 1 and 2. The present study was planned to assess the vascular endothelial function by studying the metabolic products of NO viz. nitrites and nitrates and the NO inactivating factor namely plasma hemoglobin, present freely in plasma. Essential hypertension is characterized by impaired endothelium-dependent vasodilation [29]. In our study nitrite and nitrate values were similar in controls of both age groups slightly higher in the higher age group controls (Group II). In the present study the patients in Group III who suffered only from hypertension showed diminished values of NO₂ and NO₃ as compared to the control group, Group II subjects, which indicates an impaired or malfunction of NO system in patients with essential hypertension [33]. The present study showed significantly high concentrations of NO₂ and NO₃ in the diabetes with hypertension (Group IV) group when compared to Group II, age-matched controls.

Increased hemolysis leading to hemoglobinemia is now considered as a novel mechanism of human disease. Hemoglobin is released into plasma from erythrocytes during intravascular hemolysis. In our study significantly high levels of hemoglobin in plasma were seen, not only in hypertensives and diabetic hypertensives but also in middle aged controls.

Table 1 Endothelial function markers in subjects

Study groups	Group I <i>n</i> = 30	Group II <i>n</i> = 56	Group III <i>n</i> = 50	Group IV <i>n</i> = 43
Age range (in years)	18–24	25–60	25–60	25–60
Sex				
Female	15 (50%)	37 (66%)	25 (50%)	24 (56%)
Male	15 (50%)	25 (50%)	19 (44%)	19 (44%)
Plasma Hb (mg/dl)				
Range	0.32–1.3	0.49–4.71	0.49–7.2	0.81–7.4
	0.76 ± 0.32	1.74 ± 0.82*	3.35 ± 1.61**	3.3 ± 1.45***
hsCRP (mg/L)				
Range	0.19–1.39	0.36–3.53	0.68–5.83	0.78–6.44
	0.62 ± 0.37	1.76 ± 0.83*	3.10 ± 1.38**	3.15 ± 1.30***
NO ₂ (μmol/l)				
Range	3.43–7.75	3.12–6.94	2.09–6.94	3.24–8.15
	5.03 ± 1.65	4.44 ± 1.45	3.95 ± 1.46	5.65 ± 1.63
NO ₂ + NO ₃ (μmol/l)				
Range	21.85–46.23	11.0–67.25	10.16–46.32	11.13–84.28
	31.55 ± 8.01	33.27 ± 11.93	21.98 ± 10.89**	48.20 ± 20.42***
NO ₃ (μmol/l)				
Range	15.05–51.37	7.57–63.82	3.44–45.59	4.5–79.6
	27.57 ± 8.75	28.67 ± 11.81	19.61 ± 11.73**	46.57 ± 18.17***

All values expressed as mean ± SD

* $P < 0.001$ (Group I vs. Group II)

** $P < 0.001$ (Group II vs. Group III)

*** $P < 0.001$ (Group II vs. Group IV)

Table 2 Lipid profile values of subjects

Study groups	Group I <i>n</i> = 30	Group II <i>n</i> = 56	Group III <i>n</i> = 50	Group IV <i>n</i> = 43
Age range (in years)	18–14	25–60	25–60	25–60
Sex				
Female	15 (50%)	37 (66%)	25 (50%)	24 (56%)
Male	15 (50%)	19 (44%)	25 (50%)	19 (44%)
BMI (kg/m ²)				
Range	16.5–24.5	21.1–28.1	21.74–28.04	23.11–29.11
	20.5 ± 4.0	24.6 ± 3.5*	24.89 ± 3.15	26.11 ± 3.0
FBS (mg/dl)				
Range	83.6–97.6	87.74–102.72	89.31–106.21	113.89–201.67
	90.6 ± 7.03	95.23 ± 6.78	97.76 ± 8.45	156.00 ± 25.14
Serum TG (mg/dl)				
Range	54.91–84.15	77.16–160.62	59.08–192.38	95.23–179.31
	69.53 ± 14.62	118.89 ± 41.73*	118.42 ± 57.13	147.80 ± 51.52
Serum TC (mg/dl)				
Range	144.54–176.46	162.78–242.48	149.36–236.5	169.06–229.38
	160.5 ± 15.96	201.85 ± 34.80*	189.37 ± 35.24	199.22 ± 30.77
HDL-C (mg/dl)				
Range	42.59–56.69	33.38–51.76	36.71–50.53	36.02–52.34
	48.49 ± 5.41	42.33 ± 8.78*	43.62 ± 6.91	44.18 ± 8.16
VLDL-C (mg/dl)				
Range	11.0–16.84	14.95–31.97	11.82–38.48	19.47–35.65
	13.92 ± 2.92	22.74 ± 7.72*	25.15 ± 13.33	27.56 ± 8.09
LDL-C (mg/dl)				
Range	82.65–111.21	95.52–171.7	88.04–158.44	92.59–165.63
	95.86 ± 13.24	129.83 ± 32.81*	123.24 ± 35.2	129.11 ± 36.52

