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EVALUATION OF OXIDATIVE STRESS, ANTIOXIDANT ENZYMES, LIPID AND LIPOPROTEIN PROFILE IN TYPE-2 DIABETIC PATIENTS

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Abstract: Diabetes mellitus is a group of metabolic disorder characterized by hyperglycemia with glycosuria and it is well documented that increased level of blood glucose is a marker of disorder of carbohydrate metabolism and is associated with the initiation of diabetic dyslipoproteinemia and other complications. Prolonged free radical mediated lipotoxicity and abnormal glucose tolerance are involved in the pathogenesis of diabetes mellitus. Diabetic dyslipoproteinemia is characterized by the increased level of cholesterol, reduced HDL and high triglyceride (TG) levels. The present study was carried out to explore the status of oxidative stress, antioxidant enzymes, lipids and lipoprotein profile in type-2 diabetic patients. This study was conducted on type 2 diabetic patients attending the diabetes OPD, Era's Lucknow Medical & Hospital, Sarfaraz Ganj, Hardoi Road, Lucknow. All biochemical assays were carried out by the standard kit methods. A marked increase in plasma levels of fasting blood sugar, lipid peroxide, lipid profile accompanied with increase in the lipids and apo-protein levels of serum β lipoproteins following decrease in lipid and protein constituents of α lipoprotein, antioxidant enzymes and reduced glutathione were noted in type 2 diabetic patients compared to healthy controls. Type 2 diabetic patients are consistently associated with disorder of metabolism and disturb redox status.

Key words: Lipid profile-Lipoprotein profile-Oxidative stress-Antioxidants; LCAT; Oxidation; Stress

INTRODUCTION

Diabetes Mellitus Type 2 (DMT-2) is a cluster of abnormal metabolic paradigms with the essential feature of hyperglycemia and is dubbed as the disease of "premature ageing". Incidence of DMT-2 is rising all over the world at worrying rate, despite, comprehensive and coordinated effects of World Health Organization (WHO), International Diabetes Federation and Several Social Science Agencies (1). All efforts have failed till date to arrest this rising incidence. 6.6% of the world population was affected by this disease in 2010 with an estimated 285 million carriers and the number may become almost double (552 million) by 2030. India is facing an even grimmer scenario. In 2000, the number of diabetic carriers was 31.7 million which rose to 58.7 million in 2010 and 12 million more patients are expected to get added in another 20 years. On the basis of affected population, both in terms of percentage and numbers India has significantly more patients than China and other neighboring countries and is often referred to as the diabetic capital of the world. The reasons for this lopsided proclivity are still poorly understood (2).

Metabolically, DMT-2 is a hetrogenous multifactorial syndrome with environmental and pleotropic involvement in which the former are overwhelmingly significant factors. Indeed, hyperglycemia is an essential expression due to relative or absolute lack of insulin action or secretion. Pathway selective insulin resistance is a cardinal, if not essential feature. It is almost inevitably accompanied with hyperglycemic complexities such as altered lipid metabolism and raised oxidative status due to unfavorable "Cellular Redox Homeostatic Box". Several researchers have corroborated this condition by animal cell culture and *in vitro* studies and our recent animal studies also support them (3). Therefore, present study was design to assess the level of altered lipid profile, lipoprotein profile, oxidative stress and antioxidants in type -2 diabetic patients.

MATERIALS AND METHODS

The present study was carried out in the department of Biochemistry in collaboration with Biochemistry Division, Central Drug Research Institute Lucknow and Department of Medicine, Era's Lucknow Medical & Hospital, Sarfaraz Ganj, Hardoi Road, Lucknow.

Selection of healthy human volunteers

60 healthy control (Male-30, Female-30), age 25 to 35years, BMI 18-22.9 were served as Control. These individuals attended the outpatient department for their periodical health checkup.

Selection of diabetic subjects

60 type 2 diabetic subjects (Male-30, Female-30) age 45 to 55 years, BMI 23-24.9 were selected from Diabetes Outpatient Department of Medicine, Era's Lucknow Medical & Hospital, Sarfaraz Ganj, Hardoi Road, Lucknow.

Exclusion criteria: Patients with evidence of acute or chronic inflammatory conditions, infectious disease, hypertension, cancer, persons on insulin or other medications that could affect glucose metabolism were excluded. Pregnant and lactating women were also not included in the study.

Inclusion criteria: All diabetic individuals were subjected to a complete medical evaluation by a physician including recording a full medical history and physical examination. Both males and females with fasting blood glucose 145 – 165 mg/dl were included in the study.

