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NITRIC OXIDE AS AN OBJECTIVE MARKER OF OXIDATIVE STRESS MEDIATED ENDOTHELIAL DYSFUNCTION IN NEPHROPATHY: A STUDY ON TYPE 2 DIABETES MELLITUS WITH REFERENCE TO INSULIN RESISTANCE, URINARY ALBUMIN CREATININE RATIO AND E GFR

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Abstract

Background: Thenexus among urine protein and serum nitric oxide, insulin resistance has beenevaluated in type 2 diabetics by earlier workers. However, we wanted to elicit the value of nitric oxide as a solitary, reliable marker of oxidative stress mediated endothelial dysfunction in nephropathy.

Methods: Thestudy subjects (n=150 total; both genders) were those who had visited the clinics of a tertiary health care set up in South India.The groups were segregated: fifty patients with normoalbuminuria (<30 mg/g creatinine), and an equal number of patients each with microalbuminuria (30–299 mg/g creatinine), macroalbuminuria (≥300 mg/g creatinine). Fifty healthy age, gender matched subjects constituted the control group.The duration of T2DM in the experimental group was greater than five years.

Results: In comparison to the control group, the magnitude of glycated haemoglobin (HbA1c), insulin resistance and nitric oxide ($p<0.05$) were statistically significant in the study groups, as a function of albuminuria.

Conclusion: It was concluded based onthe correlation analysis of Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), glycated hemoglobin (HbA1c), fasting plasma glucose (FPG) and Postprandial blood glucose (PPBS) with nephropathy markers, namely urinary albumin creatinine ratio (UACR) and eGFR that nitric oxide could serve as a solitary, reliable and objective marker of endothelial dysfunction in oxidative stress related nephropathy. Since insulin resistance and serum nitric oxide are found to be closely related to diabetic nephropathy, they could serve as reliable reference indices for evaluating diabetic nephropathy.

Key words:HOMA-IR, HOMA –β, QUICKI, eGFR, diabetic nephropathy.

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Introduction

Clinically obvious nephropathy affects nearly one third of type 2 diabetes mellitus patients[1].Hyperglycemia associated with insulin resistance is considered a pre disposing factor to renal injury and eventually kidney failure[2].The risk is dependent on several variables that includes oxidative stress, antioxidant status, inflammation and most significantly the association with endothelial dysfunction[3].

Renal pathology is progressive, but develops as a result of pronounced oxidative stress and inflammation[4].Several key biochemical players, namely NADPH oxidase, xanthine oxidase, endothelial nitric oxide synthase, mitochondrial electron transfer machinery, besides other miscellaneous biochemical events are implicated in the genesis of reactive oxygen species in the vascular system[5,6].Of these, the role played by endothelial nitric oxide synthase (eNOS) is considered cardinal in the generation of reactive oxygen species (ROS) and Reactive Nitrogen species (RNS). This event is triggered by cytokines and stress[7-10].It is believed that chronic inflammation makes its advent necessarily as a result of characteristic biological reactionin response to vascular endothelial dysfunction[11]. It is in the fitness of things to state that Nitric oxide might play a huge role in endothelial dysfunction, culminating in diabetic nephropathy, according to several lines of evidences[12].The present study is a sincere attempt to determine as to whetheralbuminuria in T2DM patients and nitric oxide areassociated with each other in the light of insulin resistance and eGFR that could open newer vistas in the prediction of the clinical course of nephropathy after endothelial dysfunction triggered by oxidative stress. Furthermore, till date Malondialdehyde (MDA)which is used as a biochemical marker of oxidative stress is hampered by problems associated with specificity in assays (TBARS). Hence, we wanted to evaluate the utility of nitric oxide as a single, reliable, and objective marker of diabetic nephropathy in insulin resistant type 2 diabetics, with reference to oxidative stress mediated endothelial dysfunctionculminating in diabetic nephropathy.

Methodology

Subjects and Methods

150subjects were enrolled for the present study. The subjects were type 2 diabetics of both genders with a documented history of T2DMfor five years or more and between the ages of 35 and 50. The study subjects received standard care and were maintained on oral hypoglycemic agents. Due approval from the Institutional Human Ethics Committee (at the first author's place of employment following the recommendations of the Research Advisory Committee of the institute where he had registered as a doctoral candidate) was obtained and the study was carried out in compliance with the guidelines laid down in the Helsinki declaration of 1975.

