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Serum Nitric Oxide and Plasma HbA1c Levels in Type 2 Diabetes Mellitus Patients

Biochemistry Section

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ABSTRACT

Introduction: Diabetes Mellitus (DM) is a metabolic disorder characterised by hyperglycaemia and insufficiency of secretion or action of endogenous insulin. Glycated haemoglobin (HbA1c) is commonly used as reliable index to determine cumulative glycaemic history of preceding 2-3 months. Nitric Oxide (NO) is a potent vasodilator and an endothelium-relaxing factor. Low serum Nitric Oxide (NO) levels are known to be associated with endothelial dysfunction in Type 2 Diabetes Mellitus (T2DM).

Aim: To estimate the serum nitric oxide levels in type 2 diabetic patients and correlate it with serum HbA1c levels.

Materials and Methods: The cross-sectional study comprised of 100 (50 male and 50 female) T2DM patients who attended KLE'S Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi, Karnataka, India. Fasting Plasma Glucose (FPG) was measured by Hexokinase method using Roche/Hitachi COBAS 6000 Fully Automated Analyser. HbA1c was estimated using a Bio-Rad D-10 HbA1c program and serum NO was estimated by Classical Griess Reaction. Data was analysed using independent t-test within the groups and Karl Pearson Correlation Coefficient in between the groups. The $p<0.05$ was considered as statistically significant.

Results: Statistically significant positive correlation was observed between NO and with HbA1c ($r=0.7674$) and NO with FBS ($r=0.5688$).

Conclusion: Hyperglycaemia increases NO levels in T2DM patients. Measurement of nitric oxide in early course of diabetes may help in preventing the progression and development of complications like endothelial dysfunction.

Keywords: Diabetic complications, Endothelial dysfunction, Glycated haemoglobin, Griess reaction

INTRODUCTION

DM is a metabolic syndrome characterised by insufficiency in insulin secretion or decreased utilisation/sensitivity of insulin by the tissues. It occurs due to impaired fat, protein or carbohydrate metabolism [1]. Diabetic management prevents the development of acute and chronic complications of DM. Cardiovascular disorders, neuropathy, retinopathy and nephropathy are the most prevalent complications, and are the main cause of death in a diabetic population [2]. The level of HbA1c in diabetes is used as a reliable index of glycaemic control over the preceding 6-8 weeks [3]. Normally, the level of HbA1c is less than 5.5%, but in diabetic patients, the value is increased up to 15% as per ADA guidelines [4]. An elevated level of HbA1c indicates poor control of DM. Increase in HbA1c values by 0.1% occurs with every 10 years of age; therefore elderly patients have significantly higher values from young adults [5].

NO is a vasodilator and an endothelium-relaxing factor. Due to endothelial Nitric oxide synthase (eNOS), vascular NO is produced. In diabetic patients, decreased activity of eNOS results in increased super anion production and decreased NO availability that leads to endothelial dysfunction [6].

Based on the available literature, the study was designed to correlate levels of serum nitric oxide and HbA1c in DM patients, and to assess the difference between genders.

MATERIALS AND METHODS

The cross-sectional study was conducted at the Department of Biochemistry, Jawaharlal Nehru Medical College, KLES Dr Prabhakar Kore Hospital & Medical Research Centre, Belagavi, Karnataka, India from January 2018 to December 2018. Before commencement of study, ethical clearance was obtained from Institutional Ethics Committee on Human Subject's Research, Jawaharlal Nehru Medical College, Belagavi (MDC/DOME/94). After obtaining written consent, 100 (50 male and 50 female) (10% CI) patients were recruited for the study.

Inclusion Criteria

Clinically diagnosed cases of T2DM (aged 20 years or above) as per ADA guidelines were selected [3]. Patients on oral hypoglycaemic agents and insulin treatment were also included.

Exclusion Criteria

Critically-ill patients admitted in the Intensive Care Unit (ICU), patients below the age of 20 years, pregnant women, active smokers, alcoholic individuals, patients with a history of chronic illness such as hypertension, inflammatory diseases, cardiac or renal disorder, Type 1 DM, and patients on long term meditation such as antioxidant supplementation or lipid lowering drugs were excluded from the study.

