Serum Nitric Oxide as a Biochemical Marker for Diabetic Nephropathy

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ISSN: 2231-9123

ABSTRACT

The realization that diabetic nephropathy is associated with nitric oxide production, create a great incentive to study the level of nitric oxide in diabetic patients and to ascertain its relation with renal impairment. This study aimed to find out whether serum nitric oxide can be considered as a biochemical marker in diabetic nephropathy. A total of 240 subjects (age and sex matched) were enrolled in this study and divided into two groups; diabetic group included 120 diabetic patients (type 1 and type 2) and control group included 120 apparently healthy subjects. Blood samples were collected from all the individuals and laboratory measurements for HbA1c, serum glucose, urea, creatinine and nitric oxide were done. Urine samples were also collected for measurement of microalbuminuria. The patients were classified into three main groups on the basis of blood pressure and nephropathy. Group 1: Diabetic, normotensive patients; Group 2: Diabetic, hypertensive patients; Group 3: Diabetic, hypertensive patients with nephropathy then group 3 was divided into 3 subgroups according to the stage of diabetic nephropathy: Diabetic incipient nephropathy, diabetic overt nephropathy and diabetic with end stage renal disease. The mean value of serum nitric oxide level was significantly lower in diabetic patients when compared to controls **(**26.1±1.37 µmol/l) for diabetic normotensive patients, **(**15.2±0.97 µmol/l) for diabetic hypertensive patients, and (17.4±17.5 µmol/l for diabetic hypertensive patients with nephropathy versus 30.6±2.18 µmol/l for healthy controls). The mean serum nitric oxide (50.9±1.89 µmol/l) and glomerular filtration rate (132±13.9 ml/min), were significantly higher in diabetic patients with early nephropathy (Diabetic incipient nephropathy) than diabetic overt nephropathy (10.1±1.32µmol/l and 44.5±6.36 ml/min), and diabetic with end stage renal disease ((4.9±0.63 µmol/l and 10.5±3.16 ml/min). It is evident from the present study that serum nitric oxide is the major biochemical marker for microvascular complications of diabetes mellitus such as nephropathy. The data support the use of raised serum nitric oxide as a marker for early incipient diabetic nephropathy and a low serum nitric oxide for overt and end stage diabetic nephropathy. The decreases being proportionate to the degree of renal impairment.

Keywords: *Diabetic nephropathy, hypertension, nitric oxide*

1. Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action or both (Alberti and Zimmet. 2004). It is widely considered that persistent hyperglycemia is the primary causal factor in the development of

diabetic nephropathy (Sugimoto et al. 1999). Diabetic nephropathy is the leading cause of chronic renal disease in patients starting renal replacement therapy and affects approximately about 40% of type 1 and type 2 diabetic patients (Jorge et al. 2005). Diabetic nephropathy has been classically defined as increased protein excretion in urine. Early stage is characterized by a small increase in urinary albumin excretion, also called microalbuminuria or incipient diabetic nephropathy. More advanced disease is defined by the presence of macroalbuminuria or proteinuria and is classically named overt diabetic nephropathy (Zelmanovitz et al. 2009). Advanced glycation end products considered being one of the causes of endothelial dysfunction in diabetes and binding of these molecules to their cellular receptors will enhance oxidative stress (Yan et al. 1994). One of the main effects of oxidative stress is the decrease in the biological activity of nitric oxide and this effect is expressed through the endothelial dysfunction (Bonetti et al. 2003). Endothelium derived relaxing factor was identified as the free radical gas, Nitric oxide which is synthesized from the amino acid L-arginine, catalyzed by nitric oxide synthase (NOS) (Palmer and Ashton. 1988). Nitric oxide diffuses from the endothelium to the vascular smooth muscle cells, where it increases the concentration of cGMP by stimulating soluble guanylate cyclase leading to vascular relaxation (Moncada and Higgs. 1993). NO-dependant vasodilation has shown to be an important factor in the maintenance and regulation of vascular tone in the renal microcirculation. Glomerular arteriolar resistances are regulated by basal nitric oxide level and are supported by observations of vasoconstrictions in afferent and efferent arterioles of both superficial cortical and juxtamedullary nephrons following nitric oxide synthesis inhibition (Zats and Nussi. 1991). Altered nitric oxide levels have been described in diabetic mice with nephropathy, including increased nitric oxide expression in early diabetic nephropathy, followed by marked down regulations (Azakimori et al. 2001). On the other hand, advanced nephropathy leading to severe proteinuria, declining renal function and hypertension is associated with a state of progressive nitric oxide deficiency (Prabhakar, 2004). In Iraq, as well as in many other developing countries, the diabetic patients have a poorly or uncontrolled disease course and presented in many of them as a complicated manner. Furthermore, early detection of diabetic nephropathy is still a significant medical problem especially in our locality.

