

Research article

3-Nitrotyrosine (NT) levels in serum and its association with insulin resistance in patients with type 2 diabetes mellitus: Biomarker role of NT in the assessment of oxidative stress mediated impending vascular complications in nephropathyMeka Farid Babu¹, Srinivasan A.R.², Benerji G.V.¹¹Department of Biochemistry, Konaseema Institute of Medical Sciences and Research Foundation, Amalapuram, 533 201, East Godavari district, Andhra Pradesh, India²Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry 607 402, India

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ABSTRACT

Introduction and Aim: 3-Nitrotyrosine (NT) has been recognized as a marker of oxidative stress in diabetes mellitus. NT has also been studied in diverse metabolic conditions. The aim of our study was oriented towards the role of NT as a predictor of oxidative stress mediated impending nephropathy in diabetes mellitus and that with reference to albuminuria.

Materials and Methods: A total of 150 type 2 diabetics in the age group 35 - 50 years were enrolled as three groups, comprising 50 each, based on albuminuria. 50 healthy age and gender matched subjects constituted the control group. Serum NT and Insulin were assessed by ELISA. HbA1c was quantitated by immunoturbidimetric method and microalbumin was assessed by turbidatex method. Routine biochemistry was enabled through ERBA EM-200 fully automated analyzer. Stringent quality control was affected. The study was begun following approval accorded by the competent committees.

Results: NT levels were positively correlated with albumin-creatinine ratio and insulin resistance. NT could be used as a predictor of impending vascular complications in diabetic nephropathy.

Conclusion: NT levels could act as a predictor of oxidative stress mediated diabetic nephropathy in the light of albuminuria.

Keywords: 3-Nitrotyrosine (NT); Type 2 diabetes mellitus (T2DM); Glycemic control; Insulin resistance (IR); HOMA-IR; nephropathy.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is an endocrine disorder which is presently experiencing an alarming increase in developing countries. The prevalence of T2DM in developed countries is 1.2%, whereas in developing countries, the prevalence is believed to be fourfold higher (1,2). The aggressive multipronged approach would result in an attenuated risk for microangiopathy, coronary events and mortality. However, the incidence of end stage renal disease (ESRD) is progressively on the rise globally. To evaluate the progression of diabetic nephropathy, monitoring of albuminuria and glomerular filtration rate (GFR) are appropriately indicated by the KDIGO: Kidney Disease Improving Global Outcomes CKD Work Group (3). Repeated monitoring of albuminuria is emphatically recommended in routine clinical practice in diabetes and pronounced decline in GFR in both type 1 and type 2 diabetes has been documented (4). However, it is practically difficult to predict the progression of diabetic nephropathy by quantitating albuminuria and eGFR (5,6). Precisely for this reason, the authors of the present paper had embarked on a

study to look for sensitive and specific biochemical marker for assessing impending vascular complications in diabetic nephropathy. Presently, very few reports with the aforesaid focus are available in the literature.

Acute hyperglycemia increases the levels of proinflammatory cytokines including tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), (7-9). Nitrotyrosine (NT) is indirectly inferred as a marker of oxidative stress because of the production of peroxynitrite, also called as peroxonitrite (ONOO⁻) during acute hyperglycemia (10). Experimental studies have also revealed that elevated NT is associated with coronary artery disease (CAD) (11-13). It is well documented that oxidative stress and the pathogenesis of insulin resistance through insulin signal inhibition and adipocytokines dysregulation are considered cardinal (14,15). However, very few reports are available from South India pertaining to our present attempt, namely, to look out for a biomarker, with reference to imminent nephropathy in type 2 diabetics. Hence, we decided to explore the NT levels in the serum of patients with T2DM and its association with microalbuminuria and insulin

resistance with a view to envisaging a biochemical predictor of impending vascular complications in diabetic nephropathy.

MATERIALS AND METHODS

A total of 150 patients of both genders with a diabetic history of five years or more and aged between 35-50 years were included. They were on a standard regimen comprising oral hypoglycemic drugs. The study was begun only following the approbation of the Research Advisory Committee at the institute following clearance from the Institutional Human Ethics Committee (IHEC) at the workplace of the first author.

The study was conducted in accordance with Helsinki declaration of 1975. Three groups were established, accordingly: 50 patients with normoalbuminuria (<30 mg/g creatinine), 50 patients with microalbuminuria (30–299 mg/g creatinine), and 50 patients with macroalbuminuria (≥ 300 mg/g creatinine). Care was taken to exclude the patients on insulin therapy. Smokers, tobacco chewers and alcoholics were not included. Patients with a known history of urinary tract infections and other kidney pathology and inflammatory disorders were excluded. Patients possessing neoplastic conditions, hepatic dysfunction, thyroid abnormality, acute myocardial infarction, cerebrovascular stroke, and peripheral vascular diseases were also excluded. Fifty healthy age, gender matched subjects denoted the controls.