Study design

Subjects were divided in to two groups of 60 subject each: Group 1: Healthy Control (n=60), Group 2: Diabetic Control (n=60). The study proposal was approved

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by the Institutional Ethics Committee of Era's Lucknow Medical & Hospital, Sarfaraz Ganj, Hardoi Road, Lucknow.

Collection of blood samples

Fasting blood samples were collected, from the ante median cubital vein of the subjects following overnight fasting, using disposable plastic syringes with all aseptic precautions. Blood was transferred immediately in to a dry clean plastic test tube with a gentle push to avoid hemolysis. Blood was collected from both groups (Control & Diabetic), for biochemical estimations in fluoride (sodium fluoride and potassium oxalate, 5.4 mg NaF and 3.0 mg K-oxalate in each vial), EDTA (3 mg/ vial) and plain vials.

Separation of serum and plasma

Plasma was separated by centrifuging anticoagulant mixed whole blood at 1500 rpm for 15 minutes at 4 °C in Eppendorf centrifuge machine. On the other hand, for separating serum, the whole blood was kept in plain vacuutainer at 37°C for 30 minutes after which this coagulated blood was centrifuged at 1500 rpm for 15 minute at 4°C in Eppendorf centrifuge machine. The supernatant was pipette out in a new tube and kept at -20°C till analysis.

Preparation of RBC lysate

3ml whole blood of EDTA vacuutainer was taken and centrifuged at 1500 rpm for 15 minutes at 4°C in Eppendorf centrifuge machine. The whole supernatant from the tubes was pipette out, then to this supernatant was added 1 ml of normal saline (0.9% Nacl, isotonic solution). It was then again centrifuged at 1500 rpm for 15 minutes at 4°C in Eppendorf centrifuge machine. This step was repeated for three times for proper washing of RBC. Then 1.0 ml of washed RBC was taken in a new test tube, to which 3 ml of chilled Tripled Distilled Water (TDW) was added to lyses RBC. It was mixed/shaked well for 1 minute. This step followed by centrifugation at 10,000 rpm for 15 minutes at 4°C in Eppendorf centrifuge machine to settle down cell ghost of RBC. The supernatant was pipette out in a new tube and stored it at -20°C till analyzed.

Biochemical analysis of blood and plasma

The blood was centrifuged and plasma was separated. The fasting blood sugar (FBS) (4) was analyzed in plasma while glycosylated hemoglobin (HbA1C) (5), Super oxide dismutase (SOD) (6), Catalase (CAT)(7), Glutathione peroxidase (GPx) (8) and Glutathione reductase (GR) (9) were estimated in RBC lysate, serum totalcholesterol (TC) (10), triglyceride (TG) (11), high density lipoprotein total cholesterol (HDL-TC) (12) were assayed by standard spectrophotometric methods. Low density lipoprotein total cholesterol (LDL-TC) and very low density lipoprotein total cholesterol (VLDL-TC) were calculated by Friedewald's equation (13). Serum was also used for the assay of lecithin cholesterol acyl transferase activity (LCAT) (14), lipid peroxide (LPO) (15), and reduced glutathione (GSH) (16). A portion of serum was fractionated into very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) by polyanionic precipitation methods (17). Lipoproteins were measured for their total cholesterol (TC) (10), phospholipids (PL) (18), triglyceride (TG) (11) and apoprotein (19) by standard spectrophotometric methods.

Statistical analysis

One-way-analysis of variance (ANOVA-Newman's student test) was performed by comparison of values for diabetic group with control. All hypothesis testing were two-tailed. $P<0.05$ was considered statistically significant and the results were expressed as mean ± SD. The Graph pad INSTAT 3.0 software was used to carried out the statistical analysis (20).

RESULTS

Status of blood sugar fasting, HBA1C, LCAT and serum lipid profile in type 2 diabetic patients

The data in Table-1 shows that, in type 2 diabetic patients showed markedly increased levels of in fasting blood sugar 57%, HbA1c 40%, serum; TC, TG, LDL-Cholesterol and VLDL- Cholesterol levels 34%, 59%, 58%, 60% respectively. On the other hand type 2 diabetic patients showed decreased levels of HDL- Cholesterol by 39% and LCAT levels17%. With respected to healthy control.

Table 1: Status of fasting blood sugar, glycosylated hemoglobin, serum lecithin cholesterol acyl transferase and serum lipid profile in type-2 diabetic patients.