2. Materials and Methods

A total of 120 patients with type 1 and type 2 diabetes mellitus of either sex visiting Sulaimani Diabetes Center were selected randomly (every 3rd) and divided into three groups. The aim and procedures were explained both to the patients and controls and informed consent was obtained. The mean age of patients was 50.7±9.5 (mean±SD) years. Their diabetic age was more than 5 years. The diagnosis of diabetes mellitus was made according to the World Health Organization's (WHO) criteria (American Diabetic Association. 2011). Patients who were newly diagnosed (less than 5 years duration of diabetes mellitus), patients suffering from gestational diabetes, any known mental illness, macrovascular disease, chronic liver disease and urinary tract infections prior to diagnosis of diabetes mellitus, or patients who refused to participate in the study were excluded. The study protocol was approved by the ethical committee of the general director of health in Duhok governorate in collaboration

with that in Sulaimani for the use of human subjects in research. The groups of patients and control subjects were as follows:

Group 1: Diabetic, normotensive patients
Group 2: Diabetic, hypertensive patients
Group 3: Diabetic, hypertensive patients with nephropathy then group 3 was divided into 3 subgroups according to the stage of diabetic nephropathy: Diabetic incipient nephropathy
Diabetic overt nephropathy and
Diabetic with end stage renal disease

A structured questionnaire was used to record the demographic characteristics of all subjects. Height and weight were noted for the calculation of Body Mass Index [(BMI=weight in kilograms/height in meters) 2] (Buchholz and Bugaresti. 2005). Blood pressure was measured with the help of standard mercury sphygmomanometer while the patient was sitting after resting for 5-10 minutes. Hypertension was defined as blood pressure140/90 mm Hg (Ethel et al. 2011). The blood samples of patients and control subjects were collected after the patients have been overnight fasting for 12-14 hours. Blood samples for sera were collected in BD Vacutainer System CAT-plain (5 ml); and one ml was collected immediately into DMD-DISPO tubes containing K_3 EDTA as anticoagulant for estimation of HbA1c. Blood samples were processed same day for estimations, in accordance with the ethical guidance and regulation of institution and with generally accepted guidelines governing such work. The serum nitric oxide metabolites (nitrate+nitrite) were measured by routine spectrophotometric method (Sastry et al. 2002). The fasting blood glucose, serum urea and creatinine were measured by routine spectrophotometric methods. The HbA1c was measured by immunoassay technique method, using DCA™-Analyzer (Knowles et al. 1986). The glomerular filtration rates (GFR) were estimated by Cockcroft-Gault formula, which in turn estimates GFR in ml/min (Gault et al. 1992). Urine samples were collected in the morning (9-11 A.M), avoiding heavy physical activity for more than 2 hours before the test. Then, the sample was processed for measuring microalbuminuria by using (i-chroma) reader based on fluorescence immunoassay technology (Oh et al. 2005).

All data were analyzed using the Statistical Package for Social Sciences SPSS version 19 software for windows 7. Independent student t-test was used to compare differences between the different groups. Analysis of data was performed and expressed as (mean ± SD), quartile and ranges. Pearson correlation coefficient (r) was used for calculating the correlation of serum nitric oxide with glomerular filtration rate, microalbuminuria, HbA1c, serum sugar, creatinine, urea, duration of disease and age in all patients. Level of statistical significance was set at P value < 0.05.

3. Results

The tables 1-4 summarize the results in terms of mean±SD. In diabetic patients BMI, systolic and diastolic blood pressures levels were significantly higher ($p<0.05$), blood glucose, serum urea, serum creatinine, HbA1c and microalbuminuria levels were found to be significantly higher ($p<0.001$), while mean value of serum nitric oxide level and glomerular filtration rate were lower ($p<0.001$), as compared to control subjects (Table 1).The mean value of serum nitric oxide level, microalbuminuria and glomerular filtration rate were observed to be