Control and test groups

Group I: 50 healthy age and gender matched subjects (Control)

Group II: 50 patients with normoalbuminuria (<30 mg/g creatinine)

Group III: 50 patients with microalbuminuria (30–299 mg/g creatinine)

Group IV: 50 patients with macroalbuminuria (≥ 300 mg/g creatinine)

Biochemical analysis

Eight ml of blood samples were withdrawn from the antecubital vein of the subjects, following an overnight fast, under well-defined and aseptic conditions. Standard laboratory investigations were enabled immediately, and other aliquots were stored appropriately at -80°C until further quantitation of insulin and NT were undertaken.

Blood samples in the fed state were collected appropriately for estimating plasma glucose. Extreme care was exercised to collect the first(morning) urine samples in sterile container and these samples were utilized for the determination of microalbumin and creatinine.

Routine laboratory investigations including glucose, lipid profile were performed on ERBA EM-200 fully

automated analyzer. Glucose was estimated by an enzymatic method using Glucose Oxidase-Peroxidase. Cholesterol in serum was quantitated by the enzymatic method based on Cholesterol oxidase/ Peroxidase (CHOD/POD). The quantitation of triacylglycerols was based on Glycerol phosphate oxidase/ Peroxidase (GPO/POD) method. HDL cholesterol was estimated by direct enzymatic method and LDL cholesterol was computed by employing Friedwald formula. Glycated Hemoglobin (HbA1C) was measured by immunoturbidimetry. Microalbumin was quantitated by turbidimetric method, whereas fasting insulin and Nitrotyrosine were estimated using ELISA kits supplied by M/s Dia Metra, Spello, Italy and Sincere Biotech Ltd, Beijing, China respectively.

Homeostatic model assessment for insulin resistance (HOMA-IR), a surrogate marker of insulin resistance (IR) was computed, from the values pertaining to fasting glucose and insulin by employing the formula:

$$\text{HOMA} - \text{IR} = \frac{\text{Fasting venous plasma insulin (mIU/L)} \times \text{Fasting glucose (mM/L)}}{22.5}$$

Statistical analysis was carried out using SPSS software, version-22.

RESULTS

The data for NT and other biochemical parameters and their statistical significance when examined under the following scenario: Control vs normoalbuminuria; Control vs microalbuminuria; Control vs macroalbuminuria; Normoalbuminuria vs microalbuminuria; Normoalbuminuria vs macroalbuminuria; Microalbuminuria vs macroalbuminuria is depicted in Table 1.

Table 2 denotes the correlation among NT and ACR, FBS, PPBS, HbA1C, HOMA-IR, TAG, HDL cholesterol. From the results depicted in Table 2, it is conspicuous that NT correlates pronouncedly with ACR, thereby confirming the diagnostic significance of nitro-tyrosine in the light of impending nephropathy, as observed in this study on insulin resistant T2DM. Also, good correlation was observed between NT and insulin resistance, as measured through the surrogate marker, namely HOMA-IR.

ROC curves

ROC curves were elaborated based on the sensitivity and specificity with reference to the independent variable, namely NT of normoalbuminuria, microalbuminuria and macroalbuminuria groups as against HOMA – IR (insulin resistance), the dependent variable. We analyzed the data accordingly as Control vs. Normoalbuminuria (Fig.1), Control vs Microalbuminuria (Fig.2), and Control vs Macroalbuminuria (Fig.3). ROC curves' results clearly indicate that NT could be used as a predictor of impending vascular complications in diabetic nephropathy, in the light of albuminuria.

Table 1: Comparison of all groups by ANOVA for determining statistical significance of biochemical parameters

S.No.	Parameters	Comparison of Groups for obtaining 'p' values						All groups for the purpose of computing ANOVA
		I vs. II	I vs. III	I vs. IV	II vs. III	II vs. IV	III vs. IV	
1.	Microalbumin (mg/dl)	0.0038	0.0040	0.0045	0.0013	0.0019	0.0013	0.0029
2.	Urinary Creatinine(mg/dl)	0.0193	0.0071	0.0032	0.0049	0.0036	0.0076	0.0017
3.	ACR (mg/g of creatinine)	0.0074	0.0013	0.0087	0.0021	0.0010	0.0038	0.0025
4.	FBS (mg/dl)	0.0026	0.0046	0.0053	0.0023	0.0032	0.0027	0.0018
5.	PPBS (mg/dl)	0.0072	0.0078	0.0049	0.0067	0.0018	0.0011	0.0094
6.	HbA1c (%)	0.0080	0.0012	0.0078	0.0002	0.0043	0.0019	0.0033
7.	Insulin(μ Iu/ml)	0.0014	0.0035	0.0051	0.0048	0.0013	0.0041	0.0073
8.	HOMA-IR	0.0011	0.0020	0.0038	0.0075	0.0012	0.0013	0.0041
9.	Cholesterol (mg/dl)	0.0024	0.0094	0.0015	0.0443	0.0016	0.0094	0.0018
10.	TAG (mg/dl)	0.0066	0.0069	0.0013	0.0025	0.0026	0.0044	0.0015
11.	HDL (mg/dl)	0.0054	0.0093	0.0032	0.1477	0.0022	0.0037	0.0068
12.	LDL (mg/dl)	0.0037	0.0035	0.0034	0.1370	0.0001	0.0446	0.0010
13.	TAG/HDL ratio	0.0050	0.0028	0.0099	0.0018	0.0089	0.0013	0.0020
14.	NT (μ Mol/L)	0.0072	0.0036	0.0035	0.0048	0.0015	0.0070	0.0036

