Original article

STUDY OF PROTEIN CARBONYL GROUP, NITRIC OXIDE AND MDA (INDEX OF LIPID PEROXIDATION) AS BIOMARKERS OF OXIDATIVE STRESS IN TYPE 2 DIABETES MELLITUS

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Financial Support: None declared

Conflict of interest: None declared

How to cite this article:
Chavan VU, Melinkeri RR. Study of protein carbonyl group, nitric oxide and MDA (index of lipid peroxidation) as biomarkers of oxidative stress in type 2 diabetes mellitus. Natl J Community Med 2013; 4(2): 294-299.

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Date of Submission: 04-12-12

Date of Acceptance: 11-05-13

Date of Publication: 30-06-13

ABSTRACT

Background: Diabetes mellitus (DM) is metabolic disorders characterized by hyperglycemia and abnormalities in lipid and protein metabolism. The free radicals and oxidative stress may act as a common pathway to diabetes itself, as well as to its later complications.

Objectives: The present study was planned to study the biomarkers of oxidative stress, such as protein carbonyl (CO) group, nitric oxide (NO·) in the form of total nitrite (NOx) and malondialdehyde (MDA) as an index of lipid peroxidation in type 2 DM patients and healthy individuals.

Methodology: We studied 60 cases of type 2 DM and 30 healthy individuals as control. Serum protein carbonyl estimated by dinitrophenyl hydrazine (DNPH) method, nitrate (NO_3^-) and nitrite (NO_2^-) concentrations in terms of total nitrites (NOx) by Griess reaction and MDA by thiobarbituric acid reagent test.

Results: We found highly significant increase in the level (Mean ± SD) of protein carbonyl (3.28 ± 0.75 nmol/mg), NOx (total nitrites) (176 ± 68.12 µmol/L) and MDA (8.3 ± 1.19 nmol/ml) in type 2 DM patients compared to healthy subjects (1.51 ± 0.22 nmol/mg), (46.6 ± 17.5 µmol/L) and (3.12 ± 0.77 nmol/ml) respectively ($P < 0.0001$).

Conclusion: Biomarkers of oxidative stress such as protein carbonyl, NOx (total nitrites) and MDA are significantly increased in type 2 DM compared to healthy subjects. There is increased protein oxidation, lipid peroxidation, and increased conversion of NO· to its end products like nitrite and nitrates in type 2 DM. This suggests that there is increased production of reactive oxygen species (ROS) and oxidative stress in type 2 DM patients compared to healthy subjects.

Keywords: Diabetes mellitus, oxidative stress, protein carbonyl (CO) group, nitric oxide (NO·) malondialdehyde (MDA)

INTRODUCTION

Diabetes mellitus (DM) is characterized by chronic hyperglycemia and disturbances of carbohydrate, lipid and protein metabolism due to deficiencies in insulin secretion and /or insulin action. Diabetic patients have defect in antioxidant defence mechanism, free radical and oxidative stress may responsible for diabetes itself, and its complications[1,2].

A free radical is a highly reactive molecule possessing an unpaired electron in its outer orbit having independent existence. Free radicals can be positively charged, negatively charged or electrically neutral. Free radicals are of different types like, the reactive oxygen species (ROS), reactive nitrogen species (RNS), can damage all types of biological molecules by oxidation[3,4]. Oxidative stress results from imbalance between the production of oxidants and antioxidant defence system of an organism[1-4]. Generation of free radicals and oxidative stress in vivo can be estimated by measuring products formed by free radical reactions. ROS and oxidative stress causes damage to proteins directly or indirectly leading to formation of protein carbonyl group (CO). Carbonyl groups are oxidised product of proteins and relatively stable in plasma, used as marker of oxidative damage[3,5].

Nitric oxide ($NO\cdot$) is a reactive diatomic gaseous molecule with an unpaired electron (a free radical). $NO\cdot$ is labile molecule, half life of which is 5-6 seconds. $NO\cdot$ it is also called as endothelium derived relaxing factor (EDRF) which function as relaxation of vascular smooth muscle[1], neurotransmitter, and inhibition of platelet aggregation. $NO\cdot$ reacts with oxygen or superoxide and converted to reactive nitrogen species (RNS) such as $NO_2\cdot$, $ONOO^-$ and N_2O_3 which are more reactive species[3,4,6]. Being $NO\cdot$, highly reactive and labile, endogenous production of $NO\cdot$ is evaluated by measurement of relatively stable metabolites such as nitrite (NO_2^-) and nitrate (NO_3^-) collectively called as total nitrites (NO_x)[6-8].

