Research Article

Status of serum nitrate levels in type II diabetic patients with hypertension

Zaheera Sultana S[1*], Lakshmi T[2], Abdul Shabeer[3], Mohammed Zaheer Pasha[4]

[1,2]Assistant Professor Department of Physiology, Chamrajnagar Institute of Medical Sciences, Chamrajnagar, Karnataka, INDIA.
[3]Postgraduate, Department of Anaesthesia, Vijayanagar institute of Medical sciences,Bellary, Karnataka, INDIA.
[4]Postgraduate,Department of Anaesthesia, SDM College of Medical sciences and Hospital, Dharward, Karnataka, INDIA.
Email: dranjumshaik786@gmail.com

Abstract

Background and objectives: Diabetes mellitus is a long term disease with variable clinical manifestation and progression. The prevalence of diabetes mellitus in India was found to be 2.4 % in rural and 4 - 11.6 % in urban. It leads to number of complications like cardiovascular, renal, neurological, ocular and others such as intercurrent infections. Therefore understanding the pathophysiology of hypertension and diabetes is important.Hence this study is taken up to estimate Serum Nitrate levels in Type II Diabetic Patients with Hypertension 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 Type II Diabetic Patients with Hypertension (35) between 35 to 65 yrs age and Thirty nine (39) healthy individuals, controls between 38 yrs to 65 yrs age visiting Al-Ameen Medical College Hospital, Bijapur and District Hospital Bijapur were selected. Serum Nitrate was estimated by GRIESS METHOD. Statistical analysis was done by ANOVA and unpaired t test. **Results:** Statistically significant variations were found in parameters like age, Ht, Wt, BSA, BMI, PR, SBP, DBP, Serum Nitrate levels in controls and Primary Hypertension patients. **Interpretation and conclusion:** In the present study the mean ± SEM of Serum Nitrate in controls was found to be 49.93 ± 1.01 µ mol / lt, and Type II Diabetic patients with Hypertension was 29.36 ± 1.29 µ mol / lt. It was found Serum Nitrate levels of Type II Diabetic patients with Hypertension was lower when compared with the controls, these difference were found to be statistically significant (t =15.324, p= 0.0000). Mechanism which can reduce NO levels in Diabetic patients with Hypertension are decreased eNOS activity and thus reduces the bioavailability of NO and also increased oxidative stress. ADMA is an endogenous inhibitor of eNOS thus elevated ADMA will further decrease the bioavailability of NO. Elevated CRP has been reported to decrease the stability of mRNA for eNOS, thus further reducing the synthesis of NO.

Key Word: Type II Diabetic Patients with Hypertension, Nitric oxide, Nitrate.

[*]**Address for Correspondence:**
Dr. Zaheera Sultana S, Assistant Professor Department of Physiology, Chamrajnagar Institute of Medical Sciences, Chamrajnagar, Karnataka, INDIA.
Email: dranjumshaik786@gmail.com
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INTRODUCTION

Diabetes mellitus is one of the most common endocrine disease in all populations and all age groups. It is a syndrome of disturbed intermediary metabolism caused by inadequate insulin secretion or impaired insulin action, or both.[1] Diabetes mellitus is a long term disease with variable clinical manifestation and progression. The prevalence of diabetes mellitus in India was found to be 2.4 % in rural and 4 - 11.6 % in urban. It leads to number of complications like cardiovascular, renal, neurological, ocular and others such as intercurrent infections. Therefore understanding the pathophysiology of hypertension and diabetes is important.[2] 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).[3] Vascular tone is intricately regulated by a number of vasorelaxant and contractile factors synthesized and released from endothelial cells: vasodilators (NO, prostacyclin, endothelium-derived hyperpolarizing factor, bradykinin, adrenomedullin, C-natriuretic peptide) and vasoconstrictors (endothelin-1, angiotensin-II, thromboxane A 2, prostaglandins, hydrogen peroxide (H 2 O 2), reactive oxygen species (ROS).[4] Endothelium-