Experimental schedule	BMI (Kg/m²)	Fasting Blood sugar	Glycosylated Hemoglobin (g%)	*Serum LCAT* (mmol/L/hr)	Serum lipid profile				
					TC (mg/dl)	TG (mg/dl)	LDL-TC (mg/dl)	VLDL-TC (mg/dl)	HDL-TC (mg/dl)
Healthy Control (n=60)	18-22.9	92.26 ± 9.05	4.98 ± 0.53	79.54 ± 14.97	200.26 ± 21.67	110.06 ± 20.18	127.24 ± 24.57	21.89 ± 7.56	49.80 ± 9.17
Type 2 Diabetic Control (n=60)	23-24.9	144.40 ± 10.86* (+ 57%)	6.95 ± 0.78* (+ 40%)	65.78 ± 13.18* (- 17%)	268.53 ± 11.36* (+34%)	175.00 ± 28.01* (+59%)	201.17 ± 14.42* (+58%)	35.00 ± 5.60* (+60%)	30.53 ± 4.44* (-39%)

Values expressed as mg/dl are mean ± SD of 60 subjects. Values in the parenthesis are percent change. Type 2 Diabetic control is compared with Healthy Control*p<0.001.

Table 2: Status of serum lipoprotein profile in type-2 diabetic patients

Experimental schedule	VLDL				LDL				HDL			
	TC (mg/dl)	PL (mg/dl)	TG (mg/dl)	Apo-protein (mg/dl)	TC (mg/dl)	PL (mg/dl)	TG (mg/dl)	Apo-protein (mg/dl)	TC (mg/dl)	PL (mg/dl)	TG (mg/dl)	Apo-protein (mg/dl)
Control (n=60)	21.89 ± 7.56	37.50± 3.99	39.10 ± 3.95	16.85± 1.04	124.16± 14.42	29.55± 2.11	27.12± 1.11	27.13± 1.61	46.80± 9.17	82.55± 9.35	18.47± 1.79	183.83± 11.34
Type 2 Diabetic (n=60)	35.00 ± 5.60** (+ 60%)	87.50± 7.48** (+ 133%)	58.76± 5.70** (+ 59%)	18.00NS±0.57 (6.8%)	201.17± 14.42** (+ 62%)	70.59± 7.43** (+ 139%)	33.21± 6.29** (+ 23%)	30.55±1.46* (12.6%)	30.53± 4.44** (-34%)	62.52±6.27** (- 24.26%)	16.55 NS±1.28 (-10.39%)	137.42±12.33** (-25.31%)

Values are expressed as mean ± SD of 60 subjects, Type 2 Diabetic group was compared with control. **p<0.001, *p<0.05, NS= Non-significant.

Table 3: Status of GSH, serum lipid peroxide; SOD, catalase, GPX and GR in type-2 diabetic patients

Experimental schedule	Status of markers used for oxidative stress in Serum		Status of Antioxidant Enzymes in RBC Lysate			
	GSH (mg/ dl)	Lipid peroxide (nmol MDA/ml)	SOD (Unit/minute/mg protein)	Catalase (Unit/minute/mg protein)	GPx (nmoleNADPH Oxidase/min/mg protein)	GR (µmole/mg protein)
Control (n=60)	38.56 ± 3.76	2.17 ± 0.36	2.78 ± 0.19	3853 ± 251.36	366.38±160.00	245.00±34.88
Type 2 Diabetic (n=60)	19.79±1.63* (- 49%)	7.65 ± 1.36* (+253%)	2.12 ± 0.18NS (-8%)	3432 ± 267.08NS (-11%)	280.00±87.56* (-24%)	145.00±38.13* (-41%)

Values are expressed as mean ± SD of 60 subjects, Type 2 Diabetic group was compared with control *p<0.001, NS= Non-significant.

Status of serum lipoprotein profile in type 2 diabetic patients

Analysis of hyperglycemic serum (Table 2) showed marked increase in the levels of lipids and apoprotein constituting α-lipoproteins (VLDL and LDL) and these effects were pronounced for VLDL-TC 60 %, PL 133%, TG 59% and apoproteins 7%. There was increase in LDL-TC, PL, TG 62 %, 139 %, 23% respectively and apoprotein 13 %. There was a decrease in HDL-TC, PL, TG and apoprotein (34 %, 24 %, 10 % and 25 %) respectively with respected to healthy control

Status of GSH, LPO, SOD, Catalase, GPX and GR in type 2 diabetic patients

The data in table-3 show that in type 2 diabetic patients there was decrease in the levels of GSH, SOD, CAT by 49%, 8%, 11% respectively and increase in level of plasma LPO by 252% with respect to healthy control.

