Original Research Article

Assessment of serum nitric oxide level in cigarette smoking

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Abstract

Background: Cigarette smoke has been implicated as a major risk factor in various diseases associated with endothelial dysfunction like essential hypertension, atherosclerosis and related cardiovascular dysfunction. Nitric oxide, an important second messanger plays a pivotal role in maintaining vasomotor tone and its level is also found to be altered in many chronic vascular disorders. The particulates in cigarette smoke is presumed to affect the production NO in vascular endothelium. Hence, it is proposed to study the effect of cigarette smoking in influencing Nitric oxide level. The study sample comprised of 138 chronic smokers and144 apparently healthy nonsmokers . Plasma glucose, total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and triglyceride concentration (TGL) were determined enzymatically and serum NO index (NOx) estimated by Griess method. It was found that serum NOx level was not influenced by biochemical parameters like plasma glucose and lipid profile. The estimated mean NOx levels was slightly lower in smokers(15.92 with SD 5.4) than in nonsmokers (16.66with SD 6.73) . However the difference was not statistically significant (P= 0.31). It is concluded that there is no significant correlation between serum nitric oxide level and smoking, based on this study.

Keywords: Essential hypertension, Nitric oxide, Endothelial dysfunction, Vasomotor tone, Nitric oxide index, Frees radicals.

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INTRODUCTION

The endothelial lining of blood vessels is critical to vascular health and constitutes a major defense against hypertension. It helps in the regulation of vascular tone and blood flow, by the secretion and capture of paracrine vasoactive substances, which includes vasodilator substances (NO, prostacyclin and endothelium-derived hyperpolarizing factor) and vasoconstrictor substances (Endothelin-1, thrombaxane A2 and platelet-activating factor). Endothelial dysfunction appears to play a pathogenic role in the initial development of atherosclerosis[1-3] and of unstable coronary syndromes[4]. Recent clinical studies have demonstrated that, some drugs well known to reduce the incidence of cardiovascular events, improve endothelial function.[5-8] NO is the main mediator of vasomotor tone regulation in physiological situations, small amounts being continuously secreted by the eNOS (endothelial nitric oxide synthase)[9,10] to maintain a reduced arterial tone in the systemic and pulmonary circulation.[11] The vasodilator activity of NO is due to its interaction with the iron atom of the heme prosthetic group of guanylyl cyclase, causing its activation and increasing the intracellular levels of cyclic guanosine monophosphate (cGMP).[12] In smooth muscle cells, this decreases intracellular calcium concentration, causing vascular relaxation.[13] NO prevents binding of leucocytes to the endothelium and decreases inflammation, thereby preventing endothelial damage. Various studies suggest that smoking causes endothelial dysfunction by influencing level of serum nitric oxide and is one of the major risk factors for chronic vascular disorders like hypertension and atherosclerosis Nitrite and nitrates are the

oxidative breakdown product of NO[14] and it represents a major storage form of NO in blood and tissues.[15] The most commonly used nitrite assay is based on the Griess diazotization reaction, which is specific for nitrite and does not detect nitrate. Therefore, nitrate in samples is first be reduced to nitrite; subsequent nitrite determination thus yields nitric oxide index (NOx)that is the total nitrite + nitrate concentration of the sample. Hence, it is proposed to study the level of serum Nitric oxide index in chronic smokers and compared with age matched nonsmokers.

AIMS AND OBJECTIVES

Smoking plays a pivotal role in endothelial dysfunction and is proposed to do so by altering serum nitric oxide level. In this study, the aim is to elucidate the role of smoking in altering the level of Nitric oxide, the second messenger involved in maintaining the endothelial function and to compare it with normal non-smoking individuals.

MATERIALS AND METHODS

It is a cross-sectional study, single centered study conducted in a tertiary health center over a period of 10 months.138chronic smokers of mean age 49.53 $\pm$ 9.6 years and 141 apparently age matched healthy nonsmokers were included in this study. Those with hypertension, diabetes mellitus, fever, acute infections, chronic inflammatory states and those on drugs like oral contraceptive pills, steroids were excluded from study group. Standard anthropometric data (height, weight) and resting blood pressure was recorded in each subject, after a thirty minutes rest on a couch. Blood samples were collected by venipuncture after an overnight fasting. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and triglyceride concentration (TGL) were determined enzymatically. Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's formula[304]. Cadmium based reduction of nitrate to nitrite followed by estimation of total nitrite by Griess method is used in this study.