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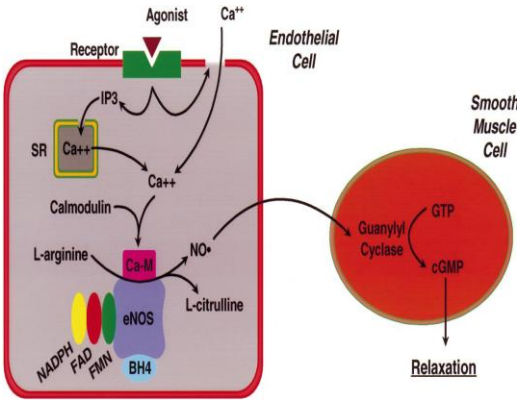
derived NO and other vasodilators oppose such vasoconstrictor effects, and thus act in a homeostatic fashion to maintain normal arterial patency and compliance despite local production of vasoconstrictors. When the endothelium is dysfunctional, the vasoconstrictor effects are unopposed and arterial tone is increased. In addition, pathological states are associated with increased endothelial production of endothelin-1 and other endothelium-derived vasoconstrictors that may further promote vasospasm and increase arterial stiffness.[3] 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.[5,6] Nitric oxide (NO), has a major influence on basal arteriolar tone and blood pressure. Interestingly, the contribution of NO to resting tone is greater in larger (>200 μm) than in smaller (resistance;< 200 μm) vessel**.** NO is formed continuously by endothelial NO synthase (eNOS) in the low nanomolar concentration range.[7,8] Elevated asymmetric dimethylarginine levels cause eNOS uncoupling, a mechanism which leads to decrease NO bioavailability. The endothelium dysfunction associated with diabetes has been attributed to lack of bioavailable NO due to reduced ability to synthesize NO from L – arginine.[9] Because the stimulation of NOS activity by insulin is impaired in muscle of type 2 diabetic patients, investigations on the response to the hormone of whole-body NO production in type 2 diabetes is of key relevance. An impaired NO generation in type 2 diabetes may be another feature of insulin resistance.[10]

Biosynthesis and Release of Nitric oxide



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. [11] 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 monocleotide), HEME and BH4 (Tetrahydrobiopterin). The activation of NO synthase depends on the intracellular calcium ions in the endothelium cells, is calmodulin dependent.[12, 11] When nitric oxide synthase (NOS) containing cells are provided with adequate stimuli, such as acetylcholine in endothelial cells or glutamate in neural cells, receptor activation leads to an increase influx of cytosolic calcium, When agonists activate the endothelial cells, an increase in inositol phosphate (IP3) may contribute to the increase in cytoplasmic Ca 2+ by releasing it from the sarcoplasmic reticulum (SR). Following interaction with calmodulin, Ca^{2+} activates nitric oxide synthase (NOS**)**. Thus NOS converts L- arginine to L – citruline and NO. [11, 13] NO reacts with heme moiety of a soluble guanylyl cyclise, which produces cyclic guanosine monophosphate (cGMP) from GTP.This reduces the levels of cytosolic calcium and also phosphorylates myosin light chain kinase causing relaxation**.**[14]

MATERIAL AND METHODS

The present study was conducted in the Department of Physiology, Al-Ameen Medical College, Bijapur and District Hospital, Bijapur. Thirty five (35) Type II Diabetic Patients with Hypertension (17 male, 18 female) between 45 yrs 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 Type II Diabetic Patients with Hypertension were studied. Type I diabetes mellitus were excluded from the study.The study protocol was explained to the Type II Diabetic Patients with Hypertension and Controls, who volunteered for the study. Informed consent was obtained from each of the participant.A detailed history of subjects was taken.

