Original Article

Serum nitric oxide status in patients with type 2 diabetes mellitus in Sikkim

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Abstract

Background: Serum nitric oxide (NO) has emerged as a fundamental signal associated with the endothelial dysfunction in type 2 diabetes. **Aims:** To compare serum nitric oxide level among type 2 diabetic patients along with other biochemical parameters and to compare it with that of normal population in Sikkim. **Settings and Design:** This prospective study was carried out in the Biochemistry Department in a tertiary care teaching hospital in Sikkim on 50 type 2 diabetics compared to 100 non-diabetics. **Materials and Methods:** The un-hemolyzed blood sample was collected for estimation of biochemical parameters. Griess reaction was used for indirect assay of stable decomposition products in serum (serum nitrite and nitrate levels) as an index of NO generation. The comparison of different parameters between cases and control was done by using Student's *t*-test. **Results:** There was significant difference when age- and sex-matched cases and controls were compared in regard to waist circumference and body mass index. The values of fasting and postprandial serum glucose, and lipid profile between study group and control group differed significantly. The mean serum level of NO in the study and control group was 43.83 ± 11.3 µmoles/L and 58.85 ± 12.8 µmoles/L respectively, and this difference was statistically significant. **Conclusion:** To sum up, serum NO was observed significantly low in diabetic participants as compared to control, along with difference in other biochemical parameters.

Key words: Griess reaction, nitric oxide, type 2 diabetes

Introduction

Free radical nitric oxide (NO) has emerged as a fundamental signaling device regulating virtually every critical cellular function and is a potent mediator of cellular damage in many conditions.[1]

Vascular injury in diabetes consequential from hyperglycemia has been associated with oxidative stress that leads to depletion of intracellular glutathione with an augmented plasma extracellular superoxide dismutase which intervenes lipid peroxidation and diabetic complications.[2-4] Elevated concentration of superoxide dismutase causes impairment of endothelial isoform of nitric oxide synthase (eNOS) by triggering advanced glycation end products and poly (ADP-ribose) polymerase.[5] NO is synthesized as a byproduct of conversion of its physiological precursor L-arginine to L-citrulline. This reaction is catalyzed by a family of enzymes known as NO synthases (NOS).[6] Nitric oxide is produced in endothelial cells from the substrate L-arginine via eNOS. Elevated asymmetric dimethylarginine levels cause eNOS uncoupling, a mechanism which leads to decreased NO bioavailability. The endothelial dysfunction associated with diabetes has been attributed to lack of bioavailable NO due to reduced ability to synthesize NO from L-arginine. New basic research insights provide possible mechanisms underlying the impaired NO bioavailability in type 2 diabetes. So, the nitric oxide is reduced in the course of vascular disease (e.g., diabetes and hypertension).[7-11]

With this idea, we conducted the study to compare the levels of serum triacylglycerol (TG), total cholesterol (TC), high density lipoproteins (HDL), and low density

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lipoproteins (LDL) and evaluate the oxidative stress marker NO in the serum of diabetics and non-diabetics of Sikkim.

Materials and Methods

This prospective study was conducted in a tertiary care teaching hospital in Sikkim on participants suffering from type 2 diabetics for more than 1 year, aged 30 years and above; 100 age- and sex-matched apparently healthy caregivers without any history or symptoms of diabetes and other metabolic disorders were chosen as the control.

The participants with co-morbidities, viz. febrile illness, suffering from any other hormonal disorders, benign or malignant disorders, diabetic ketoacidosis, infection, gastroenterological conditions, hypertension, renal and cardiac disease, inflammatory diseases, transplant rejection, central nervous system disorders, and other chronic diseases, were excluded from the study; none were on antioxidant supplementation or lipid lowering drugs. The diabetics were allowed to pursue their intervention schedules and regular lifestyles; smokers were excluded from the study.

The study conformed to the Helsinki Declaration. Institutional ethics committee approved the study and the study was conducted from January 2008 to June 2009. An informed written consent was taken from each participant. For the biochemical investigations, fasting un-hemolyzed blood sample (after a minimum of 12 hours of fasting) was collected and used, except for the postprandial serum glucose estimation.[12] Blood was collected from the antecubital vein, following universal precautions. The sample was then allowed to clot in the aliquot at room temperature for about 2 hours and was then centrifuged at 3000 rpm for 10 minutes to separate the serum.

