ESTIMATION OF SERUM NITRITE IN INDIVIDUALS WITH PRIMARY HYPERTENSION
Zaheera Sultana S¹, Lakshmi T², Farquana Qushnoon³, Salim A Dhundasi⁴, K. K. Das⁵

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ABSTRACT: BACKGROUND AND OBJECTIVES: Hypertension is the commonest cardiovascular disorder, posing a major health challenge. It is one of the major risk factors for cardiovascular mortality which accounts for 20-50% of all deaths. Evidence suggests that Nitric oxide (NO) plays a major role in regulating blood pressure and that impaired NO bioactivity is an important component of hypertension. Hence this study is taken up to estimate nitrite levels in Primary Hypertension patients compared with healthy individuals. METHODOLOGY: The present study was conducted in the Department of Physiology, Al-Ameen Medical College, Bijapur and District Hospital, Bijapur. Thirty five (35) Primary Hypertension Patients (17 male, 18 female) between 35 to 65 yrs age and thirty nine (39) healthy individuals, controls (20 male, 19 female) between 38 yrs to 65 yrs age visiting Al-Ameen Medical College Hospital, Bijapur and District Hospital Bijapur were selected. Serum Nitrite was estimated by GRIESS method. Statistical analysis was done by ANOVA. RESULTS: Statistically significant variations were found in parameters like age, Ht, Wt, BSA, BMI, PR, SBP, DBP, Serum Nitrite levels in controls and Primary Hypertension patients. INTERPRETATION AND CONCLUSION: In the present study the mean±SEM of Serum Nitrite in controls was found to be 18.001±0.306 µ mol / lt, and primary hypertension patients was 13.06±0.005 µ mol / lt. It was found Serum Nitrite levels of PHTN patients were lower when compared with the controls, these difference were found to be statistically significant (t =12.212, P=0.0000). Hypertension can produce 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. Increased production of superoxide anions, which rapidly deactivate NO, is a characteristic feature of experimental models of hypertension, and plasma indexes of lipid peroxidation are increased in patients with hypertension.

KEYWORDS: Primary Hypertension, Nitric oxide, Nitrite.

INTRODUCTION: Hypertension is the commonest cardiovascular disorder, posing a major health challenge. It is one of the major risk factors for cardiovascular mortality which accounts for 20-50% of all deaths. In Indians hypertension is the predominant risk factor for coronary artery disease. Primary or essential hypertension is the most prevalent form of hypertension accounting 90% of all cases of hypertension. The prevalence of hypertension is 59.9 and 69.9 per 1000 in males and females respectively in urban population and 35.5 and 35.9 per 1000 in males and females respectively in rural population.¹ Reactive nitrogen species comprises of nitric oxide (Nitrate and Nitrite), peroxynitrous acid, S-nitrosothiols. The term “RNI” refers to oxidation states and adducts of the nitrogenous products of nitric oxide synthases, ranging from nitric oxide (NO) to nitrate (NO₃⁻), that arise in physiological environments, including NO −, NO₂, NO₂⁻, N₂O₃, N₂O₄, S-nitrosothiols, peroxynitrite (OONO⁻) and dinitrosyl-iron complexes.²
Endothelium was once thought to be simply a passive lining for blood vessels, it is now recognized that the vascular endothelium is a key determinant of vascular health. Broadly speaking, the term “endothelial dysfunction” refers to an impairment of the ability of the endothelium to properly maintain vascular homeostasis. Although the term is often used in reference to a loss of bioavailable nitric oxide (NO), endothelial dysfunction also reflects increased production of vasoconstrictors and disturbed regulation of inflammation, thrombosis, and cell growth in the vascular wall.

The fact that the endothelium can profoundly affect the function of vascular smooth muscle cells has been recognized only recently. In 1980, Furchgott and Zawadzki demonstrated that the relaxation of isolated arteries induced by acetylcholine requires the presence of endothelial cells.

NO has a very short half-life in tissues (three to ten seconds) because it reacts with oxygen and superoxide and then is converted into nitrate and nitrites. The tiny diatomic gas nitric oxide has an unpaired electron in its outer orbit and in pure form, in either solid or liquid phases, achieves chemical stability by forming dimmers. The unpaired electron makes the molecule highly reactive. It is soluble both in water (upto 2 mmol/L at 20°C and one atmosphere) and lipid and therefore freely diffusible into the cell. Many cell types other than endothelium that is platelets, brain, adrenal cells, non-adrenergic non cholinergic nerve fibres, neutrophils, monocytes, mast cells while macrophages also produce nitric oxide as part of their immunological response.