Table 2: Correlation data among NT and other biochemical parameters

Parameters	Correlation Coefficient(r)
ACR	0.587**
FBS	0.314*
PPBS	0.347*
HbA1C	0.325*
HOMA-IR	0.595**
Cholesterol	0.235
TAG	0.247
HDL	-0.227
LDL	0.173

ACR-Albumin- creatinine ratio FBS-Fasting blood sugar(glucose); PPBS-Post prandial blood sugar(glucose); HbA1c- Glycated hemoglobin; HOMA-IR- Homeostatic model assessment-Insulin resistance; TAG –Triacylglycerol; HDL-High Density Lipoprotein; LDL-Low Density Lipoprotein.

**Correlation significant at 0.01 level (2-tailed). *Correlation significant at 0.05 level (2-tailed)

DISCUSSION

Oxidative stress has been implicated in T2DM and this appears to signal the imminent vascular disease and other associated complications attributed to insulin resistance. The imbalance between the prooxidants and antioxidants in the human body, as exemplified by the magnitude of the state of oxidative stress culminates in cellular disruption and damage (16). The free-radicals which are highly reactive chemical species endowed with a lone electron in the outer orbital act on polyunsaturated fatty acids and other biomolecules including carbohydrates and proteins. Lipid peroxidation products include conjugated dienes and malondialdehyde (MDA) that are increased in

T2DM patients (17). In the current study, we observed that levels of Nitrotyrosine (NT), a product of free radical induced protein modification were significantly increased in T2DM subjects.

A pronounced difference was observed in macro and microalbuminuric diabetic patients in comparison to normoalbuminuric diabetic patients. Peroxynitrite, a chemical product formed by the reaction between superoxides and nitric oxide (NO) modifies tyrosine, an aromatic amino acid in proteins to form NT. NT is involved in the inactivation of mitochondrial and cytosolic proteins, synonymous with the damage inflicted on the various cellular constituents (18).

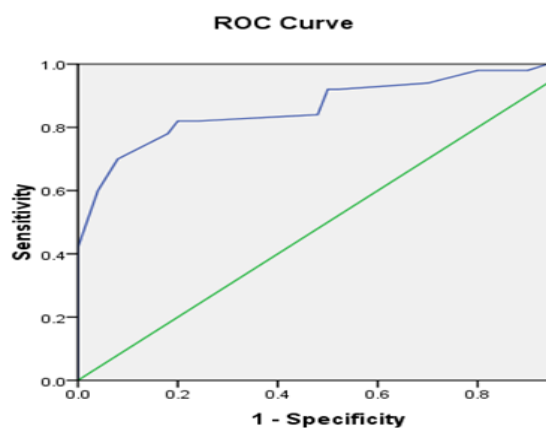


Fig.1: Control vs. Normoalbuminuria
Area Under Curve (AUC)=0.865; Confidence interval=0.793-0.938; Standard error=0.037; Sensitivity=800; specificity= 0.820; Cut-off minimum)=0.245

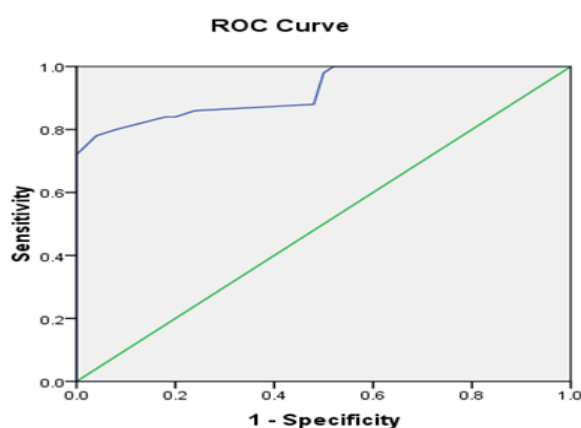


Fig.2: Control vs. Microalbuminuria
Area Under Curve (AUC) =0.922; Confidence interval =0.870 – 0.974; Standard Error=0.027; Sensitivity= 0.920; Specificity= 0.800; Cut-off (minimum)=0.265