GRIESS METHOD - A) Materials

1) Griess reagent [Sulphanil amide, N-(1-Naphthyl) ethylene diamine dihydrochloride)]
2)Vanadium(III) Chloride: 8mg dissolved in DDW upto 1ml.
3)Sodium nitrite($NaNO_2$): 1mM $NaNo_2$/ L
4)Sodium nitrate ($NaNO_3$): 1mM $NaNO_3$ / L
5) Double Distilled Water (DW)
6) Ethanol

PROCEDURE

Blood (5 ml) for analysis was obtained from the antecubital vein of 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)
↓
500µl serum + 1000µl Ethanol (1ml)
↓
Vortexed well for 2 to 3 min
↓
Centrifuge (10000rpm for 10min)
↓
Take 0.5mL Supernatant

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.[15]

Sl no	Reagent	Test	Blank	
1	Supernatant	500µl	-	
2	DDW	-	500µl	
3	VCl3	500µl	500µl	INCUBATION FOR 30 MIN AT 37 ^{0}C
4	Greiss Reagent (Sulphanil amide + NED)	500µl (250µl+250µl)	500µl (250µl+250µl)	

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 nitrate. By taking OD of the serum sample SERUM NITRATE are calculated by using the following formula from the standard curve. [16]

$$NITRATE = \frac{OD + 0.0076}{0.0058}$$

RESULTS

Thirty five (35) Type II Diabetic Patients with Hypertension (17 male, 18 female) between 45 yrs to 65 yrs age were selected for the study. Thirty nine healthy individuals, controls between 38 to 65 yrs age from Bijapur city were the volunteers. All Type II Diabetic Patients with Hypertension underwent history taking and a thorough clinical examination. The ANOVA was used to analyse the variation in the parameters of controls and Type II Diabetic Patients with Hypertension. $P < 0.05$ was considered as a level of significant in all the statistics tests.. The unpaired ' t ' test was used to analyse the variation in FBS and PPBS of controls and Type II Diabetic patients with Hypertension.

Table 1: Mean ± SEM of Age, Ht, Wt, BMI, BSA in controls and Type II Diabetic patients with Hypertension

Parameter	Controls (n = 39) Mean±SEM	Type II Diabetic patients with Hypertension.(n = 35)MEAN±SEM	One way ANOVA
Age (yrs)	53.69 ± 1.69	54.94 ± 1.31	P =0.4944 (NS)
Ht (cms)	154.38± 0.905	159 ± 1.43	P =0.0209 (S)
Wt (kgs)	51.51 ± 1.01	66.23 ± 0.238	P = 0.0000 (S)
BMI (kg/m^2)	21.61 ± 0.174	26.24 ± 0.423	P= 0.0000 (S)
BSA (m^2)	1.48 ± 0.018	1.68 ± 0.011	P = 0.0000 (S)

Table 2: Mean ± SEM of PR, SBP, DBP, Serum Nitrate in controls and Type II Diabetic patients with Hypertension

SL No	Parameter	Controls	Type II Diabetic patients with Hypertension	One way ANOVA
		MEAN ±SEM	MEAN ±SEM	
1	PR (bpm)	74.63 ± 0.113	81.34 ± 0.835	P=0.0000 9 (S)
2	SBP (mmHg)	123.12 ± 1.58	161.65 ± 0.956	P= 0.0000 (S)
3	DBP (mmHg)	79.64 ±0.451	103.2 ± 1.19	P= 0.0000 (S)
4	Serum Nitrate (µmol / lt)	49.93 ±1.01	29.36 ± 1.29	P= 0.0000 (S)

Table 3: Shows the mean ± SEM of FBS, PPBS in controls and Type II Diabetic patients with Hypertension

SL No	Parameter	Controls	Type II Diabetic patients with Hypertension.	Unpaired ' t ' test
1	FBS (mg/dl)	87.15 ± 0.79	129.22 ± 0.597	P < 0.0001 (S)
2	PPBS (mg/dl)	125.64 ± 0.905	170.14 ± 2.51	P < 0.0001 (S)

A. Physical parameters

Table No. 1 shows the mean ± SEM of age, Ht, Wt, BSA, BMI in controls and Type II Diabetic patients with Hypertension. The height of Type II Diabetic patients with Hypertension.was found to be numerically more than the controls and was found to be statistically significant (t = — 2.362, p = 0.020) when compared with controls. The weight of Type II Diabetic patients with Hypertension was more than the controls and which were statistically significant (t = — 11.323, p = 0.0000). The mean value of BMI and BSA of Type II Diabetic patients with hypertension was more as compared to that of controls. There was a statistically significant difference (t= — 12.406, p = 0.0000) and (t = — 7.297, p = 0.0000) respectively.