The major immediate breakdown product of NO in human plasma is nitrite (NO$_2^-$). Autoxidation of NO by reaction with O$_2$ results in the formation of nitrite (NO$_2^-$) as the primary end-product, although at physiological concentrations of NO and O$_2$, this reaction may be too slow to be of major importance in vivo. In the vascular system, NO is rapidly oxidized by reaction with oxyhemoglobin (HbO$_2$), resulting in formation of methemoglobin (Hb$_3^+$) and nitrate (NO$_3^-$). NO also reacts with Hb$_3^+$ to form a complex (Hb-NO), which can hydrolyze to Hb$_2^+$ and NO$_2^-$. Nitric oxide is formed from the guanidine-nitrogen terminal of L-arginine by an enzyme called NO synthase (NOS) which is constitutive in normal endothelial cells, through a metabolic route called L-arginine-nitric oxide pathway. L hydroxyl-arginine is an intermediate product that remains tightly bound to the enzyme. The reaction being oxidative in nature consumes five electrons and requires molecular oxygen in addition to several cofactors. Nitric oxide synthase is a very complex enzyme, employing five redox cofactors; NADPH (nicotinamide adenine dinucleotide phosphate), FAD (flavine adenine dinucleotide), FMN (flavin mononucleotide), HEME and BH4 (Tetrahydrobiopterin). The activation of NO synthase depends on the intracellular calcium ions in the endothelium cells, is calmodulin dependent.

MATERIAL AND METHODS: The present study was conducted in the Department of Physiology, thirty five (35) Primary Hypertension Patients (17 male, 18 female) between 35 to 65 yrs age and Thirty nine (39) healthy individuals, controls (20 male, 19 female) between 38 yrs to 65 yrs age visiting Al-Ameen Medical College Hospital, Bijapur and District Hospital Bijapur were selected. All known Primary Hypertension were studied. Patients diagnosed with causes of secondary hypertension viz pheochromocytoma, hyperthyroidism, coarctation of aorta, renovascular diseases, vasculitis, any liver disorder were excluded. The study protocol was explained to the Primary
Hypertension and Controls, who volunteered for the study. Informed consent was obtained from each of the participant. A detailed history of subjects was taken.

**GRIESE METHOD –**

**A) Materials**

1. Griess reagent \([\text{Sulphanil amide, } N-(1\text{-Naphthyl}) \text{ ethylene diamine dihydrochloride}]\)
2. Vanadium (III) Chloride: 8mg dissolved in DDW upto 1ml.
3. Sodium nitrite\([\text{NaNO}_2]\): 1mM \text{NaNO}_2/ L
4. Sodium nitrate \([\text{NaNO}_3]\): 1mM \text{NaNO}_3 / L
5. Double Distilled Water (DW)
6. Ethanol

**PROCEDURE:** Blood (5 ml) for analysis was obtained from the antecubital vein of the primary hypertension and type II Diabetic patients with hypertension as well as from the controls. Blood was allowed to clot and serum was separated by centrifugation at 2500 rpm for 15 minutes.

**Serum;**

Deproteinization: (Serum: Ethanol, 1: 2)

\[
\begin{align*}
500\mu l \text{ serum} + 1000\mu l \text{ Ethanol (1ml)} \\
\text{Vortexed well for 2 to 3 min} \\
\text{Centrifuge (10000rpm for 10min)} \\
\text{Take 0.5mL Supernatant}
\end{align*}
\]

The supernatant was taken for Nitric oxide determination. 500µl of supernatant was mixed with 500µl of vanadium chloride. [Vanadium chloride acts as a chemical catalyst, which leads to reduction of sodium nitrate to sodium nitrite], 500µl of Greiss reagent was added into the mixture. Mixed well by vortexing it for 1 to 2 min. This sodium nitrite reacts with Griess reagent.13

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supernatant</td>
<td>500µl</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>DDW</td>
<td>-</td>
<td>500µl</td>
</tr>
<tr>
<td>3</td>
<td>VCl3</td>
<td>500µl</td>
<td>500µl</td>
</tr>
<tr>
<td>4</td>
<td>Greiss Reagent</td>
<td>500µl</td>
<td>500µl</td>
</tr>
<tr>
<td></td>
<td>(Sulphanil amide + NED)</td>
<td>(250µl+250µl)</td>
<td>(250µl+250µl)</td>
</tr>
</tbody>
</table>

Finally the absorbance of the product read spectrometrically by using 540nm filter.