The groups were divided as follows: fifty patients with normoalbuminuria (<30 mg/g creatinine), and an equal number of patients with microalbuminuria (30–299 mg/g creatinine), and macroalbuminuria (≥300 mg/g creatinine). Patients receiving insulin as well as such of those subjects who had a history of tobacco and alcohol consumption were not included in the study.

Patients with a known history of urinary tract infections, malabsorption syndrome, inflammatory disorders, malignancy,and patients with other endocrine, metabolic and organ dysfunction were excluded. The controls consisted of fifty healthy volunteers of similar age and gender.

Control and Test groups

Group-1 - Fifty healthy age, gender matched subjects (controls)

Group-2 - Fifty T2DM patients with normoalbuminuria (<30 mg/g creatinine)

Group-3 - Fifty T2DM with microalbuminuria (30–299 mg/g

creatinine)

Group-4 -Fifty T2DM with macroalbuminuria (≥300 mg/g creatinine)

Biochemical analysis: Following an overnight fast, venous blood samples were withdrawn from the antecubital vein. Care was taken to ensure that the subjects rested in a well-ventilated collection area, maintained at a temperature of 22°C to 24°C, for a minimum period of fifteen minutes. The study subjects were explained about the little discomfort associated with the withdrawing of blood samples. Informed consent was elicited from all the study subjects.

For Nitric Oxide estimation,the blood was placed immediately in an ice bath and centrifuged (within 30 seconds). The centrifugation was carried out for a period 5 minutes at 2000g. One ml of Glycine-NaOH buffer (pH=9.7),one ml of protein free filtrate, two 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 were added in order to reduce the nitrate to nitrite. To two ml of supernatant oneml of Griess reagent A (consisting of 0.1% N-(1-Naphthyl) ethylenediamine Dihydrochloride in distilled water) was added. Following this, one ml of Griess Reagent – B (consisting of 1% sulfanilamide in 5% H3PO4,) was added to the solution. The solution was incubated for 20 minutes and the absorbance maximum was monitored at 540 nm [13]so as 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 prior to and following sample measurement[14,15].Fasting blood glucose (FBS) was measured promptly using an autoanalyzer.

Pertaining to the estimation of microalbumin and creatinine, first-morning urine samples were collected in sterile containers. Glucose (FBS) was estimated by enzymatic method using Glucose Oxidase-Peroxidase. Glycated Hemoglobin (HbA1C)was measured by immunoturbidimetry. Microalbumin was by turbilatex method and urinary creatinine was estimated by Jaffe's kinetic method. The biochemical analyses were performed on ERBA EM-200 fully automated analyzer, whereas fasting insulin were estimated using ELISA kits supplied by M/S Diametra, Spello, Italy. LISA SCAN (ERBA) ELISA reader was employed for the purpose of enabling estimations of analytes.

Insulin Resistance

Homeostatic Model Assessment for Insulin Resistance (HOMA-IR):

HOMA -IR, a surrogate for insulin resistance was calculated based on the fasting glucose and insulin values [16,17]

HOMA – IR = Fasting insulin (µU/ml) X fasting glucose (mg/dl)/405

HOMA –BETA

HOMA-beta=(360 × fasting insulin (µU/mL))/(fasting glucose (mg/dl)-63).Glucosein mg/dl, Insulin (Fasting) in µU/ml [18,19]

Quantitative Insulin-Sensitivity Check Index(QUICKI)

It wascomputed as per the formula put forth by**Katz et al**[20]

QUICKI = 1/[log(Fasting Insulin in µU/ml) + log(Fasting Glucose in mg/dl)]

eGFR

It was calculated in compliance with CKD-EPI (Chronic Kidney Disease Epidemiology) formula[21]

Table 1-Computation of eGFR

	Serum Creatinine (mg/dl)	**eGFR**
Females	**≤0.7**	**144 x $(Scr/0.7)^{-329}$ x $(0.993)^{age}$**
	>0.7	**144 x $(Scr/0.7)^{-1.209}$ x $(0.993)^{age}$**
Males	**≤0.9**	**141 x $(Scr/0.9)^{-0.411}$ x $(0.993)^{age}$**
	>0.9	**141 x $(Scr/0.9)^{-1.209}$ x $(0.993)^{age}$**

Statistical analysiswas carried out by SPSS software, version-22.