All values expressed as mean ± SD

* *P* < 0.001 when Group I was compared to Group II

In the present study, Group II higher age group controls have hsCRP values within the normal range but higher as compared to the lower age group (Group I) controls suggesting that age itself is a major risk factor to most health problems. In our study, Group III patients who suffered from hypertension showed significantly high values of hsCRP as compared to the Group II age-matched controls. Type 2 diabetic patients have increased CRP values. In the present study, patients who suffered from both hypertension and diabetes mellitus (Group IV) had significantly higher hsCRP values as compared to Group II age-matched controls. Their (Group IV) hsCRP values were also higher than that of Group III patients who suffered from only hypertension.

Lipid profile of each subject was done and the results are summarized in Table 2. This included TC, TG, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C). Group II was compared with Group I in order to assess age related changes in lipid levels. Group I subjects were well within the recommended ranges of lipid profile values of which the TG reference value is 50–150 mg% [17], TC is 150–250 mg% [13], HDL-C is

30–70 mg% [18], LDL-C is 90–160 mg% and VLDL-C is 10–30 mg%. Whereas Groups II, III and IV who were subjects of the age group 25–60 years, had lipid profile values on the higher side of the reference ranges and HDL-C was found to be on the lower side of the reference range in these subjects.

Discussion

The ranges of nitrite and nitrate concentrations in plasma as established in a study by Moshage et al. [30] are 1.3–13 µmol/l (mean 4.2 µmol/l) and 4.0–45.3 µmol/l (mean 19.7 µmol/l), respectively. Kawakatsu et al. [31] found that nitrite/nitrate gradually increased with aging in healthy male subjects. In humans endothelium-dependent vasodilation is reduced in essential hypertensive patients compared to normal control subjects, suggesting that endothelial function can be impaired in human hypertension [32]. Panza et al. [33] and Koichi et al. [34] reported decreased NO products in patients with essential hypertension.

Evidence demonstrates a relationship between hypertension and type 2 diabetes mellitus, and several vascular

and metabolic abnormalities [35]. Yugar-Toledo et al. [36] found that patients with diabetes showed an increase in plasma nitrite/nitrate levels, suggesting an association with excessive production and/or inactivation of NO. Ouviaña et al. [37] analyzed endothelial dysfunction and NO levels in patients with hypertension and in patients who suffered from both hypertension and diabetes. They found an increase in the NO levels of patients with hypertension and diabetes together, hence indicating that there is increased release of NO but maybe, low NO bioavailability.

When the capacity of protective hemoglobin-scavenging mechanisms gets saturated, levels of cell free hemoglobin increase resulting in consumption of NO and clinical sequelae [7]. Increased plasma hemoglobin levels are responsible for impairment of EDRF (NO) function and may be the cause in setting up a disease process. Exact mechanism of inactivating EDRF (NO) by hemoglobin is not known [3]. Thus, it may be noted that as part of the aging process, more hemoglobin is released in plasma. Atre et al. [38] also reported high values of plasma hemoglobin in patients suffering from hypertension as compared to normal controls.

hsCRP levels of <1, 1–3, and >3 mg/l should be interpreted as low, moderate and high vascular risk, respectively, based on population studies [39]. Elevated CRP is also associated with endothelial dysfunction [40]. Saito et al. [41] suggested that variations in circulating hsCRP, even within the normal range is involved in the interrelation of cardiovascular risk factors, like age, smoking, obesity, high blood pressure and dyslipidemia, which are supposed to promote atherosclerosis and ultimately provoke cardiovascular diseases, such as coronary artery disease. Lima et al. [42] observed that patients who suffered from both hypertension and diabetes had higher levels of hsCRP than normal subjects; indicating that patients with two associated diseases have a more active inflammatory state.

This could indicate that lipid profile values may not be affected by the presence of diseases such as hypertension or diabetes alone, but may be strongly affected by escalating age. Thus our study showed that, plasma hemoglobin and hsCRP are associated with endothelial dysfunction and increase both with age as well as in disease condition whereas NO₂ and NO₃ decrease in hypertension and increase in patients with diabetes mellitus and hypertension together.

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