The concentration of Nitric oxide in serum sample was determined from standard curve established by 0 to 120µmol/L of sodium nitrite. By taking OD of the serum sample, SERUM NITRITE is calculated by using the following formula from the standard curve.14
RESULTS: Thirty five Primary Hypertension patients between 35 to 65 years were selected for the study. Thirty nine healthy individuals, controls between 38 to 65 yrs age from Bijapur city were the volunteers. All Primary Hypertension patients underwent history taking and a thorough clinical examination. The ANOVA was used to analyse the variation in the parameters of controls and Primary Hypertension patients. P < 0.05 was considered as a level of significant in all the statistics tests.

![Image](image_url)

TABLE NO.1: Shows the mean ± SEM of Age, Ht, Wt, BMI, BSA in controls and PHTN patients

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CONTROLS (n = 39) MEAN±SEM</th>
<th>PHTN patients (n = 35) MEAN±SEM</th>
<th>ONE WAY ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>53.69 ± 1.69</td>
<td>56.97 ± 0.358</td>
<td>P = 0.0757 (NS)</td>
</tr>
<tr>
<td>Ht (cms)</td>
<td>154.38 ± 0.905</td>
<td>157.83 ± 1.79</td>
<td>P = 0.0820 (NS)</td>
</tr>
<tr>
<td>Wt (kgs)</td>
<td>51.51 ± 1.01</td>
<td>64.51 ± 1.19</td>
<td>P = 0.0000 (S)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.61 ± 0.174</td>
<td>25.91 ± 0.111</td>
<td>P = 0.0000 (S)</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.48 ± 0.018</td>
<td>1.65 ± 0.026</td>
<td>P = 0.0000 (S)</td>
</tr>
</tbody>
</table>

TABLE NO. 2: Shows the mean ± SEM of PR, SBP, DBP, Serum Nitrate in controls and PHTN patients

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>PARAMETER</th>
<th>CONTROLS (GROUP 1) MEAN±SEM</th>
<th>PHTN patients (GROUP 2) MEAN±SEM</th>
<th>ONE WAY ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PR (bpm)</td>
<td>74.63 ±0.113</td>
<td>80.77 ±0.119</td>
<td>P = 0.0000 9 (S)</td>
</tr>
<tr>
<td>2</td>
<td>SBP (mmHg)</td>
<td>123.12 ±1.58</td>
<td>157.08 ±1.91</td>
<td>P = 0.0000 (S)</td>
</tr>
<tr>
<td>3</td>
<td>DBP (mmHg)</td>
<td>79.64 ±0.451</td>
<td>97.48 ±0.717</td>
<td>P = 0.0000 (S)</td>
</tr>
<tr>
<td>4</td>
<td>Serum Nitrite (μmol / lt)</td>
<td>18.001 ± 0.306</td>
<td>13.06 ± 0.005</td>
<td>P = 0.0000 (S)</td>
</tr>
</tbody>
</table>

A. Physical parameters: Table No. 1 shows the mean ± SEM of age, Ht, Wt, BSA, BMI in controls and Primary Hypertension patients. The height of Primary Hypertension patients was found to be numerically more than the controls and was found to be not significant statistically (t = — 1.764, p = 0.0820) when compared with controls. The weight PHTN patients was more than the controls and which were statistically significant (t = — 10.000, p = 0.0000). The mean value of BMI and BSA of PHTN patients was more as compared to that of controls. There was a statistically significant difference (t = — 11.5222, p = 0.0000) & (t = — 6.203, p = 0.0000) respectively.

B. Physiological parameters: Table No. 2 shows the mean ± SEM of PR, SBP, DBP controls and Primary Hypertension patients. The PR of PHTN patients was found to be more compared to that of controls and which was
significant statistically \( (t = -9.227, \ p = 0.0000) \). The mean value of SBP levels PHTN patients where higher when compared with the controls, this difference was found to be statistically significant \( (t = -15.698, \ p = 0.0000) \). The mean value of DBP levels in PHTN patients where higher when compared with the controls, this difference was found to be statistically significant \( (t = -15.431, \ p = 0.0000) \).

