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Original Research Article

Role of serum nitrogen oxide in treated hypertensive and normotensive subjects

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Abstract

Background: Hypertension is a leading cause of mortality and morbidity worldwide. Essential hypertension is characterized by endothelial dysfunction and increased vascular tone and resistance. The former is the result of imbalance of endothelium derived contracting & relaxing factors. Nitric oxide which is produced locally by endothelium is crucial for maintaining vascular tone.

Aims & Objectives: To study the role of nitric oxide in hypertensive cases and also find out the normal level of serum nitric oxide in the specific population of the district East Godavari, Andhra Pradesh.

Materials &Methods: A total of 60 hypertensive and non-diabetic patients aged between 30 years to 72 years; (Male: Female =1.3:1) was taken for the study. Serum creatinine was measured and hypertensives having elevated serum creatinine (>0.3 mg/dl) were excluded from the study; this was done to rule out any effect of kidney functional status on level of serum nitric oxide. Serum nitrate was measured using colorimetric Griess assay. The data was analyzed using SAS 9.1

Results: The mean serum nitrate in hypertensive patients (43.77± 0.29 µmol /L) is higher than the controls (38.57 ±5.02 µmol /L) and is statistically significant $p< 0.0002$. The concentration of nitrogen oxide (nitrate plus nitrite) in the plasma of systemic venous blood was significantly more in the hypertension group than in the control group. The plasma nitrogen oxide concentration showed a significant positive correlation with both systolic ($r=+1.57$, $p<0.05$) and diastolic blood pressure ($r=+1.47$, $p<0.05$).

Conclusion: Elevated serum nitric oxide level in hypertensives indicates an endothelial cell response to the antihypertensive medications. Serum nitric oxide thus may be used as a marker for predicting "future hypertensive". This may allow the physicians to modulate life style of these "future hypertensive" before overt hypertension actually develops; this will not only save a probable future hypertensive from taking many medications and facing complications but will also reduce the burden of this ever-growing devastating disease. Also the mean serum nitrate in controls of this study is much different from the other studies; does it indicate that serum nitric oxide value is population specific? Anyway, the authors feel that in order to establish both these points and comment with such conviction, much larger future studies are needed.

Keywords: endothelium, hypertension, nitric oxide, non-diabetic, serum creatinine.

1.Introduction

Hypertension is a condition that presently afflicts more than 1-billion people worldwide and is hence a leading cause of morbidity and mortality. Over time it has earned the euphemism "silent killer" as it is usually asymptomatic until the damaging effects are virtually observed. Hypertension is classified by etiology as being either primary or secondary. The pathophysiology of essential hypertension is still unclear[1].

Nitric oxide (NO) is a soluble gas continuously synthesized by the endothelium. This substance has a wide range of biological properties that maintain vascular homeostasis, including modulation of vascular dilator tone, regulation of local cell growth, and protection of the vessel from injurious consequences of platelets and cells circulating in blood. Endothelium-dependent vasodilator responses are attenuated in patients with primary or essential hypertension[2][3]. Endothelin-1 (ET-1), homocysteine (Hcy), and nitric oxide (NO) presently enjoys the focus of considerable attention in this regards[4]-[7]. However, the debate regarding their role in essential hypertension still goes on[8][9]. Hyperhomocysteinaemia generates reactive oxygen species and reduces the bioavailability of NO via endothelial dysfunction[10]. Similarly, ET-1 has potent physiologic actions including vasoconstriction, cell proliferation, edema formation, and possibly a contribution to inflammation[11][12]. In addition, numerous studies have shown an interaction between ET- 1 and NO, a potent vasodilator, in the vascular endothelium[13]. Endothelium-dependent dilatation in resistance arteries is defective in patients with essential hypertension[14]. This dysfunction is a reflection of reduced bioactivity of NO[15]. Hence, essential hypertension is characterized by endothelial dysfunction and increased vascular tone and resistance[16]. The former is the result of imbalance of endothelium derived contracting & relaxing factors. Nitric oxide is produced locally by endothelium is crucial for maintaining vascular tone[17]; NO released from cells rapidly autoxidizes to yield nitrite (NO^{2-}), which interacts with hemoglobin to yield nitrate (NO^{3-}). Because nitrite plus nitrate (nitrogen oxide) is relatively stable in blood, the level of nitrate plus nitrite in blood may be an indicator of the endogenous formation of NO.