significantly higher ($p<0.001$) in diabetic incipient nephropathy patients as compared to diabetic hypertensive patients, whereas the blood pressure was significantly lower ($p<0.001$).Although the mean serum urea and creatinine levels were within the reference range for both groups, but the difference was statistically significant ($p<0.01$) for both parameters. No significant difference was observed in body mass index, serum sugar and HbA1c levels between the groups (Table 2). The mean value of serum nitric oxide level and glomerular filtration rate were observed to be significantly lower ($p<0.001$) in diabetic overt nephropathy patients as compared to diabetic hypertensive patients, whereas the microalbuminuria was significantly higher ($p<0.001$). Significantly, higher mean values of blood pressure, serum urea, serum creatinine and HbA1c were found in diabetic patients with overt nephropathy (Table 3).The mean value of serum nitric oxide level and glomerular filtration rate were observed to be significantly higher ($p<0.001$) in diabetic incipient nephropathy patients as compared to diabetic overt nephropathy patients. The mean of disease duration in years was significantly higher in overt nephropathy group ($p < 0.05$). No significant difference was observed in microalbuminuria level (Table 4). The figures 1&2 summarize the correlations between nitric oxide, microalbuminuria and glomerular filtration rate in diabetic incipient nephropathy group, in which a strong positive correlation was found between nitric oxide and glomerular filtration rate in diabetic incipient nephropathy ($r=0.86$, $p<0.001$) (Fig.1), also microalbuminuria shows a strong positive correlation with glomerular filtration rate in the same group ($r=0.84$, $p<0.001$) (Fig.2).

Table 1
Serum nitric oxide and other parameters in controls and diabetic patients

Variables	**Patients * n=120**	**Controls * n=120**	**P-value****
Body mass index	27.3±4.36	26.4±3.27	0.03
Systolic BP [mm Hg]	134±2.18	118±0.76	0.01
Diastolic BP [mmHg]	87±1.09	76±0.65	0.01
Glomerular filtration rate [ml/min]	78.6±32.7	105.8±13	0.0001
Serum sugar [mg/dl]	165.9±50.1	94.4±9.8	0.0001

Serum urea [mg/dl]	56.2±43.6	36.8±6.5	0.0005
Serum creatinine [mg/dl]	1.6±2.18	0.86±0.2	0.0001
HbA1c (%)	10±2.18	5.1±1.1	0.0002
Microalbuminuria [mg/l]	104±132.9	8.4±4.36	0.0001
Serum nitric oxide [µmol]	20±11.9	30.6±2.18	0.0001

* All data are analyzed as mean ± SD

**P-value based on independent t-test

Table 2
Serum nitric oxide and other parameters in diabetic incipient nephropathy and hypertensive diabetic patients

Variables	**Diabetic incipient Nephropathy (n=10)**	**Hypertensive diabetics (n=24)**	**P-value**
Body mass index	27.1±3.47	28.7±3.9	0.066
Systolic BP [mm Hg]	114±1.26	149±0.97	0.0001
Diastolic BP [mmHg]	73±0.94	94±0.97	0.0001
Glomerular filtration rate	132±13.9	82±14.1	0.0001

[ml/min]			
Serum sugar [mg/dl]	173.6±36.97	172.4±73.3	0.93
Serum urea [mg/dl]	35±5.68	38.9±5.86	0.00
Serum creatinine [mg/dl]	0.7±0.12	0.9±0.14	0.00
HbA1c (%)	10.3±1.58	10.3±1.95	0.81
Microalbuminuria [mg/l]	134.5±18	11±5.86	0.0001
Serum nitric oxide [µmol]	50.9±1.89	15.2±0.97	0.0001

Table 3
Serum nitric oxide and other parameters in diabetic overt nephropathy and hypertensive diabetic patients

Variables	**Diabetic overt nephropathy n=29**	**Hypertensive diabetic (n=24)**	**P-value**
Body mass index	26.6±3.7	28.7±3.9	0.022
Systolic BP[mm Hg]	155±1.06	149±0.97	0.00
Diastolic BP [mmHg]	99±1.06	94±0.97	0.00
Glomerular filtration rate [ml/min]	44.5±6.36	82±14.1	0.0001

Serum sugar [mg/dl]	180±37.1	172.4±73.3	0.6
Serum urea [mg/dl]	70.8±7.42	38.9±5.86	0.0001
Serum creatinine [mg/dl]	2.1±0.21	0.9±0.14	0.0001
HbA1c (%)	11.1±1.59	10.3±1.95	0.048
Microalbuminuria [mg/l]	220.5±50.8	11±5.86	0.0001
Serum nitric oxide [µmol]	10.1±1.32	15.2±0.97	0.0001