DISCUSSION

Interestingly the results are very encouraging. In the present study the average glycosylated hemoglobin (HbA1c) was significantly higher in patients when compare with control ($p < 0.001$) and so was the fasting blood sugar level, total cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides levels. On the contrary shown HDL cholesterol level and lecithin cholesterol acyl transferase activity (LCAT) were significantly lower. These observations clearly indicated that in these diabetic patient's lopsided dyslipidemia also existed.

In another exercise subfraction (total cholesterol, phospholipids, triglycerides and apoprotein fractions) of VLDL, LDL and HDL were examined. While lipid fractions were adversely affected in patients and required correction, the three most important features needing focus are low HDL cholesterol, low LCAT levels (Table- 1), low HDL apoprotein fraction (Table-2) and low GSH, SOD, CAT, GPx and GR (Table-3). There is consistent evidence that HDL choledterol is a potent predictor of cardiovascular events independently and also in DMT-2 patients (21). The cardio protective effect of HDL is attributed to its role in reverse cholesterol transport. It removes excess cholesterol from peripheral tissues towards the liver for excretion in to bile or else for steroid hormone synthesis in steroidogenic organs. Further effects of HDL are proteotropic as it also exerts most importantly as antioxidant and anti-inflammatory agent (22). Lecithin cholesterol aceyl transferase is an vitally important enzyme helping in reverse cholesterol transport. It transfers 2 acyl groups of lecithin to cholesterol resulting in generation of cholesterol esters which are retained in core of HDL particle for final scavenging. Incidentally glycosylated Hb negatively correlates with LCAT activity in DMT-2. Apoprotein-1 is quantitatively a major component of HDL. Glycation of apoprotein A-1 in HDL alters and reduces LCAT activity in proportion to the extent of apoprotein A-1 glycation. Indeed there is convincing evidence that hyperglycemia induces several pathways generating more ROS. These ROS increase glycation potential (22). In our study, apoprotein-1 significantly decreased (25.31%; $p<0.01$) and concomitantly OS also increased by 25.30% ($p<0.01$). Furthermore in both VLDL and LDL fractions total cholesterol and triglycerides level were consistently and considerably higher in diabetic patients indicating dyslipidemia. It is now widely accepted that dyslipidemia is a cardinal feature in dibetes**.** American Diabetes Association, 2003, had stated that DMT-2 is associated with a cluster of interrelated plasma lipid and lipoprotein fractions. Low HDL and elevated triglycerides also increase the risk of cardiovascular disease 2 -4 times in DMT-2 (23).

Although cells usually exist with reductive environment, but oxidation and reduction reactions are essential and crucial phenomenon of every cell. In normal cells at any given time oxidative processes yielding Reactive oxygen species (ROS) are slightly more than reduction processes. This oxidative potential is termed as OS. ROS and antioxidants are major determinant of oxidative stress (OS) as other cellular oxidative reductive processes are in balance. OS is raised in DMT-2 through numerous pathologies.

Our study indicates the pivotal role of oxidative stress in pathogenesis and progression of DMT-2. Although the role of OS in origin of DMT-2 is still controversial issue but it definitely abets DMT-2 and plays a central role in development of diabetic complications. One of the major oxidant is super oxide anion, that too with predominance in endothelial cells of both large and small arteries and myocardium and in convenience with dyslipidemia it increases the risk of cardiovascular events several folds. It is also postulated that O2 inactivates 2 critical artiatherosclerotic enzymes endothelial nitric oxide synthase and prostacyclin Synthase (24). In the present study, LPO, an accepted marker of OS in DMT-2 was significantly raised in diabetic patients. The average increase was more than threefold to that of controls. This clearly alluded and signified to provoke OS in diabetes. Consequently this must be disturbing the redox box. The raised OS was accompanied with reduction in GSH level (49%), and lower

SOD (8%), Cat (11%), GPx (24%) and GR (41%) activities. On the contrary endogenous antioxidants are reducible and try to balance cellular antioxidants, thereby maintaining cellular redox homeostasis[27]. In light of these report, the observation stated in Table 3 purport perturbed redox box in DMT-2. This clearly suggested that increased oxidative stress abnormal lipid and lipoprotein profile are major independent risk factors in the patho-mechanism of DMT-2.

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