Results

Table-2depicts the findings pertaining to mean and standard deviation of the different groups in terms of the biochemical parameters.

Table 2: Groups mean and standard deviation

S.No.	**Parameters**	**Control (n=50) Group- 1**		**Normoalbuminuric T2 DM (n=50) Group-2**		**Macroalbuminuric T2 DM (n=50) Group 3**		**Macroalbuminuric T2 DM (n=50) Group-4**	
		Mean	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
1.	ACR (Albumin in urine mg/gm of creatinine)	19.1	1.94	23.27	3.4	132.51	38.57	430.08	55.4
2.	FBS mg/dl	83.96	5.36	135.12	16.29	149.5	27.98	252.4	33
3.	HbA1c%	5.76	0.55	7.75	0.84	8.45	0.99	9.53	0.49
4.	Insulin(µU/ml)	6.66	0.58	10.67	2.65	15.3	3.18	18	2.27
5.	HOMA-IR	1.38	0.14	3.59	1.07	5.69	1.68	11.25	2.13
6.	HOMA –β%	122.62	35.26	55.69	18.8	46.35	17.88	35.39	8.17
7.	QUICKI	0.36	0.01	0.32	0.01	0.3	0.02	0.27	0.01
8.	eGFR(CKD–EPI Formula)	116.01	16.68	108.98	29.69	85.9	24.11	79.38	21.79
9..	Nitric oxide(µmol/L)	33.36	3.24	42.64	6.14	45.9	7.03	58.56	10.82

ACR-Albumin Creatinine ratio; FBS-Fasting blood sugar(glucose); HbA1c- Glycated Hemoglobin; HOMA-IR- Homeostatic Model Assessment for Insulin Resistance; HOMA-β- Homeostatic model assessment – Beta; QUICKI-Quantitative Insulin-Sensitivity Check Index; eGFR- Estimated Glomerular Filtration Rate

Table 3: Correlation among Nitric oxide and other biochemical parameters(ACR, HOMA-IR,HOMA-β, QUICKI, eGFR)

Parameter	Correlation Coefficient(r)
ACR	0.718**
HOMA-IR	0.751**
HOMA-β	-0.585**
QUICKI	-0.774**
eGFR	-0.466**

ACR-Albumin Creatinine ratio; **HOMA-IR**- Homeostatic Model Assessment for Insulin Resistance; **HOMA-β**- Homeostatic model assessment – Beta; **QUICKI**-Quantitative Insulin-Sensitivity Check Index; eGFR- Estimated Glomerular Filtration Rate
**Correlation is significant at the 0.01 level (2-tailed).

Scatter plots :

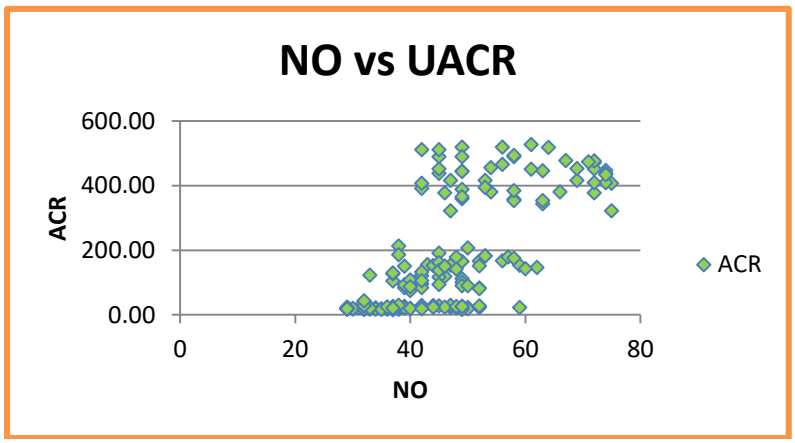

Fig. 1: Scatter plot of NO Vs UACR

If the variables between NO and UACR, the scatters follows increasing trend, so the association is positive