Table 4
Serum nitric oxide and related variables in diabetic incipient nephropathy and diabetic overt nephropathy patients

Variables	**Diabetic incipient nephropathy (n= 10)**	**Diabetic overt nephropathy (n=29)**	**P-value**
Duration of diabetes (years)	13.7±3.79	15.9±4.24	0.03
Glomerular filtration rate [ml/min]	132±13.9	44.5±6.36	0.0001
Microalbuminuria [mg/l]	134.5±18	220.5±50.8	0.93
Serum nitric oxide [µmol]	50.9±1.89	10.1±1.32	0.0001

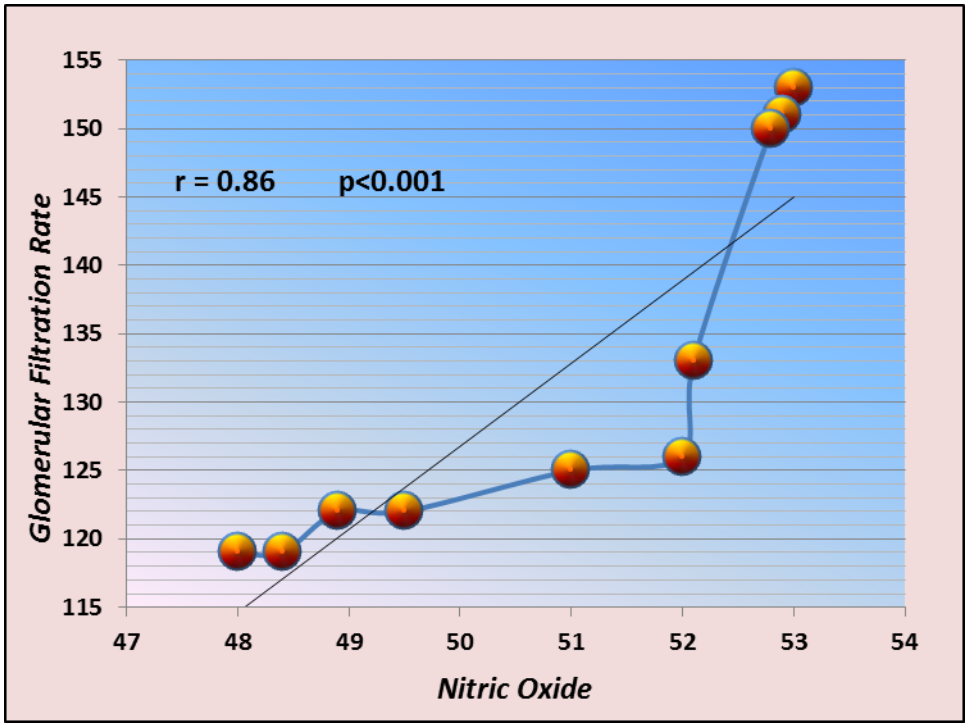

Fig. 1: Correlation between nitric oxide and glomerular filtration rate in diabetic incipient nephropathy