B. Physiological parameters

Table No. 2 Shows the mean ± SEM of PR, SBP, DBP controls and Type II Diabetic patients with Hypertension.The PR of Type II Diabetic patients with Hypertension was found to be more compared to that of controls and which was significant statistically (t = — 10.084, p = 0.0000). The mean value of SBP levels of Type II Diabetic patients with Hypertension where higher when compared with the controls, this difference was found to be statistically significant (t = — 17.810, p = 0.0000). The mean value of DBP levels in Type II Diabetic patients with Hypertension where higher when compared with the controls, this difference was found to be statistically significant (t = — 20.379, p= 0.0000).

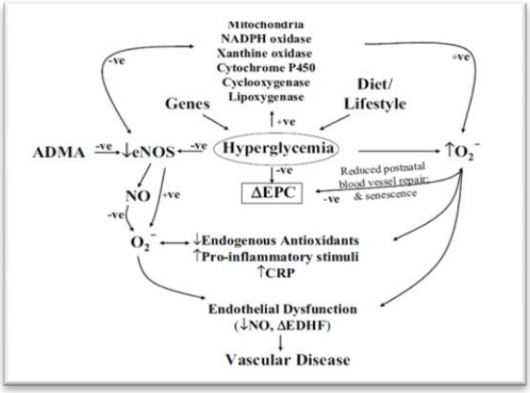
C. Biochemical Parameters

Table No. 2 Shows the mean ± SEM of Serum Nitrate in controls was found to be 49.93 ± 1.01 µ mol / lt, and Type II Diabetic patients with Hypertension was 29.36 ± 1.29 µ mol / lt. It was found Serum Nitrate levels of Type II Diabetic patients with Hypertension were lower when compared with the controls, these difference were found to be statistically significant (t =15.324, p= 0.0000). Table No. 3 Shows the mean ± SEM of FBS, PPBS in controls, and Type II Diabetic patients with Hypertension.The FBS level of Type II Diabetic patients with Hypertension was more when compared with the controls,this difference was found to be statistically significant (t = 41.711, p < 0.0001). It was found PPBS levels of Type II Diabetic patients with Hypertension was more when compared with the controls,this difference was found to be statistically significant (t = 17.386, p < 0.0001).

