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ASSESSMENT OF BIOCHEMICAL PARAMETERS IN THE PATIENTS OF CORONARY ARTERY DISEASE WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT: Impaired carbohydrate and lipid metabolism with amplified oxidative stress are well recognized factors for Coronary artery disease (CAD) which is the major reason of mortality and morbidity all over world. The incident of CAD with type 2 diabetes mellitus (T2DM) is growing and they are predicted to be the biggest cause of death by 2020 in India. Therefore, the venture of the present study was to assess the association of hyperglycemia, oxidative stress, antioxidant enzymes, lipid profile and lipoprotein components in serum of CAD with T2DM patients as well as to compare the results with age-sex matched healthy human volunteers. Five hundred participants were enrolled for the present study, with their ages ranging from 37 to 57 years from OPD of medicine, Era's Lucknow Medical College & Hospital, Lucknow, were attending OPD for routine medical checkup. Out of which two hundred fifty were clinically new diagnosed case of CAD with T2DM like hyperglycemia, angina pectoris and Myocardial infarction (MI), remaining two hundred fifty were healthy controls. All biochemical assays were carried out by the standard kit methods. Participants were investigated for serum lipid profile, Lipoprotein constituents along with levels of lipid peroxide, reduced glutathione and antioxidant enzymes. A marked impairment in levels of 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, serum reduced glutathione as well as level of antioxidant enzymes in CAD with T2DM patients in comparison to healthy human controls.

INTRODUCTION: Dyslipipoproteinemia is an independent risk factor for the development of coronary artery diseases, myocardial infarction, and hypertension in hyperlipidemic patients [1]. Clinically diabetic patients are characterized by marked increase in blood glucose level followed by normal or mild hyperlipidemia.

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Elevated level in low density lipoprotein (LDL) along with triglyceride especially in very low density lipoprotein (VLDL) and cholesterol in low density lipoprotein with free radicals mediated formation of modified LDL is recognized as a leading cause of development of Atherosclerosis in CAD with T2DM patients [2]. Furthermore hyperlipidemia may also induce abnormalities like resistance to insulin in muscle and liver cells in CAD with T2DM patients.

Treatment of hyperlipidemia with available lipid lowering drugs: fibrats and bile acid sequestrants are not free from many side effects such as

myositis, gastrointestinal upset along with elevated hepatic renal function tests [3].

Coronary artery diseases (CAD) and CAD with T2DM are the most frightening of the health prediction for the new millennium worldwide. According to world health report 2002, CVD will be the largest death causing disease in India. In India by 2020AD, 2.6 million Indian are predicted to die due to CAD, which constitutes 54.1 % of all CVD death [4]. CAD, the most common form of heart disease is characterized by atherosclerosis and the development of fibro-fatty plaques, which is followed by the formation of occlusive thrombi and the precipitation of acute events that interrupts the blood flow [5]. This condition leads to a disparity between oxygen supply and demand. If this imbalance is exceeds, it results in myocardial infarction (MI) [6]. Type 2 Diabetes Mellitus (T2DM) is a group of abnormal metabolic paradigms with the essential feature of hyperglycemia and is dubbed as the disease of "premature ageing". Incidence of CAD with T2DM 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 [7].

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 [8].

Metabolically, CAD with T2DM 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 those findings [9]. Therefore, present study was design to assess the level of altered lipid profile, lipoprotein sub fractions, oxidative stress and antioxidants in CAD with T2DM patients.

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

Selection of Healthy Human Volunteers: 250 healthy control (Male-135, Female-115), age 37 to 57 years, BMI 18-22.9 were served as healthy control. These individuals attended the outpatient department for their periodical health checkup.

Selection of CAD with T2DM Patients: 250 CAD with T2DM patients (Male-135, Female-115) age 37 to 57 years, BMI 23-24.9 were selected from Diabetes Outpatient Department (OPD) of Medicine, Era's Lucknow Medical & Hospital, Sarfaraz Ganj, Hardoi Road, Lucknow.

Exclusion Criteria: Patients with evidence of acute or chronic inflammatory conditions, rheumatoid arthritis, renal disease, infectious disease, cancer, persons on insulin or other medications that could affect glucose metabolism and who have been taken up steroidal hormone (oral contraceptive drug and other medications contradictory to CVD) were excluded. Pregnant and lactating women were also not included in the study.