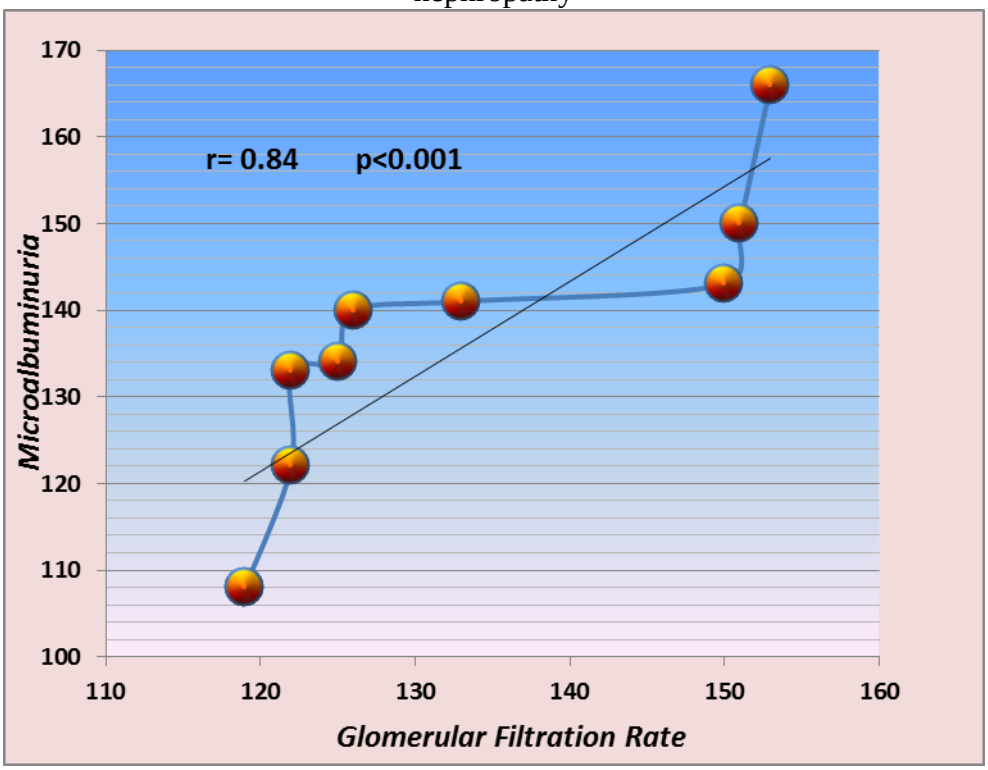

Fig. 2: Correlation between microalbuminuria and glomerular filtration rate in diabetic incipient nephropathy

4. Discussion

The endothelial dysfunction associated with diabetes has been attributed to a lack of bioavailable nitric oxide, which may contribute to vascular disease in diabetes (Segal et al.

2006). The present study finds support in the observation that diabetes affects nitric oxide metabolism as a successive and significant decrease was observed in the level of endothelial nitric oxide at the onset of diabetic complications, such as hypertension and nephropathy. Several mechanisms could account for the low nitric oxide bioavailability in diabetes. 1) Glucose can scavenge and capture the NO to form unspecified additional product, 2) there is an impairment of eNOS activation, 3) oxidative stress, which quenches NO to form peroxynitrite, 4) formation of advanced glycation end products in diabetes may result in endothelial NO deficiency and finally , NO may bind to glycosylated deoxyhemoglobin (Nakagawa, 2007). Evidence that glomerular arteriolar resistances are regulated by nitric oxide levels is supported by observations of vasoconstriction in afferent and efferent arterioles of both superficial cortical (Lockhart et al. 1994) and juxtamedullary nephrons (Zats and Nussi. 1991) following nitric oxide synthesis inhibition. The same effects were also observed in diabetic patients with incipient nephropathy during the present study. The latter is supported by evidence that increased level of nitric oxide was positively correlated with GFR values ($r=0.86$, $p<0.001$), and the level of serum nitric oxide as well as the glomerular filtration rate was significantly higher in patients with microalbuminuria (incipient nephropathy 50.9±1.89 µmol/l and 132±13.9 ml/min) respectively, than in diabetic overt nephropathy, end stage renal disease patients and healthy control subjects. Therefore, the most likely explanation for the present finding is that nitric oxide has a crucial role in determining the hyperfiltration found in diabetic patients with microalbuminuria. The significant positive correlation between nitric oxide and microalbuminuria ($r=0.92$, $p<0.001$) and the positive correlation between GFR and microalbuminuria ($r=0.84$, $p<0.001$) may indicate that glomerular hyperfiltration could contribute to the increased albumin excretion found in diabetic patients with early nephropathy in this study. The present study demonstrates the inverse relationships between serum nitric oxide and kidney function tests such as urea and creatinine. Baylis (2008) observed that arginine transport to endothelial cells is inhibited and nitric oxide will decrease in uremic plasma. In another study, Tarnow et al. (2004) measured the mean plasma level of ADMA (Asymmetric dimethyl arginine, an endogenous nitric oxide inhibitor) in patients with overt diabetic nephropathy and found significant higher levels of this marker than the diabetic control subjects, and reported that plasma concentration of ADMA increases with age, blood pressure and serum creatinine. In the current study, we found a correlation between nitric oxide reduction and elevation in urea, creatinine and microalbuminuria. This study may give an additional explanation for the deficiency of nitric oxide in overt diabetic nephropathy and diabetic hypertensive patients, also may explain the negative correlation which was found with age in diabetic patients ($r=-0.23$, $p<0.05$).

5. Conclusions

1. There is very high differentiated correlation between serum nitric oxide and the different stages of diabetic nephropathy.

2. The data support the use of raised serum nitric oxide as a marker of early (incipient) diabetic nephropathy and a low serum nitric oxide for overt and end stage diabetic nephropathy. The decreases being proportionate to the degree of renal impairment.

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