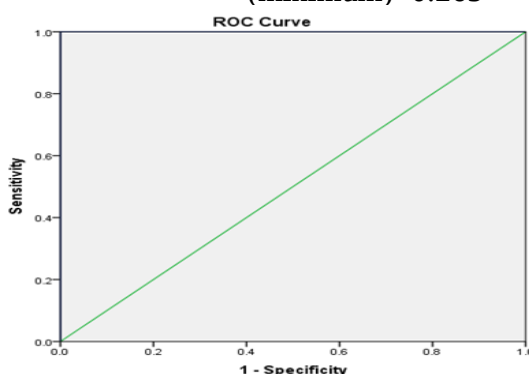


Fig.3: Control vs. Macroalbuminuria
Area Under Curve (AUC) =1-000; Confidence interval =1-000-1-000; Standard Error=0.000; Sensitivity= 1.000; Specificity= 1.000; Cut-off (minimum)=0.290

Studies have revealed that chronic hyperglycemia, dyslipidemia and oxidative stress lead to inflammation by aggravating insulin resistance (19). Furthermore, an enhancement in the expression of adipokines and macrophage inflammatory proteins are implicated in macrophage infiltration, as a manifest in the adipose tissue. This eventually leads to an exaggerated production of reactive oxygen species (ROS) and inflammatory cytokines (20). It might be of contemporary interest and relevance as well to note that adipose tissue dysfunction could culminate in adipocyte-specific deletion of a redox-sensitive transcription factor binding to the designated promoter region of defined genes whose gene products are antioxidant enzymes (21).

In our study, we had noticed pronouncedly high serum cholesterol, LDL and TAG in type 2 diabetics with reference to healthy controls. Hyperglycemia is pronouncedly synonymous with alterations in lipid metabolism which inflicts adverse effects typified by enhanced HDL clearance, decreased apoA-1 transcription and accelerated HDL glycation. These biochemical events predispose to atherosclerosis/arteriosclerosis (22). A positive association between NT and albumin creatinine ratio

(ACR), PPBS, glycemic control, insulin resistance, serum triacylglycerols and negative association with HDL -cholesterol were elicited from our study.

The results emerging from the ROC analysis reveal the biochemical utility of NT as a reliable predictor of impending vascular complications in nephropathy as observed in insulin resistant type 2 diabetics.

Oxidative stress continues to be the common nexus for the major biochemical pathways involved in the genesis and progression of diabetic micro- and macrovascular complications (23). Several events of cardinal significance are mediated through ROS. To mention a few: inflammation, extracellular matrix accumulation, endothelial dysfunction and abnormal angiogenesis (24). In these processes culminating in cellular injury, a plethora of other key players are involved that include molecules and transcription factors such as mitogen-activated protein kinases (MAPKs) (25). The highly labile and reactive species, namely ROS has already been implicated in renal injury. Such reactive species are associated with ischemia-reperfusion injury and diabetic nephropathy (26). Several studies undertaken during the past have also demonstrated that protein nitration is manifest in

tissues inflicted by atherosclerosis (27). But very few studies are there citing the utility of an objective biomarker in predicting future vascular complications in nephropathy associated with insulin resistance. Hence, our study strives to acquire significance in this context.

Nitrosative stress and peroxynitrite are not only implicated in diabetes-induced cardiovascular aberration, but also would contribute to the pathogenesis and progression of diabetic nephropathy. Accordingly, neutralization of peroxynitrite could afford a novel approach in the prevention and management of diabetic complications (28). Hence, it must be said that NT levels could be a novel biochemical predictor of nephropathy in type 2 diabetic patients, especially at a time when not many simple, economically viable, specific and sensitive predictors of insulin resistance induced diabetic nephropathy are not available in the armamentarium.

Novelty of the study

To the best of our knowledge and belief, reports are not available concerning the predictive capacity of a sensitive and specific biomarker such as Nitrotyrosine (NT) to cite impending vascular complications in diabetic nephropathy. Furthermore, our study has correlated NT levels with albumin creatinine ratio, an objective marker of diabetic nephropathy. Our study has revealed that no additional parameters of oxidative stress are needed other than NT to confirm the utility of a sensitive and specific biomarker of oxidative stress that would reveal impending vascular complications linked to diabetic nephropathy. Furthermore, our study would acquire relevance in pharmacogenomics and personalized medicine.

Limitations of the study

Sample size taken for the control and test groups was low.

CONCLUSION

The biochemical assessment of Nitrotyrosine could thus be useful to predict the progression of diabetic nephropathy as a biomarker. However, larger prospective studies are required to confirm our claim.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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