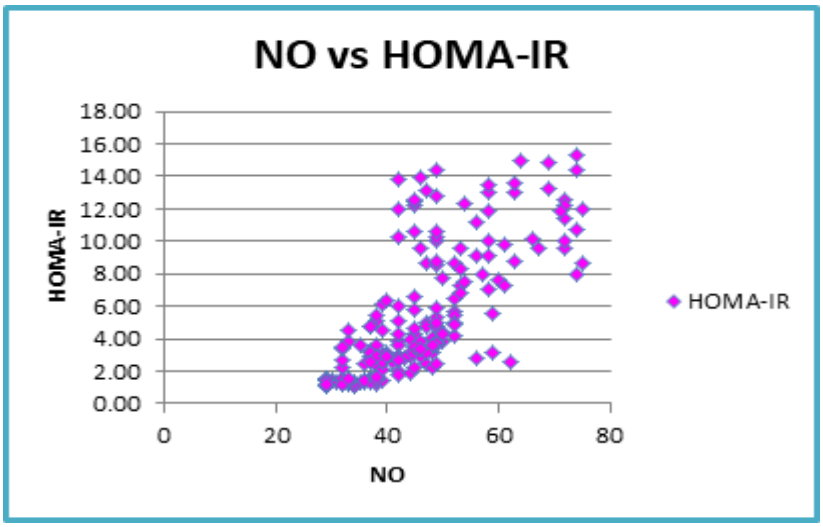

Fig. 2: Scatter plot of NO Vs HOMA-IR

If the variables between NO and HOMA-IR, the scatters follows increasing trend, so the association is positive

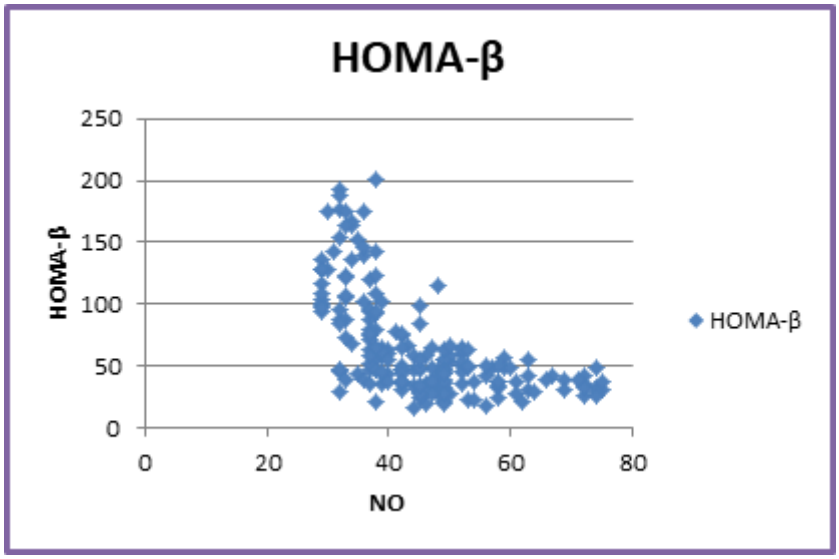

Fig. 3: Scatter plot of NO Vs **HOMA-β**

If the variables between NO and HOMA-β, the scatters follows decreasing trend, so the association is negative

The findings in Tables (3,4) and Figure (1,2,3) which demonstrate the diagnostic importance of nitric oxide in the context of imminent nephropathy, make clear that nitric oxide and albumin/creatinine ratio have a substantial correlation. This investigation on type 2 diabetes mellitus with insulin resistance found this correlation. According to the surrogate indicators, there was also a significant correlation between Nitric oxide, insulin sensitivity, and resistance

Discussion

Extracellular matrix proteins and local, systemic inflammation are related to the pathogenesis of diabetic nephropathy.Oxidative stress is a key player in nephropathy[22,23].Since humans are aerobic in nature, the tendency to oxidative stress mediated reactions is spontaneous and robust. The term "ROS" refers to the chemical species generated as a result of the incomplete reduction of oxygen. It comprises hydrogen peroxide (H2O2), hydroxyl radicals (HO•), and superoxide anions (O2-). RNS, on the other hand, stands for reactive nitrogen species, which encompasses nitroxyl (NO-), S-nitrosothiol (RSNO), and peroxynitrite (OONO-), which denote oxidation states and reactive adducts of nitrogenous nitric oxide synthase (NOS).