The free radical oxidation of polyunsaturated fatty acids (PUFA) in biological system is known as lipid peroxidation[3]. Peroxidation of PUFA in lipid membranes severely damages the cell membranes and responsible for damage to tissue where it may be a cause of inflammatory diseases. Malondialdehyde (MDA) and other aldehydes have been identified as products of Lipid peroxidation[9].

Many diseases are associated with free radical damage and oxidative stress such as diabetes mellitus, endothelial dysfunction, cardiovascular diseases, chronic renal failure, rheumatoid arthritis etc[4]. Based on present knowledge our study was aimed to find out concentration of oxidative stress markers, such as protein carbonyl (CO) group, nitric oxide ($NO\cdot$) in terms of total nitrites and malondialdehyde (MDA) in type 2 DM patients compared to healthy individuals.

MATERIALS AND METHODS

The study was conducted in the department of Biochemistry, B.J. Medical College, and Sassoon General Hospitals, Pune, over a period of one year from January 2005 to December 2005. Study was approved by ethical committee and informed consent was taken from all participants. We studied 60 patients of type 2 DM and 30 age and sex matched healthy individuals as control group. DM patients attending Medicine OPD of Sassoon General Hospital, B.J. Medical College, Pune were recruited for study. Controls were selected from general populations (e.g. friends, staff of dept, their friends and relatives, known persons who were not suffering from any disease, healthy people). Control group samples were tested for blood glucose to rule out DM.

Inclusion criteria: Clinically diagnosed cases of type 2 DM from case paper records, without any complications, aged between 30 to 60 years, males and females were recruited for study.

Exclusion criteria: Diabetes mellitus other than type 2, history of smoking, alcoholism and Pregnant women. Same criteria were used for cases and controls.

Blood sample was collected in fluoride bulb (fasting and post prandial) for glucose estimation and in plain bulb for other parameters. Blood glucose was estimated by glucose oxidase peroxidase (GOD-POD) enzymatic method. Serum protein carbonyl estimated by dinitrophenyl hydrazine (DNPH) method[10], total nitrite (nitrate and nitrite) concentrations by Griess reaction[11] and Malondialdehyde (MDA) by thiobarbituric acid (TBA) method[12]. Quality was checked by method standardization, calibration of methods and controls run. The principal investigator himself did the laboratory analysis and, hence blinding to subject's case / control status was not possible.

Statistical Analysis: Data is expressed as Mean ± SD. Comparison of various biomarkers of oxidative stress between diabetes and normal group was done by applying 'Z' test. The difference was said to be significant when P was < 0.001. All the calculations were done manually with the help of statistician, Department of PSM, BJMC, Pune.

RESULTS

We studied 60 patients, 37 males (61.7%) and 23 females (38.3%) of type 2 DM and 30 age and sex matched healthy individuals as control group. The mean (Mean ± S.D.) age of study subject was 52 ± 7.6 years. Blood glucose estimation was done as baseline investigation (Table 1). Blood glucose was significantly higher in diabetic patients as compared to control group.

Table 1: Blood glucose level in diabetic and control group

	Control (n = 30) Mean ± S.D.	Diabetic Patients (n = 60) Mean ± S.D.	Mean difference (CI)	P value
Blood glucose level (fasting) (mg/dl)	90.63 ± 7.03	140.4 ± 50.41	49.77 (31.34-68.20)	P < 0.001*
Blood glucose level (Post-prandial) (mg/dl)	121.26 ± 10.76	202.32 ± 65.25	81. 06 (57.16-104.96)	P < 0.001*

*P value significant

Table 2- Serum levels of protein carbonyl, nitric oxide and MDA in control and diabetic patients

Biochemical Parameter	Control group (n= 30) (Mean±SD)	Diabetic Patients (n=60) (Mean±SD)	Mean difference (CI)	P value
Protein Carbonyl (CO) (nmol/mg protein)	1.51±0.22	3.28 ± 0.75	1.77 (1.49-2.05)	P<0.0001**
Total nitrites (NOx) (µmol/L)	46.6 ± 17.5	176.1 ± 68.12	129.4 (104.21-154.58)	P<0.0001**
MDA (nmol/ml)	3.12 ± 0.77	8.3 ± 1.19	5.18 (4.70-5.65)	P<0.0001**

** P value significant

Table 2 shows the levels of various biomarkers of oxidative stress in type 2 DM and control group.