Inclusion Criteria: The patients were diagnosed as having CAD with T2DM by clinical cardiologist on the basis of clinical symptoms, a positive stress test with chest pain, echocardiography results,

electrocardiogram and trade mill test as well as hyperglycemia by fasting blood glucose test. All CAD with T2DM patients 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 – 225 mg/dl, blood pressure more than 140/90 were included in the study.

Study Design: Subjects were divided in to two groups of 250 subject each: Group 1: Healthy Control (n=250), Group 2: CAD with T2DM (n=250). The study proposal was approved 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 (Healthy Control & CAD with T2DM\), 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: 3 ml 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, and then 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 Serum, Plasma and Lysate: The blood was centrifuged and plasma was separated. The fasting blood sugar (FBS) [10] was analyzed in plasma while glycosylated hemoglobin (HbA1C) [11], Super oxide dismutase (SOD) [12], Catalase (CAT) [13], Glutathione peroxidase (GPx) [14] and Glutathione reductase (GR) [15] were estimated in RBC lysate, serum totalcholesterol (TC) [16], triglyceride (TG) [17], high density lipoprotein total cholesterol (HDL-TC) [18] 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 [19]. Serum was also used for the assay of lecithin cholesterol acyl transferase activity (LCAT) [20], lipid peroxide (LPO) [21], and reduced glutathione (GSH) [22]. 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 [23](20). Lipoproteins were measured for their total cholesterol (TC) [16], phospholipids (PL) [24], triglyceride (TG) [17] and apoprotein [25] by standard spectrophotometric methods.

Statistical Analysis: One-way-analysis of variance (ANOVA- Newman's student test) was performed by comparison of values for CAD with T2DM 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 [26].

RESULTS:

Status of Blood Sugar Fasting, HbA1c, LCAT and Serum Lipid Profile in Cad with T2DM Patients: The data in **Table 1** shows that, in CAD

with T2DM patients showed markedly increased levels of in fasting blood sugar 106 %, HbA1c 103 %, serum; TC, TG, LDL- Cholesterol and VLDL-Cholesterol levels 44%, 69%, 71%, 64 % respectively. On the other hand T2DM with CAD patients showed decreased levels of HDL-Cholesterol by 45% and LCAT levels 20%. With respected to healthy control.

TABLE 1: STATUS OF FASTING BLOOD SUGAR, GLYCOSYLATED HEMOGLOBIN, SERUM LECITHIN CHOLESTEROL ACYL TRANSFERASE AND SERUM LIPID PROFILE IN CAD WITH T2DM PATIENTS

Experimental schedule	BMI (Kg/m^2)	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=250)	18-22.9	90.00 ± 10.50	4.90 ± 0.33	75.00 ± 16.00	180.00 ± 23.67	110.06 ± 21.18	117.24 ± 26.57	22.00 ± 8.56	53.00 ± 9.17
CAD with T2DM Patients (n=250)	23-24.9	185.40 ± 24.86* (+106 %)	9.95 ± 0.88* (+ 103%)	60.00 ± 14.18* (- 20%)	258.53 ± 12.36* (+44 %)	186.00 ± 28.01* (+ 69 %)	201.00 ± 15.42* (+ 71%)	36.00 ± 5.60* (+64%)	29.00 ± 4.44* (-45%)

Values expressed as mg/dl are mean ± SD of 250 subjects. Values in the parenthesis are percent change. T2DM with CAD Patients were compared with Healthy Control,*p<0.001.

Status of Serum Lipoprotein Constituents in Cad with T2DM Patients: Analysis of hyperglycemic serum (**Table 2)** showed marked increase in the levels of lipids and apoprotein constituting β-lipoproteins (VLDL and LDL) and these effects were pronounced for VLDL-TC 65 %, PL 134 %, TG 64 % and apoproteins 46 %. There was increase in LDL-TC, PL, TG 54 %, 86 %, 70 % respectively and apoprotein 49 %. There was a decrease in HDL-TC, PL, TG and apoprotein (46 %, 41 %, 30 % and 34 %) respectively with respected to healthy control.