It is still unclear whether NO is a marker or a mediator for endothelial dysfunction in hypertension and also whether changes in NO concentration occur as primary or secondary events. Literature reveals many conflicting reports regarding the association between serum nitrogen oxide and essential hypertension. Moreover, in a recent study conducted by Zhang *et al*[18] in hypertensive women in Suzhou revealed that serum NO level was not associated with hypertension in women in Suzhou.

The Konaseema belt of East Godavari district of Andhra Pradesh, in development transition, has witnessed a marked increase in cardiovascular disease (CVD) morbidity and mortality. Nonetheless, little is known about the levels of circulating biomarkers in this population. We therefore, aimed to carry out a case control study to evaluate plasma levels of nitric oxide (NO) among patients with essential hypertension and healthy normal control subjects (normotensives) in a heterogeneous sample population from this belt of Konaseema.

2. Materials and Methods

2.1. Source of data

This study was approved by Institutional Ethical Committee, Konaseema Institute of Medical Sciences and Research Foundation, Amalapuram, Andhra Pradesh, India. All subjects gave their written informed consent for participation.

2.2. Measurement of blood pressure

The present study was done in Department of Biochemistry, Konaseema Institute of Medical Sciences & Research Foundation, Amalapuram, Andhra Pradesh. Blood pressure was measured conventionally by sphygmomanometer and the blood pressures of both groups were recorded. Resting BP measurements were performed in the sitting position between 8:00 AM and 10:00 AM on at least two separate days for 1-week apart. Caffeinated beverages were avoided for at least 30-min prior to measurement. The recordings were made under quiet, comfortable ambient (~24°C) laboratory conditions. An appropriately sized cuff (cuff bladder encircling at least 80 percent of the arm) was used. Recordings were made in triplicate in the upright sitting position and the average recorded.

2.3. Diagnosis of hypertension

Hypertensive cases were diagnosed as per the guidelines of JNC-7 (2003). Normal blood pressure was defined as systolic pressure <140 mm Hg and diastolic pressure <90 mm Hg. Hypertension was defined as either systolic pressure ≥160 mm Hg or diastolic pressure of ≥95 mm Hg, or both, with a well-documented history of long-term high blood pressure. Sixty hypertensive Patients (30 years to 72 years; M:F=1.3:1) not having other diseases like diabetes mellitus, renal disorder, cardiovascular

disorders, cerebrovascular disorders etc. were included in the study. Cases and controls having serum creatinine more than 0.3 mg/dl were excluded from study. All the patients were under treatment of antihypertensive medications. Causes of secondary hypertension, such as pheochromocytoma, renovascular disease, hyperthyroidism, and aortic contraction were excluded in all patients by the primary physician on the basis of conventional clinical and laboratory criteria before the initiation of antihypertensive therapy. Total number of 60, age and sex matched persons, ranging from 30 years to 72 years; with male: female ratio of 1.3:1, having normal blood pressure and no significant ailments were included in this study as controls. Participants were instructed to refrain from eating for 18 hours, drinking beverages containing alcohol or caffeine, or smoking for at least 24 hours before blood sampling.

2.4. Estimation of serum nitrogen oxide

Specimens (1.5 mL) of peripheral venous blood from the median cubital vein were collected into heparinized tubes after the subjects had been sitting at rest for 15 minutes in a quiet room maintained at a temperature of 22°C to 24°C. The blood was placed immediately in an ice bath and centrifuged within 30 seconds for 5 minutes at 2000g. 1 ml of Glycine - NaOH buffer (pH=9.7) ,1 ml of protein free filtrate, 2 ml of distilled water and 2-3 gms of Cu-Cd granules were sequentially added in a tube; The tubes were placed on a rotor/shaker and mixed vigorously for 20-25 minutes; the Cu-Cd granules reduced nitrate to nitrite. To 2 ml of supernatant 1 ml of Griess reagent A (consisting of 0.1% naphthylethylenediaminedihydrochloride in distilled water) was added. Then, 1 ml of Griess Reagent – B (consisting of 1% sulfanilamide in 5% H_3PO_4,) was added to the solution. The solution was incubated for 20 minutes and pink colour was observed. The absorbance was then read at 540 nm [19] to provide the total amount of plasma NO end products (nitrate plus nitrite). The efficiency of the cadmium column in the conversion of nitrate to nitrite was confirmed to be 100% by measuring both nitrate and nitrite standards before and after sample measurement[19].