Data Collection

The HbA1c and FBS reports along with serum sample of OPD and IPD patients enrolled in the study were collected from the Biochemistry Laboratory.

Biochemical Analysis

Overnight fasting venous blood samples (5 mL) were collected, and serum obtained by centrifugation was stored at -4°C until further analysis.

- Fasting Plasma Glucose (FPG) was estimated by the Hexokinase method using Roche/Hitachi COBAS 6000 Fully Automated Analyser. Reference value: 70-110mg/dL as per ADA guidelines [4].
- Plasma HbA1C was measured using a Bio-Rad D-10 HbA1c program [7].

Reference value: (as per ADA guidelines) [4].

4.3%-5.6%=Non-Diabetic/Normal

5.7%-6.4%=Increased Risk for Diabetes/Prediabetic

>6.4%=Diabetes

- NO levels were estimated by using Classical Griess Reaction. The Griess reagent was prepared by using 1% sulphanilamide, 1% naphthylethylene diamine dihydrochloride and 2.5% phosphoric acid. The samples of the subjects were added to Griess reagent, transferred to the spectrophotometer and their optical densities were recorded. The standard graph was plotted and optical densities of patients sample were taken and correlated over the standard curve. NO estimation was done as the sum of NO_2 and NO_3 as mentioned by Miranda KM et al., [8]. Reference value: Male- 11.5-76.5 µM/l and Female- 10.1-65.7 µM/l.

STATISTICAL ANALYSIS

Data analysis was done by using Statistical Package for the Social Sciences (SPSS) Software version 16. Data was analysed using independent t-test within the groups and Karl Pearson Correlation Coefficient in between the groups. The p-value <0.05 was considered statistically significant.

RESULTS

Clinically diagnosed 50 T2DM men and 50 women were categorised into 5 age groups. The minimum number of subjects (n=8) belonged to 31-40 years age group and the maximum number of subjects (n=33) to 51-60 years age group. The mean and standard deviation age range was 53.22±12.81 years [Table/Fig-1].

Age groups (years)	21-30	31-40	41-50	51-60	>=61	Mean±SD
Male (n=50)	5 (10)	6 (12)	10 (20)	13 (26)	16 (32)	52.24±13.51
Female (n=50)	4 (8)	2 (4)	8 (16)	20 (40)	16 (32)	54.22±12.13
Total	9	8	18	33	32	53.23±12.81

[Table/Fig-1]: Gender and age-wise distribution of patients.
Values in parentheses are prevalence percentage

Gender-wise analysis showed Mean±SD in men was 52.24±13.51 years, 21-30 years age group had the least prevalence of n=5 (10%) and the highest prevalence (n=16 (32%)) was found in ≥61 years. Mean±SD in women was 54.22±12.13 years, 31-40 years group had the least prevalence of n=2 (4%) and the highest prevalence (n=20 (40%)) was found in 51-60 years group [Table/Fig-1].

Comparison of FBS, HbA1c, and NO was done between the genders using an independent t-test. However, no clinically or statistical significant results were obtained [Table/Fig-2].

	Male	Female	t-value	p-value
Fasting blood glucose **(FBS)**	154.20±45.49	162.90±60.87	0.8096	0.4201
Glycated haemoglobin **(HbA1c)**	8.33±1.64	8.19±1.33	0.468	0.6408
Nitric oxide **(NO)**	87.08±22.83	91.12±29.03	0.7728	0.4415

[Table/Fig-2]: Comparison of Fasting blood glucose (FBS) (mg/dL), glycated haemoglobin (HbA1c) (%) and Nitric oxide (NO) (µM) in males and females.
Independent t-test; *p<0.05 was considered statically significant

Highly significant positive correlation was observed between FBS and HbA1c, FBS and NO and HbA1c and NO [Table/Fig-3].