The diagnosis of diabetes mellitus was based on World Health Organization (WHO) criteria, i.e. a fasting blood glucose (FBG) of 126 mg/dL (7.0 mmoles/L) after a minimum 12-hour fast, with symptoms of diabetes, and a 2-hour postprandial glucose (PPG) level of more than or equal to 200 mg/dL (11.1 mmoles/L) after 75 g oral glucose load. Postprandial sample was drawn 2 hours following ingestion of 1.75 g/kg body weight with a maximum of 75 g of oral glucose in 300 mL of water.

All the biochemical estimations (except nitric oxide) were done by using RFCL kit on the spectrophotometer (Ranbaxy Hospitex REF, LIHD116-SN-161252). FBG and PPG were estimated quantitatively by GOD/POD technique as described by Trinder.[13] Total cholesterol was estimated quantitatively by CHOD-PAP technique as described by Allian.[14] TG was estimated quantitatively by GPO-ESPAS technique as described by Buccolo and David.[15] Estimation of HDL was done quantitatively by using PEG-PAP method.[16] The Friedewald formula was used to measure the LDL level indirectly.[17,18]

Serum nitric oxide was determined indirectly by the measurement of stable decomposition product (NO_2^-), employing the Griess reaction according to the modified method of Mathew *et al*.[19] Serum samples were processed immediately with freshly prepared equal volumes of Griess reagent and incubated for 10 minutes at 37°C. Absorbance of each sample was determined at 540 nm using spectrophotometer, Ranbaxy Hospitex.[19]

Statistical analysis

All results were summarized as mean ± SEM. The statistical analysis was done using Graph Pad 3, and the comparison between cases and control was done by using Student's *t*-test. The difference was considered to be statistically significant at an alpha error of 0.05.

Results

This study was conducted among 50 type 2 diabetics and 100 matched non-diabetic controls. In the study group, the mean duration of diabetes was 7.9 ± 1.87 years. There was significant difference when age- and sex-matched cases and controls were compared in regard to waist circumference and body mass index.

The FBG and PPG levels between study group and control group differed significantly; in lipid profiles also, the values were significantly different. The mean serum level of NO in the study group was 43.83 ± 11.3 µmoles/L; in the control group, it was 58.85 ± 12.8 µmoles/L; this difference was statistically significant [Table 1].

Discussion

In our study, there was significant difference in all the independent and dependant variables in terms of FBG and PPG with lipid profile and serum nitric oxide level.

A Turkish study compared the basal serum levels of nitric oxide in type 2 diabetes mellitus patients with different stages of diabetic retinopathy and compared them with the levels in non-diabetics using Griess reaction. The patients with type 2 diabetes had significantly higher levels of serum NOx than the non-diabetics.[20] A Turkish study on micro- and normo-albuminuric type 2 diabetics and healthy controls found that serum and urine NO levels were higher in both micro-albuminurics and normo-albuminurics than controls in early diabetes.[21] A Japanese study showed that plasma NO_x

Table 1: Clinico-social correlates of study and control participants and laboratory findings

Variables	Groups	Mean ± SD	SEM	95% Confidence interval	*P* value *t* value
Age (years)	Study	49 ± 7.91	1.12	46.75–51.25	*P* = 0.1791
	Control	47 ± 9.73	0.97	45.07–48.93	*t* = 0.13
Waist circumference (cm)	Study	34.61 ± 4.22	0.59	33.41–35.80	*P* = 0.005
	Control	32.84 ± 3.18	0.32	32.21–33.47	*t* = 2.88
Body mass index (kg/m^2)	Study	26.2 ± 4.15	0.59	25.02–27.38	*P* < 0.0001 *t* = 5.17
	Control	23.8 ± 1.49	0.15	23.50–24.10	
Fasting glucose (mg/dL)	Study	117.69 ± 47.20	6.67	104.26–131.12	*P* < 0.0001 *t* = 7.47
	Control	81.15 ± 9.43	0.94	79.28–83.02	
Postprandial glucose (mg/dL)	Study	173.8 ± 61.95	8.76	156.18–191.42	*P* < 0.0001 *t* = 9.21
	Control	114.2 ± 13.61	1.36	111.50–116.91	
Total cholesterol (mg/dL)	Study	197.5 ± 37.25	5.26	186.92–208.08	*P* < 0.0001 *t* = 7.21
	Control	154.73 ± 32.66	3.27	148.24–161.22	
Triacylglycerol (mg/dL)	Study	241.3 ± 64.93	9.178	222.84–259.76	*P* < 0.0001 *t* = 7.22
	Control	179.73 ± 39.35	3.930	171.91–187.53	
HDL (mg/dL)	Study	35.2 ± 10.86	1.53	32.13–38.27	*P* < 0.0001 *t* = 6.53
	Control	43.1 ± 4.42	0.44	42.23–43.97	
LDL (mg/dL)	Study	158.3 ± 30.72	4.34	149.57–167.03	*P* < 0.0001 *t* = 11.51
	Control	103.3 ± 25.92	2.59	98.15–108.45	
NO (μmoles/L)	Study	43.83 ± 11.31	1.13	41.50–66.08	*P* < 0.0001 *t* = 12.20
	Control	58.85 ± 12.81	1.81	45.21–72.49	