2.5. Statistical analysis

The data was analyzed using SAS 9.1 Software. Data was expressed as mean ± SD. Standard deviation has been used to indicate whether the variation of difference of an individual from the mean is by chance. Independent t-test / Mann-Whitney U-test was used based on the normality assumption to compare the groups. For Categorical variables, Chi-square/Fisher's exact test was used to compare the groups. Statistical analysis was performed applying independent sample 't' test to the data of independent samples for equality of means between the groups and "Levenes Test for Equality of variances" within the group. The probability value of less than 0.05 was considered statistically significant because such a difference could commonly occur due to chance and the factors under study may have no influence on the variables.

3. Results

Demographic characteristics of the subjects are summarized in table 3.1. Statistical analysis revealed that cases and controls were not significantly different with respect to their age are presented in table 3.1. (independent't' test: t=0.77; p=0.4404) and sex (chi square test; p=0.2599).

Table 3.1: Demographic analysis of male and female subjects

Characteristics	Cases (N=60)	Control (N=60)	P Value
Age	53.98± 10.98	52.58 ± 8.69	0.4404
Male	34(56.67%)	40(66.67%)	0.2599
Female	26(43.33%)	20(33.33%)	

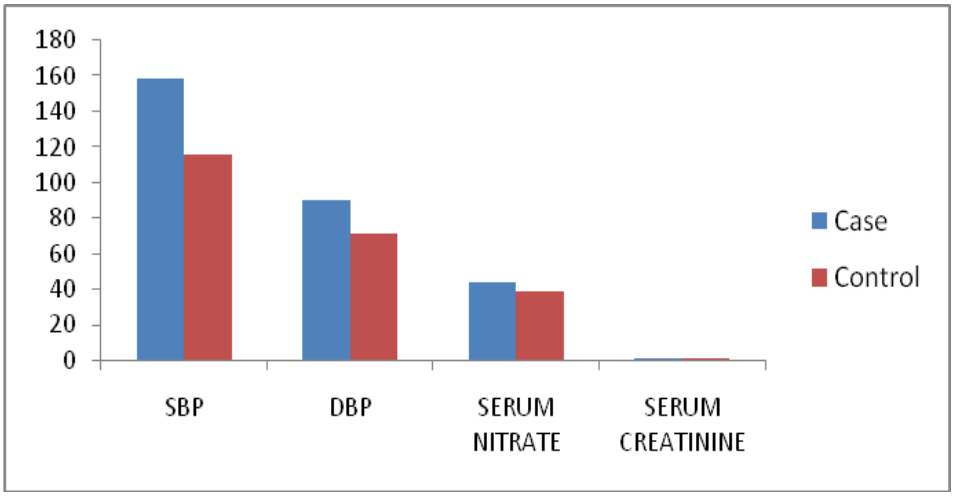
The respective values of systolic blood pressure, diastolic blood pressure and serum nitric oxide of both the groups are tabulated in table 3.2. Mann-Whitney U test used based on the normality assumption to compare the two groups revealed that all the findings are significantly different among the two groups.

Table 3.2: The respective values of systolic blood pressure, diastolic blood pressure and serum nitric oxide

Characteristics	CASES (N=60)	Control (N=60)	Z Value	P VALUE
SBP	158.17± 16.82	116.00 ± 7.24	9.57	<0.0001
DBP	89.97± 10.86	71.50 ± 9.36	7.69	< 0.0001
NITRIC OXIDE	43.77± 0.29	38.57 ±5.02	3.70	0.0002

The concentration of nitrogen oxide (nitrate plus nitrite) in the plasma of systemic venous blood was significantly more in the hypertension group than in the control group as shown in the figure 3.1. The plasma nitrogen oxide concentration showed a significant positive correlation with both systolic (r=+1.57, p<0.05) and diastolic blood pressure (r=+1.47, p<0.05).

Figure 3.1: Effect of Serum NO and serum creatinine level and on Hypertension.



4. Discussion

Hypertension can produce structural damage to aortic endothelial cells in animals, and pressure overload is associated with a direct toxic effect on human endothelium; impairment of the release of NO from vascular endothelial cells may thus contribute to the reduced plasma nitrogen oxide concentrations in patients with essential hypertension. In a study of hypertension on serum Nitric Oxide (NO) and Vascular Endothelial Growth Factor (VEGF) concentrations in DOCA-Salt hypertensive ovariectomized rats, reduced serum NO and increased serum VEGF concentrations in hypertensive animals supported the concept of endothelial dysfunction in hypertensive subjects[20].