It is imperative that high glucose environmentprompts ROS. This has a direct bearing on then complications. The enhanced synthesis of RNSaugments oxidative stress. It is strongly felt that metabolic changes in target tissue molecules, and oxidative stress acquire relevance in hyperglycemia (insulin resistance)associated with microvascular complications of T2DM.A study from South India cited the Griess reaction with reference to the comparison of basal serum levels of nitric oxide in T2DM as linked to the various stages of diabetic nephropathy. Our study has revealed that serum nitric oxide levels were noticeably greater in T2DM than in non-diabetics. In diabetic patients, nitric oxide was pronouncedly linked with levels of proteinuria, particularly macroalbuminuria and microalbuminuria. Nitric oxide levels were found to be greater in obese patients

than controls in an Iranian study comparing them to healthy controls. Adipose tissue inflammation had an impact on higher nitric oxide levels[24]. Urine nitric oxide levels were higher in micro- and normo-albuminuric type 2 diabetics than controls in early diabetes, according to Turkish research of healthy controls and those with micro- and normo-albuminuria[25].According to a Japanese study, when plasma NOx levels were assessed, the levelsin the diabetics were substantially greater than those of controls[26].In a study undertaken in Pakistan, the levels of nitric oxide metabolites including nitroxyl (NO-), S-nitrosovthiol (RSNO), and peroxynitrite (OONO-) were found to be higher in diabetic patients compared to non-diabetics, but significantly higher in diabetic patients with hypertension compared to controls. However, levels were not significantly different between patients with and without hypertension[27].In T2DM, pro-inflammatory cytokines are found to be responsible for inducing nitric oxide generation[28].The purported mechanism to increase serum Nitric oxide level in T2DM was hyperglycemia driven and endothelial dysfunction through increased oxidative stress is a hallmark of diabetics. Hyperglycemia accelerates the formation of advanced glycosylated end products, boosts the polyol pathway, and activates several cellular machinery including protein kinase C, resulting in oxidative stress induced Nitric oxide production.

Conclusion

Our research corresponds to a basic attempt to comprehend as to how insulin resistance and nitric oxide relate to diabetic nephropathy, with reference to proteinuria. Nitric oxide (NO) levels were perceptibly pronounced in T2DM. The most important finding is that NO correlates positively with Albumin Creatinine ratio and HOMA-IR, the surrogate marker of insulin resistance.

Significance of the study

It is becoming increasingly relevant that several clinical conditions are characterized by subtle as well as pronounced aberrations in nitric oxide (NO). These changes are synonymous either with altered production of NO or less than optimal signaling process and subsequent effects. In the light of the above mentioned points, it is imperative to design pharmacologic preparations to regulate NO balance. lt is worthy to mention that select targets for the development of therapeutic modalities are presently available for pulmonary, cardiovascular diseases and neoplastic conditions. However, our present study has provided an insight into the suggested role of drugs that could modulate NO production and action with reference to the management of diabetic nephropathy(DN). This would usher in a fresh approach to treat DN, besides expanding the horizons in the pharma sector and clinical pharmacology. In addition, promulgation of the relatively safe use of NO in therapeutics might open newer vistas in confronting the perils of DN.

Novelty of the study

The present study acquires special significance for justifying the inclusion of nitric oxide as a single, reliable marker of oxidative stress mediated endothelial dysfunction in diabetic nephropathy, in the light of the following facts:-

1). With reference to the usual measurement of Malondialdehyde (MDA) as a product of oxidative stress mediated lipid peroxidation, the non-specificity of Thiobarbituric acid Reactive Substances(TBARS) assay reactivity on MDA and production of MDA from reactions other than lipid peroxidation continues to be a major hampering factor.Furthermore, the low stability of MDA is attributed to its enhanced tendency for reacting with other biomolecules besides rapid enzymatic degradation.

2). Our study has convincingly demonstrated that Nitric oxide can be considered as a single reliable marker of endothelial dysfunction and could replace MDA in monitoring oxidative stress related endothelial dysfunction in insulin resistant T2DM.

Limitations of thestudy*:* The study did not include other conventional markers of oxidative stress in type 2 Diabetes mellitus.

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Conflict of interest: The authors declare that the present study was not funded by any agency and is not bound by any conflict of interest whatsoever.

Ethics approval

The study was begun only following the approval of the Research Advisory Committee at the institute where the first author had registered himself as a doctoral candidate and also essentially following clearance that was duly obtained from the Institutional Human Ethics Committee (IHEC),with reference to the workplace of the first author.

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