The mean protein carbonyl level (nmol/mg protein) of type 2 diabetic patient was significantly higher (3.28 ± 0.75) than that of control group (1.51 ± 0.22), (P < 0.0001). The mean total nitrites (NOx) (µmol/L) of diabetic patients was statistically higher (176.1 ± 68.12) than that of control group (46.6 ± 17.5), (P < 0.0001). The mean MDA level (nmol/ml) of diabetic patients was statistically higher (8.3 ± 1.19) than that of control group (3.12 ± 0.77), P < 0.0001. All biomarkers of oxidative stress in type 2 DM were statistically higher (P < 0.0001) than that of control group.

DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, hyperlipidemia, increased production of reactive oxygen species (ROS) and oxidative stress. Free radicals are constantly being produced in the body, as a result of normal metabolic processes. Under physiological conditions damage due to free radicals is countered by antioxidants. Sometimes, excessive free radical formation occurs in the body, and the antioxidant system in the body cannot cope with the situation, results into oxidative stress[1-4].

Free radicals are formed disproportionately in diabetes and many mechanisms seen to be involved in the genesis of oxidative stress in diabetes mellitus, namely, glucose auto-oxidation, protein glycation, advanced glycation end products (AGE) formation and the subsequent oxidative degradation of glycated proteins[13,14]. In DM because of hyperglycemia, there is activation of sorbitol system and functional limitation of HMP shunt, leading to decrease in glutathione synthesis[13-15].

Non-enzymatic glycation is a chemical reaction of glucose and amino group of proteins which forms sugar protein complex which is called as glycated proteins[16]. Glycated protein undergoes irreversible modification leading to Maillard products or AGEs. Glycated proteins can give an electron to the molecular oxygen and are capable of producing oxygenated free radicals[6,13,16].

Glucose at high concentration is preferentially metabolized via the polyol pathway leading to functional limitation of HMP shunt pathway and depletion of NADPH. This negatively influences glutathione synthesis and other enzymes and systems involved in antioxidant defensive mechanism[13,15].

When proteins are oxidised, carbonyl group (CO) are produced on protein side chain. CO groups are chemically stable, useful for detec-

tion, storage and established marker of protein oxidation[5, 17].

In our study, there was increased level of protein carbonyl (CO) group (3.28 ± 0.75 nmol/mg) in type 2 diabetic patients compared to control subjects (1.51±0.22 nmol /mg). Increased level of protein carbonyl as marker of oxidative stress was found by Odetti P, et.al[5], Cakatay U[18], Martin-Gallan P, et.al[19], and Telci A, et.al[20] supports our observation. Martin-Gallan P, et.al[19], found significantly higher protein carbonyl levels in DM and higher still in DM with complications compared to controls and suggested the potential role of oxidative stress in pathophysiology of complications in diabetes patients.

Nitric oxide (NO·) is synthesized from L-arginine by a family of nitric oxide synthases (NOS) by variety of cells. The reaction of NO· production is catalysed by one of the three isoform of NOS i.e. neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Molecular oxygen and NADPH are co-substrate for NO· synthesis. NO· is biological mediator, which mediates variety of physiological functions such as relaxation of vascular smooth muscle, neurotransmitter, inhibition of platelet aggregation[3,4,6,16]. NO·, highly reactive and labile, endogenous production of NO· is evaluated by measurement of relatively stable metabolites such as nitrite (NO_2^-) and nitrate (NO_3^-) collectively called as total nitrites (NOx)[1,6-8]. Metabolic fate of NO· in the body is complex, in plasma NO· is oxidised to nitrite, which is stable for several hours, in whole blood nitrite is rapidly converted to nitrate. Thus nitrite is minor and nitrate is major end product of nitric oxide metabolism. Therefore, the concentration of nitrite and nitrate together indicates NO· production in vivo[21].