The plasma concentration of nitrogen oxide in systemic venous blood is determined by synthesis, degradation, and clearance of NO. As for the synthesis of NO, NO is continuously synthesized from L-arginine in a reaction catalyzed by NO synthase. NO is produced from endothelial cells, leukocytes, platelets, nerves, cardiomyocytes, and muscles. In the present study, both cases and controls having elevated serum creatinine (>0.3 mg/dl) was excluded from the study; hence, the serum nitrogen oxide measured in both cases and controls reflects the endothelial status correctly. Daily activity and the consumption of food or water may also affect nitrogen oxide concentration. This was taken care of by collection of blood samples only after 8 hours of fasting or 6 hours of abstaining from drinking.

Most of the endothelium dependant vasodilatation is done by release of nitric oxide and common molecular variants of endothelial "nitric oxide synthase" gene are involved in essential hypertension; hence, impairment of nitric oxide production by specific inhibitors increases blood pressure in humans[21].

Several reports are suggestive that hypertensive subjects have a blunted endothelium dependant vasodilatation which might be secondary to decreased nitric oxide production from the vessel wall.[22]-[24] Besides its role as the most potent endogenous vasodilator, NO reduces the risk of heart disease by several mechanisms including decreased platelet aggregation, decreased low-density lipid oxidation, and decreased monocyte migration. Further, reduced NO bioavailability is common in patients at high cardiovascular risk[25]. Deterioration of endothelial function was observed in patients with hypertension with risk factors of atherosclerosis, especially coronary artery disease[26]. Both arterial hypertension and endothelial dysfunction plays a central role in development of atherosclerosis; biochemical evidences of marked endothelial dysfunction are present in hypertension in chronic renal failure[27].

Hypercholesterolaemia and other lipid abnormalities may promote subclinical endothelial dysfunction by suppressing bio-available NO; in patients with both hypertension and homocysteinemia, treatment of latter disease could improve blood pressure control because hyperhomocysteinemia per se disrupts the endothelial activity of release of nitric oxide [28].

Endothelial dysfunction is indicated by decreased serum nitrate concentration as has been shown by various studies where essential hypertensive subjects were documented with endothelial dysfunction by the virtue of decreased serum nitrate values[29]-[32]. Hypertensive patients living in high mountains revealed low concentrations of nitric oxide metabolites in plasma, erythrocytes and blood which aggravated with progress of hypertension and its duration [33].

Preeclampsia is associated with hypertension; Serum nitrate and nitrite levels are higher in preeclamptic women than in the normotensive pregnant women and have positive correlation with the severity of the disease[34]. In

women with preeclampsia, a higher maternal nitric oxide level may act as a compensatory mechanism against hemoconcentration and platelet aggregation[35]. In a comparative study of pre-eclampsia and essential hypertension patients, it was observed that in patients with pre-eclampsia, the plasma concentrations of nitric oxides, lipid peroxides and glutathione peroxidase were elevated with a decrease in catalase and superoxide dismutase; On the other hand, patients of essential hypertension, had very low serum nitric oxide and superoxide dismutase and very high lipid peroxides values[36].

NO, anyway is not the only way to reverse hypertension; in a study on effect of hypertension and its reverse on serum nitric oxide (NO) concentration and endothelial permeability in two-kidney one-clip (2K1C) hypertensive rats, lower serum NO concentration in 2K1C hypertensive rats even after reversal of hypertension suggested that in addition to NO, other mechanisms could be involved in surgical reversal of hypertension [37].

There are two important points which the authors want to highlight through this study.

1). The normal serum NO(x) concentration, as was found in blood drawn from controls was 38.57 ±5.02 micromol/L; this value is much different from the value obtained in other studies. In fact, the normal range of NO showed a wide variation in different studies[38]-[43]; hence, it necessitates working out the normal range of serum NO for a particular population. However, we stress that the reference value for NO in normal Indian subjects remains to be established.

2). Mean serum nitrate of hypertensive cases was higher than that of controls and is statistically significant (p=0.0002); this is an adaptation of the blood vessel endothelium in response to antihypertensive medications; it may also reflect a protective mechanism against the action of vasoconstrictor compounds, including ET-1[44]. Similar results have also been reported by Enyioma *et al*[44]. Sainani *et al*[45] revealed elevated levels of vasoconstrictors and reduced level of vasodilators in untreated essential hypertension subjects which confirmed the presence of endothelial dysfunction, even in mild cases of hypertension. They concluded that early detection of endothelial dysfunction may be a useful measure to guide therapy before the damaging effects of hypertension manifests.

In the present study, NO levels showed a significant difference between patients and controls. This suggests that an estimation of NO levels could be included as a routine lab investigation to screen people at risk and to devise appropriate individualized therapeutic strategies.

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