Variables	Correlation		
	r-value	t-value	p-value
FBS and HbA1c	0.7314	10.6179	0.0001*
FBS and Nitric oxide	0.5688	6.8467	0.0001*
HbA1c and Nitric oxide	0.7674	11.8466	0.0001*
Male			
FBS and HbA1c	0.7498	7.8505	0.0001*
FBS and Nitric oxide	0.4359	3.3552	0.0016*
HbA1c and Nitric oxide	0.7158	7.1011	0.0001*
Female			
FBS and HbA1c	0.7732	8.4458	0.0001*
FBS and Nitric oxide	0.6429	5.8152	0.0001*
HbA1c and Nitric oxide	0.8701	12.2283	0.0001*

[Table/Fig-3]: Correlation between Fasting blood glucose (FBS) (mg/dL), Glycated haemoglobin (HbA1c) (%) and Nitric oxide (NO) (µM).
Karl Pearson's correlation coefficient method; *p<0.05 was considered statically significant

DISCUSSION

DM is generally associated with increased risk of cardiovascular disorders and other co-morbidities throughout the world. The vascular complication of diabetes such as micro and macrovascular depends on hyperglycaemia. These complications were developed due to production of Reactive Oxygen Species (ROS) via various pathways. The ROS accumulation and activation of stress-induced intercellular signal pathways induces cellular damage, while the disease progresses due to inappropriate endogenous antioxidant defence mechanism [9]. In the present study, mean±SD FBS in males and females was found to be 154.20±45.49 mg/dL and 162.90±60.87 mg/dL.

NO is lipid soluble gas released from the vascular endothelium and inflammatory mediator. It plays a vital role in vascular tone and inhibition of cell proliferation. It is an effective inflammatory mediator because of its strong reactivity with oxygen, superoxide and iron-containing compounds [6]. Currently, various studies on glucose-dependent increase in NO production in case and control groups are available but none had compared in between the genders. Therefore, the present study is first of its kind in which the levels of FBS, HbA1c and NO among males and females belonging to different age groups were compared. However, no significant difference was observed in between the genders.

In the present study, statistically significant positive correlation was observed between FBS and HbA1c levels. These findings were in accordance with Monnier L et al., and Shrestha L et al., studies, which reported the relative contribution of fasting glucose increased gradually with increasing levels of HbA1c: 30.3% in the lowest vs. 69.5% in the highest (p<0.001), and both postprandial and FBS were significantly correlated with HbA1c [10,11]. Postprandial glucose showed better correlation to HbA1c than fasting blood glucose (r=0.630, p=0.05 vs r=0.452, p=<0.001) [11].

In the present study, statistically significant positive correlation was observed between HbA1c and NO levels. Similar findings were reported by various other studies [12-15]. The reason for increased NO levels in Type 2DM is oxidative stress and inflammation. Endothelium releases NO in response to different inflammatory mediators and it results in vasodilation. Such increase of NO can be a compensatory mechanism or defensive response [14]. Another theory for increased NO levels in Type 2DM is chronic hyperglycaemia, which leads to overflow of polyol pathway products along with depletion in the NADPH. NADPH plays a vital role for the enzymes in the metabolism of the ROS by acting as a co-factor. Increased inflammation and endothelial injury in diabetes, lead to increased synthesis of NO through inducible nitric oxide synthase enzyme in endothelium [15]. On the contrary, Hsu YC et al., and Kumar S et al., reported a decrease of NO [16,17]. These variations observed can be accounted to differences in the duration of diabetes. Initially, endothelium produces NO in higher amount to protect the organism from oxidative stress and inflammation. But after exhaustion, NO decreases in later stages.

Previous studies have correlated FBS, HbA1c and NO with each other in all subjects as one group, but in the present study FBS, HbA1c and NO were compared separately for both the gender. While there was significant increase in FBS, HbA1c and NO levels

in both males and females, no statistical significance or clinical relevant results were obtained.

CONCLUSION

Serum NO was correlated with FBS and HbA1c in both males and females indicating that hyperglycaemia increases NO levels in type 2 DM patients. Since abnormal NO metabolism is associated with diabetes macrovascular and microvascular complications, early assessment of NO levels can prevent development and progression of diabetes related complications.

LIMITATION

Serum NO levels were estimated by manual Classical Griess Reaction method, which can be replaced by fully automated methods like HPLC for more accuracy. There are lacunae of studies about the level of NO in medication adherent and non-adherent type 2 DM. Further cohort studies required to assess the effect of treatment on nitric oxide level in type 2 DM.

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