TABLE 2: STATUS OF SERUM LIPOPROTEIN CONSTITUENTS IN CAD WITH T2DM 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=250)	20.00 ± 9.56	35.00 ± 3.97	36.00 ± 3.97	13.00 ± 2.44	130.00 ± 16.47	38.00 ± 3.18	37.12 ± 2.19	27.13 ± 1.62	56.80 ± 9.17	88.55 ± 9.35	19.40 ± 1.79	179.00 ± 11.34
CAD with T2DM Patients (n=250)	33.00 ± 6.60* (+ 65 %)	82.00 ± 8.48* (+ 134 %)	59.00 ± 5.70* (+ 64 %)	19.00 ± 0.76* (46 %)	200.17 ± 14.42* (+ 54%)	70.59 ± 8.42* (+ 86%)	63.21 ± 7.27* (+70 %)	40.55 ± 1.46* (49%)	30.53± 5.47* (- 46%)	52.53 ± 6.28* (- 41%)	13.55 ± 1.28* (-30 %)	119.44± 14.33* (- 34%)

Values are expressed as mean ± SD of 250 subjects, CAD with T2DM patients group was compared with Control, *p<0.001.

Status of GSH, LPO, SOD, Catalase, GPX and GR in CAD with T2DM patients: The data in **Table 3** show that in T2DM with CAD patients, there was decrease in the levels of GSH, SOD, CAT, GPx and GR by 47 %, 50 %, 21 %, 24 % and 45 % respectively and increase in level of plasma LPO by 208 % with respect to healthy control.

TABLE 3: STATUS OF GSH, SERUM LIPID PEROXIDE; SOD, CATALASE, GPX AND GR IN CAD WITH T2DM 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 (n mole NADPH Oxidized/min/mg protein)	GR (n mole NADPH Oxidized/min/mg protein)
Control (n=250)	30.00± 5.76	2.27 ± 0.56	4.00 ± 0.19	3800 ± 252.00	344.38±170.00	240.00±38.88
CAD with T2DM patients (n=250)	15.79± 3.63* (- 47%)	7.00 ± 2.36* (+208%)	2.00 ± 0.18* (-50%)	3000 ± 267.08* (-21%)	260.00±97.56* (-24%)	135.00±40.13* (-45%)

Values are expressed as mean ± SD of 250 subjects, CAD with T2DM patients group was compared with control, *p<0.001.

DISCUSSION: Interestingly the results are very stirring. In the present study the average glycosylated hemoglobin (HbA1c) was significantly higher in patients when compare with healthy 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 CAD with T2DM patient's lopsided dyslipidemia also existed.

In another exercise constituent (total cholesterol, phospholipids, triglycerides and apoprotein) 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 CAD with T2DM patients [27]. 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 [28]. Lecithin cholesterol aceyl transferase is a 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 CAD with T2DM patients. 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 [28].

In this clinical study, apoprotein-1 significantly decreased and concomitantly OS also incresesd. Further more in both VLDL and LDL components 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 CAD with T2DM**.** American Diabetes Association, 2003, had stated that CAD T2DM 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 T2DM [29].

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 CAD with T2DM patients through numerous pathologies.

Our study indicates the pivotal role of oxidative stress in pathogenesis and progression of CAD with T2DM. Although the role of OS in origin of CAD with T2DM is still controversial issue but it definitely abets T2DM 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 [30].

In the present study, LPO, an accepted marker of OS in CAD with T2DM patients was significantly raised. The average increase was more than threefold to that of controls. This clearly alluded and signified to provoke OS in CAD with T2DM patients. Consequently this must be disturbing the redox box. The raised OS was accompanied with reduction in GSH level and lower SOD, Catalase, GPx and GR activities.

On the contrary endogenous antioxidants are reducible and try to balance cellular antioxidants, thereby maintaining cellular redox homeostasis. In light of these report, the observation stated in Table 3 purport perturbed redox box in CAD with T2DM patients. This clearly suggested that increased oxidative stress abnormal lipid and lipoprotein profile are major independent risk factors in the patho-mechanism in patients suffering from CAD with T2DM.

CONCLUSION: Our study indicates the pivotal role of oxidative stress, impaired carbohydrate and lipid metabolism in pathogenesis and progression of CAD with T2DM. This study shows a significant boost in oxidative stress, β lipoproteins, blood glucose, glycosylated hemoglobin following with decrease in α lipoproteins, antioxidant enzymes, reduced glutathione and lecithin cholesterol acyl trans ferase activities were observed in CAD with T2DM patients with respect to healthy control. This is clearly suggested that increased oxidative stress, hyperglycemia, impaired lipid profile, abnormal lipoprotein constituents and decreased activity of antioxidant enzymes, reduced glutathione and lecithin cholesterol acyl trans ferase activities are risk factors in the patho-mechanism of artherosclerosis in patients suffering from CAD with T2DM.

CONFLICT OF INTEREST STATEMENT: The authors declare that they have no conflict of interest.

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