C. BIOCHEMICAL PARAMETERS: Table No. 2 Shows the mean ± SEM of Serum Nitrite in controls was found to be 18.001 ± 0.306 μmol / lt, PHTN patients was 13.06 ± 0.005 μmol / lt. It was found Serum Nitrite levels of PHTN patients were lower when compared with the controls, these difference were found to be statistically significant \( (t = 12.212, \ P=0.0000) \)

DISCUSSION: In the present study the mean ± SEM of Serum Nitrite in controls was found to be 18.001 ± 0.306 μmol / lt, and primary hypertension patients was 13.06 ± 0.005 μmol / lt.. It was found Serum Nitrite levels of PHTN patients were lower when compared with the controls, these difference were found to be statistically significant \( (t = 12.212, \ P=0.0000) \)

A Study conducted by Bülent SÖZMEN, Cahit KAZAZ, Dilek TASKIRAN, Leyla ASLAN, Akan AKYOL Erser Yıldırım SÖZMEN entitled “Plasma Antioxidant Status and Nitrate Levels in Patients With Hypertension and Coronary Heart Disease” in 1998 on 18 patients with essential hypertension and 16 healthy, age matched controls Plasma nitrite levels were determined by a colorimetric method based on the Griess reaction. The mean±SEM of Plasma nitrite level in essential hypertension patients was 4.7±2.4 μmol/L and 8.1±3 μmol/L in controls. It was significantly lower in essential hypertension patients than controls \( (p<0.05) \).15

In the study entitled “Reduced Plasma Concentrations of Nitrogen Oxide in Individuals With Essential Hypertension” by Koichi Node et al., in 1997 conducted on 108 Hypertension patients (78 men and 30 women with a mean±SEM age of 49±3 years). A total of 127 normal subjects (81 men and 46 women) aged 50±3 years were matched with the patients for sex and approximate age and served as the control group. The mean±SEM of plasma nitrogen oxide (nitrate plus nitrite) in Essential Hypertension patients was 15.7±1.1 mmol / L and that of controls was 22.8±1.4 mmol / L. The plasma concentration of NO was reduced in individuals with essential hypertension relative to that in control subjects and which was significant statistically \( (p<0.001) \).16

Shubhangi Arora, et al., conducted a study entitled “ Nitric Oxide and eNOS Gene in Essential Hypertension ” in 2009 on 45 patients (selected from the department of Cardiology All India Institute of Medical Sciences, ages between 25 to 55 yrs and not on any antihypertensive medications) and 45 controls (healthy volunteers with normal blood pressure, ages between 25 to 55 yrs. The mean ± SEM of NO in the patients with Essential Hypertension was 4.0 +/-1.7 μM and that of controls was 6.7 +/- 3.2 μM. The NO in patients with Essential Hypertension was 42% less than that of the controls. The difference is highly significant \( (p<0.001) \).17

It has been seen that SBP and DBP inversely correlated with plasma nitrite owing to the decline of antioxidative activity (lipid peroxidation enhanced by the lack of antioxidant activities) which was associated with decreased NO production and the severity of hypertension.

In the present study it was found Serum Nitrite levels of Primary Hypertension patients were lower when compared with the controls, these difference were found to be statistically significant \( (t =12.212, \ P=0.0000) \)
The plasma concentration of nitrogen oxide in systemic venous blood is determined by synthesis, degradation, and clearance of NO. Daily activity and the consumption of food or water may also affect nitrogen oxide concentration. As for the synthesis of NO, NO is continuously synthesized from L-arginine in a reaction catalyzed by NO synthase, with most NO present in the circulation originating from endothelial and smooth muscle cells. Hypertension can produce 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. Decreased synthesis of NO might also result from abnormal handling of intracellular calcium and a consequent reduction in the activity of NO synthase. Hypertension impairs endothelium-dependent dilation of rat coronary arteries as a result of superoxide anion-mediated degradation of NO. Indeed, increased production of superoxide anions, which rapidly deactivate NO, is a characteristic feature of experimental models of hypertension, and plasma indexes of lipid peroxidation are increased in patients with hypertension.16

Potential mechanisms of decreased NO activity in essential hypertension:

1. Decreased availability of the substrate L-arginine;
2. Antagonism of NO biosynthesis (e.g. by increased levels of asymmetric dimethylarginine [ADMA], reduced dimethylarginine dimethylaminohydrolase [DDAH]);
3. Decreased availability of cofactors (e.g. reduced generation of tetrahydrobiopterin [BH4]);
4. Decreased activity of eNOS (e.g. reduced expression; decreased stimulation; phosphorylation, myristoylation, palmitoylation); and
5. Increased degradation of NO. 18

REFERENCES:
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