SD = Standard deviation; SEM = Standard deviation of mean; HDL = Serum high density lipoproteins; LDL = Serum low density lipoproteins; NO = Serum nitric oxide level

levels were significantly higher in diabetics than in controls, when measured by high-performance liquid chromatography with the Griess method.[22] In a Karachi study, a nonsignificant increase was observed in the levels of nitric oxide metabolites in diabetic patients as compared to non-diabetics, but diabetic patients with hypertension showed significantly higher levels as compared to controls, but the levels were not significantly different in patients with and without hypertension.[23] In an Iranian study, NO_x was measured in adults using the Griess reaction, which was significantly higher in subjects with type 2 diabetes, supporting the existing hypothesis that NO overproduction affects insulin's metabolic actions.[24]

Brussels study was conducted to correlate the serum level of NO in patients with acute coronary syndromes in relation to the presence or absence of diabetes mellitus. Before any therapeutic intervention, arterial blood samples were withdrawn to assess the serum NO metabolites level by the Griess reaction. Compared with the control group, patients with acute ischemic syndromes had a significantly lower level of serum NO metabolites, without any significant difference between diabetic and non-diabetic patients.[25]

A study at Karachi aimed to find the correlation between glycosylated hemoglobin (HbA1c) and NO anomalies in coexisting diabetes and hypertension found that FBG and HbA1c levels were significantly high, whereas serum NO level was significantly low in diabetic normotensive and diabetic hypertensive patients as compared to controls. A significant negative correlation was found between serum nitric oxide and serum glucose and HbA1c levels in diabetic hypertensive patients, suggesting that HbA1c can critically contribute to anomalies of NO metabolism or vice versa.[26] Researchers in Taiwan assessed the NO levels in aqueous humor and plasma using the chemiluminescence assay and observed no significant differences between any of the diabetic subgroups in the plasma NO levels.[27]

Prospective studies have established that reduction in NO bioavailability is a predictor of dyslipidemia as it is an endogenous anti-atherosclerotic molecule. By the dysfunction of the endothelial L-arginine–nitric oxide pathway, several cardiovascular risk factors impart their deleterious effects on the vascular wall, including hypercholesterolemia.[28-30]

Researchers at Harbin Medical University, China, observed that the changes in the NO level and other markers of oxidative stress in patients with type 2 diabetes mellitus did not significantly correlate with the changes in plasma lipid profile.[31]

In the West Glasgow Hospitals, the researchers observed that the subjects with type 2 diabetes displayed decreased NO production which was related to confounding factors such as age, body mass index, and lipid profile.[32] Researchers have reported that subjects with diabetes have an unfavorable lipid profile and altered plasma levels of oxidative stress markers like nitric oxide, and the NO levels were lower than in control subjects.[33-36] In the study conducted at the Lady Hardinge Medical College, India, on the effect of glipizide, metformin

and rosiglitazone on nontraditional cardiovascular risk factors in newly diagnosed patients with type 2 diabetes mellitus, NO levels were increased in all the study groups, though not significantly.[37]

To the horizon of our knowledge, this was the pioneering study in this part of India. Moreover, due to ethnic origin and geographical variation in Sikkim, this particular study has been taken up to compare the serum NO level in patients with diabetes and healthy controls and to establish a correlation between serum nitric oxide level and diabetes mellitus. The serum nitric oxide level in the control group significantly differed compared to that in cases.

Limitations of our study include its small sample size and open label design. Selection bias also limits the generalizability of our findings since only the subjects from our diabetic clinic were sampled. Our finding further goes to say that we may have to do a study in primary cases of diabetes, conduct a comparative study among different ethnic groups in Sikkim, use more sensitive methods and probably study the NOS gene expression and polymorphism in the Sikkimese population before we can establish the role of nitric oxide assay in diabetics.

To sum up, serum NO was observed to be lower in diabetic participants, which needs to be further established by prospective population-based studies. This profile for diabetic patient in our hinterland matched with some of the observations of our global peers, while other researchers noted higher levels of NO in diabetics. These wide levels of variations point to the need of the standardization of method of assessment of NO with a robust multicentric study across regions.

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