NOx Assay:

Step 1: Deproteinisation[36]- 300 µL of **serum** by adding 250µL of 75 mmol/L $ZnSO_4$ solution, stirring , and centrifuging at 10 000*g* for 1 minute at room temperature, after which 350µL of 55 mmol/L NaOH was added. Again, the solution was stirred and centrifuged at 10 000*g* for 3 minutes and the supernatant was recovered (free of turbidity). We diluted 750 µL of supernatant with250 µL of glycine buffer (45 g/L, pH 9.7).

Step 2: Activation of cadmium - Cadmium granules were rinsed three times with deionized distilled water and mixed in a shaker gently in a 200 mmol/L $CuSO_4$[39] solution in glycine -NaOH buffer (15 g/L, pH 9.7) for 5 minutes till the color of the solution fades. The solution drained off and the step repeated for another time. The copper-coated granules dried in tissue paper and are to be used within 10 minutes. After use, the granules are rinsed and stored in 100 mmol/L H_2SO_4 solution; they can be regenerated by repeating these steps.

Step 3: Reduction of nitrate - The nitrite and nitrate calibrators were diluted with glycine buffer just as the serum samples were. Calibration curves were made over a linear range of nitrite between 0 and 100 µmol/L. freshly activated cadmium granules (2–2.5 g) were added to 1 mL of pretreated deproteinized serum and calibrator. After continuous stirring for 10 minutes, the samples were transferred to appropriately labeled tubes for nitrite determination.

Step 4: Nitrite assay. Nitrite was estimated by Griess reaction

Reagent 1 consisted of 50 mg of *N*-naphthylethylenediamine dissolved in 250 mL of distilled water. Reagent 2 was prepared by dissolving5 g of sulfanilic acid in 500 mL of 3 mol/L HCl. Both solutionsare stable for at least a year at 4 °C. From the above tubes 200 µL of sample were placed into fresh glass tubes. To it 800 µL sulfanilamide solution were mixed in, followed by 750 µL NED solution. We then waited for 10 min at room temperature for a pink colour development and absorbance was read at 545 nm within 60 min. the measured OD was plotted on the standardization graph and concentration found out.(figure 1)

STATISTICAL ANALYSIS:

1. Age, BMI, plasma glucose and serum lipid levels were compared between control subjects and patients by students ' t' test and chi-square test (χ^2).
2. Serum NOx distribution between smokers and nonsmokers were compared by student independent t test. $p< 0.05$ was considered significant.Independent variables included in the analysis were age, serum levels of glucose, cholesterol, triglycerides, HDL (quantitative).
3. The influence of other biochemical parameters on serum NOx level was analysed through Pearson correlation.

RESULTS

Table 1: age, sex and BMI distribution among smokers and nonsmokers

	Group				Student independent t-test
	Smokers		Control		
	Mean	SD	Mean	SD	
Age	49.53	9.6	51.33	8.91	P= 0.1
Wt (kg)	64.75	9.88	67.05	10.3	P=0.06
Ht	163.42	7.47	162.65	7.2	P=0.37
BMI	24.24	3.44	24.38	3.84	P=0.7

Table 2: Distribution of biochemical parameters like plasma glucose and lipid profile between smokers and controls

	Group				Student Independent t-test
	Smokers		Control		
	Mean	SD	Mean	SD	
Blood Glucose	95.60	6.7	95.33	7.2	P= 0.74
T.CHOL	160.53	33.08	166.25	40.15	P= 0.19
TGL	163.37	40.1	165.58	63.27	P= 0.72
HDL	40.22	9.56	41.74	11.12	P= 0.22
LDL	90.41	35.1	95.12	39.6	P= 0,29

Table 3: Comparision of serum NOx levels of smokers with nonsmokers

	Group				Student independent t-test
	Smokers		Nonsmokers		
	Mean	SD	Mean	SD	
Serum NOx	15.92	5.4	16.66	6.73	P= 0.31

Table 4: Pearson correlation analysis

	Type of statistical analysis	Plasma Glucose	T.Chol	TGL	HDL	LDL
Serum NOx level of Controls	Pearson Correlation	-0.07	-0.005	0.08	0.09	-0.06
	Sig. (2-tailed)	0.39	0.95	0.31	0.26	0.42
	N	144	144	144	144	144
Serum NOx of Smokers	Pearson Correlation	0.1	0.13	0.10	0.08	0.05
	Sig. (2-tailed)	0.24	0.12	0.21	0.33	0.5
	N	138	138	138	138	138