Reactive oxygen species (ROS) rapidly inactivate NO· leading to the formation of reactive nitrogen species (RNS) such as NO_2·, N_2O_3 and peroxynitrite ($ONOO^-$) which are more reactive species. Peroxynitrite is a toxic oxidant capable of damaging many biological molecules. This result into more conversion of NO· to RNS causing reduced NO· bioavailability, and more formation of its end products like nitrite (NO_2^-) and nitrate (NO_3^-) collectively measured as total nitrites (NOx)[3,4,6]. The reduced NO· bioavailability may contribute to the development of insulin resistance[22]. Increased superoxide radicals generated in DM by various mechanisms causes inactivation of nitric oxide and may thus contribute to impaired endothelium-dependent vascular relaxation[23]. Endothelial dysfunction can be described as endocrine disorder, and it is genetic or acquired. In both conditions it is likely that the increased endothelial generation of superoxide anion can lead to rapid inactivation of NO· and, therefore, exacerbate endothelial injury. Endothelial dysfunction is characteristic of several diseases like diabetes, atherosclerosis, angina etc.[24]. In particular, an increased oxidative stress seems to be the main mechanism through which insulin resistance causes endothelial dysfunction[25].

We observed increased level of total nitrites (NOx) in type 2 DM (176.1 ± 68.12 µmol/L) compared to healthy subjects (46.6 ± 17.5 µmol/L). Increased level of NOx was found Abou-Seif MA et.al[1] and Cosentino F, et. al[26] which support our study. Our study suggest that there is increased conversion of nitric oxide to its end products like nitrite (NO_2^-) and nitrate (NO_3) collectively measured as total nitrites (NOx) leading to decreased bioavailability of NO· in type 2 DM.

Estimation of NO level is determined spectrophotometrically by measuring its stable degradation products, nitrite (NO_2^-) and nitrate (NO_3^-)[1,6-8]. Total nitrite (NOx) concentration is determined after reduction of nitrates to nitrites using the Griess reaction[11]. Griess reaction measures only nitrites (NO_2^-), therefore nitrates (NO_3^-) is reduced to nitrites (NO_2^-) by using nitrate reductase enzyme used in Griess reaction[11]. Serum total nitrites (NOx) concentration measured by Griess reaction includes nitrite (NO_2^-) + nitrate (NO_3^-) concentration (with prior reduction of nitrate to nitrite)[7,11]. NO_2 (nitrite) levels by Griess reaction may reflect accurately the endogenous synthesis of NO· from inducible NO· Synthase (iNOS)[11]. Moshage H, et al[27] evaluated nitrate and nitrite determination in plasma. According to them determinations of plasma nitrate and nitrite are used as marker of the activity of nitric oxide synthase and the production of nitric oxide.

We found increased level of MDA (8.3 ± 1.19 nmol/ml) in type 2 diabetic patients compared to normal subjects (3.12 ± 0.77 nmol/ml). Our study is supported by Garg MC, et al[28], Atli T, et al[29] and Likidlilid A et al[30].

Cosentino F, et al[26] demonstrated that prolonged exposure to hyperglycemia in diabetes mellitus increases eNOS gene expression, protein expression, NO_2^- release and production of superoxide. In diabetes mellitus there is increased level of NO_2^- but bioavailability of NO· is decreased[26].

We also observed increased level of NO_2^- in type 2 DM. Abou-Seif MA[1] demonstrated that the development of diabetic complications in diabetes is closely related to increased generation of superoxide anion and decreased nitric oxide (NO·).

Limitation of our study

Small sample size, duration of disease, correlation between degree and /or duration of oxidative stress in type 2 DM and development of diabetic complications is not studied. Further study is needed to study markers of oxidative stress in DM with and without complications, its correlation with duration of disease, glycemic control, different complications of DM and what is effect of antioxidant supplementation in DM treatment.

CONCLUSION

Biomarkers of oxidative stress such as protein carbonyl, NOx (total nitrites) and MDA are significantly increased in type 2 DM compared to healthy subjects. There is increased protein oxidation, lipid peroxidation, reduced bioavailability of endothelial nitric oxide (NO·) and increased conversion of NO· to its end products like nitrite and nitrates in type 2 DM. This suggests that there is increased production of reactive oxygen species (ROS) and oxidative stress in type 2 DM patients compared to healthy subjects.

Acknowledgement

Authors are very thankful to Dr. Prakash Patel, Asst. Professor, Community Medicine Dept. SMIMER, Surat for valuable help in statistics and calculations.

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