DISCUSSION

In the present study the mean ± SEM of Serum Nitrate in controls was found to be 49.93 ± 1.01 µ mol / lt, and Type II Diabetic patients with Hypertension was 29.36 ± 1.29 µ mol / lt. It was found Serum Nitrate levels of Type II Diabetic patients with Hypertension was lower when compared with the controls, these difference were found to be statistically significant (t =15.324, p= 0.0000). A study entitled ”Diabetes and Hypertension: Correlation Between Glycosylated Haemoglobin (HbA1c) and Serum Nitric Oxide (NO)” conducted by Dr. Syed Muhammad Shahid *et al*., in 2009 on patients which were divided into following groups (50 each) as follows Group I:Non-diabetic, normotensive control subjects, Group II: Diabetic, normotensive patients,Group III: Diabetic, hypertensive patients. Serum NO level was measured by measuring its metabolites (Nitrate+Nitrite) concentrations by spectrophotometric method. The mean ± SD of Serum NO in controls was 18.13 ± 2.65 µ mol / L, Diabetic normotensive patients was 17.51 ± 2.91 µ mol / L and that of Diabetic, hypertensive patients was 13.01 ± 2.49 µ mol / L. Serum NO was observed significantly low in diabetic Normotensive (p<0.01) and diabetic hypertensive patients (p<0.01) as compared to control.[17] In the study entitled ”Serum nitric oxide Status in patients with type 2 diabetes mellitus in Sikkim” conducted by Amrita Ghosh *et al*., in 2011 on 50 type 2 diabetes and 100 matched non diabetic controls. The mean ± SD of Serum level of NO in type 2 diabetes mellitus patients was 43.83 ± 11.3 µmoles / L and in the controls was 58.85 ± 12.8 µmoles / L. The serum NO was lower in type 2 diabetes mellitus patients compared with the controls, this difference was statistically significant (p< 0.0001).[18] Mikiwa KAWAKATSU, Tadashi ISHIHARA *et al* conducted a study entitled ”Plasma Nitrate/Nitrite Concentration in Healthy Population and Patients with Diabetes Mellitus - Relationships with Gender, Aging and Diabetic Complications” in 2002 on total number of subjects was 1,027: 738 consecutive, healthy volunteers (512 males and 226 females) without any disease, and 289 diabetic patients (177 males and 112 females). The mean ± SD of plasma Nitric oxide of Diabetic Hypertension patients was 45.6 ± 23 µ M / L and that of controls was 51 ± 29 µ M / L.[19] Suvarna Prasad, Ajay Kumar Sinha conducted study entitled ”Free radical activity in hypertensive type 2 diabetic patients” in 2010 on 50 type 2 diabetic patients. 19 of these type 2 diabetic patients had subsequently developed hypertension and remaining 31 type 2 diabetic patients were without Hypertension are controls. The mean ± SD of serum NO in Diabetic patients with Hypertension was 21.39 ± 4.79 u / dl and that of Diabetic patients without Hypertension was 18.54 ± 4.34 u / dl. [20]

Potential mechanisms of decreased NO activity in Type II Diabetic patients with Hypertension



Hyperglycaemia decreases endothelial nitric oxide synthase (eNOS) activity and thus reduces the bioavailability of nitric oxide (NO) and also increases oxidative stress (enhanced production of •O2 -) via, potentially, a multitude of pathways that may include the involvement of mitochondria, NADPH oxidase, xanthine

oxidase, cytochrome P450, cyclooxygenase, and lipoxygenase. Oxidative stress also further reduces the bioavailability of NO, thus reducing the inhibitory actions of NO on cytochrome P450 and other enzymes that are involved in the elevation of •O2 -.Hyperglycaemia may have direct or indirect effects to decrease the numbers and/or viability of endothelial progenitor cells (EPCs) and thus reduce postnatal blood vessel repair and enhance the rate of endothelial cell senescence. Asymmetric dimethylmethylarginine (ADMA) is an endogenous inhibitor of eNOS, and the metabolism of ADMA is decreased when oxidative stress is high and thus elevated ADMA will further decrease the bioavailability of NO. Reduced availability of NO, elevated oxidative stress, and reduced levels of endogenous antioxidants add to the proinflammatory stimuli, and plasma and possibly vascular levels of C-reactive protein (CRP) are elevated. CRP has been reported to decrease the stability of mRNA for eNOS, thus further reducing the synthesis of NO. Endothelial dysfunction results from the reduction in the local production/bioavailability of NO and, possibly, changes in the contribution of endothelium-derived hyperpolarizing factor (EDHF) to the regulation of vascular tone.[21]

CONCLUSION

we observed levels of serum Nitrate in Type II Diabetic patients with hypertension were lower compared with controls. Mechanisms which can reduce NO levels in Diabetic patients with Hypertension are decreased eNOS activity and thus reduces the bioavailability of NO and also increased oxidative stress. ADMA is an endogenous inhibitor of eNOS thus elevated ADMA will further decrease the bioavailability of NO. Elevated CRP has been reported to decrease the stability of mRNA for eNOS, thus further reducing the synthesis of NO.

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