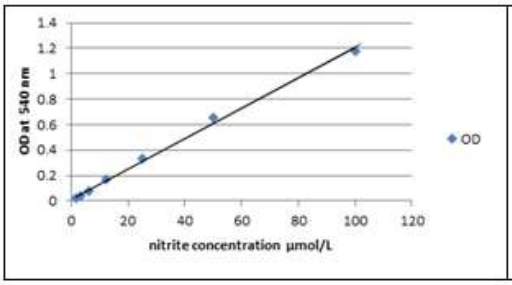

Figure 1

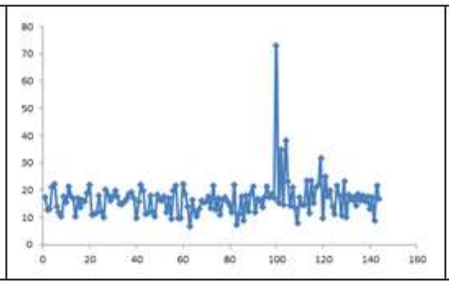

Figure 2

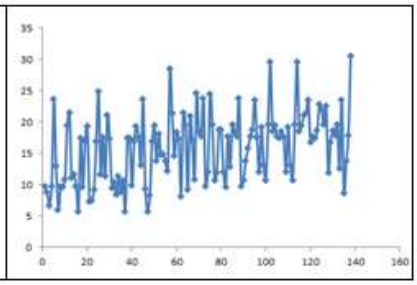

Figure 3

Figure 1: Standardization graph for nitrite; **Figure 2:** Scatter plot for serum nitrite levels among non smokers (n=144); **Figure 3:** Scatter plot for serum nitrite levels among smokers (n=138)

Table 1: Shows age, sex and BMI distribution among smokers and nonsmokers. No significant difference between the groups. Table 2: Shows the distribution of biochemical parameters like plasma glucose and lipid profile between smokers and controls. No significant difference in measured biochemical parameters between smokers and nonsmokers. Scatter diagrams 1 and 2 represents the distribution of serum NOx level in non smokers and smokers respectively (Figure 2, 3). Table 3 :Compares serum NOx levels of smokers with nonsmokers. There is no statistically significant difference in NOx levels between smokers and non smokers, with p value of 0.31 Table 4 : Pearson correlation analysis to find out the influence of other biochemical parameters on serum NOx level.

(**Correlation is significant at the 0.01 level (2-tailed). It was found that serum NOx level was not influenced by biochemical parameters like plasma glucose and lipid profile.)

DISCUSSION

Endothelium plays a vital role in regulation of vascular tone and maintaining blood pressure by secreting paracrine vasoactive substances like nitric oxide, prostacyclin, endothelin, thromboxane A2 and platelet activating factor. Among them, NO is a potent vasodilator that inhibits extracellular matrix turnover and can thus modify the mechanical properties of the arterial wall[19]. Higman *et al...* [11]reported that the release of NO from saphenous veins of nonsmokers was significantly higher than that from veins of heavy smokers. Using the NO antagonist N^G-monomethyl-L-arginine, several investigators[18] have found indirect impairment of endothelium-dependent vasodilatation in smokers with decreased NO. NO release from the endothelium, is found to be decreased in patients with established coronary atherosclerosis with hypertension[16].A reduction in vascular availability of NO determines damage to the endothelium-dependent vasodilation, an increased tendency for platelet aggregation and adhesion of monocytes to the endothelium, thus influencing the proliferation of vascular smooth muscle cells, contributing to the onset and progression of vascular diseases like hypertension and coronary artery disease. The association between smoking and vascular diseases is widely recognized, and there is a general consensus that smoking targets the vascular endothelial cells. The mechanism for the increased risk of vascular dysfunction is not well understood, but it is presumed to be due to the absorption of tobacco smoke constituents that affect endothelial cell function. Studies on exposure of chronic cigarette smoking causes an irreversible inhibition of eNOS activity in pulmonary artery endothelial cells and suggest that the decreased activity is secondary to reduced eNOS protein mass and mRNA. It was postulated that the decrease in eNOS activity may contribute to the high risk of pulmonary and cardiovascular disease in cigarette smokers[20].Another study indicates that in vitro, human coronary artery endothelial cells(HCAEC) show similar changes in NO biosynthesis as human umbilical vein endothelial cells when exposed to smokers' serum and also confirms that oxidative stress plays a central role in smoking-mediated dysfunction of NO biosynthesis in endothelial cells[21] This study was indended to measure serum nitric oxide level (NOx) in smokers and to compare with normal nonsmokers. The smokers and controls were perfectly matched with respect to confounding variables like age and BMI. Those with impaired glucose tolerance, acute infections, chronic inflammation and alcoholics were excluded from the study as these states may present with altered serum NOx level. On comparing serum NOx level of the smokers with non smoking individuals, it was found to have no significant difference.

CONCLUSION

There is no significant correlation between serum NOx level and cigarette smoking in the general population in our study.

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