

Evaluation of Oxidative Stress in Type 2 Diabetics with Vascular Complications

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Abstract: Type 2 diabetes mellitus is a chronic progressive disease characterised by hyperglycaemia and dyslipidemia which leads to the development of vascular complications. Hyperglycaemia overloads glucose metabolic pathways and increases non-enzymatic, auto-oxidative glycosylation and free radical production. Dyslipidemia favours free radical-induced lipid peroxidation and increased deposition of oxidised low density lipoprotein (LDL) cholesterol in the blood vessels resulting in atherosclerosis. Lipid peroxidation, chronic low grade inflammation and oxidative stress have been proven to be closely involved in the development and progression of type 2 diabetes mellitus and its vascular complications. Malondialdehyde (MDA) is a relatively stable lipid peroxidation end-product, frequently used as a marker of lipid peroxidation. Ceruloplasmin (CP) is an antioxidant with ferroxidase activity and an acute phase protein elevated in inflammation. Uric acid (UA) is a major chain breaking antioxidant that protects Vitamin C from oxidation by divalent ions like ferrous ion in the plasma. The purpose of this study was to evaluate oxidative stress in type 2 diabetics with vascular complications and compare the findings with healthy control group and type 2 diabetics without vascular complications. Fasting plasma glucose, glycosylated haemoglobin (HbA1c), lipid profile, MDA, CP and UA were estimated in all study subjects. This study showed statistically significant increase in the levels of fasting plasma glucose, HbA1c, serum cholesterol, serum triglycerides, LDL, VLDL, MDA, CP and UA in type 2 diabetics with vascular complications when compared to healthy control group ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). Serum high density lipoprotein (HDL) levels were significantly decreased in the type 2 diabetics with vascular complications when compared to healthy controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). The study showed statistically significant correlation of oxidative stress markers (MDA, CP and UA) with indicators of glycaemic status ($p < 0.001$) and dyslipidemia ($p < 0.001$). It can be concluded from this study that hyperglycaemia, dyslipidemia, lipid peroxidation, inflammation and oxidative stress are significantly elevated in type 2 diabetics with vascular complications than in normal healthy controls and type 2 diabetics without vascular complications, suggesting their role in the development of vascular complications.

Key Words: Type 2 diabetic vascular complications, oxidative stress, malondialdehyde, ceruloplasmin and uric acid

I. Introduction

Type 2 diabetes mellitus involves progressive development of insulin resistance and a relative deficiency in insulin secretion due to β -cell dysfunction, causing hyperglycaemia. Symptoms are often less marked delaying the diagnosis of the disease to several years after the disease onset. Diabetes increases the risk of heart disease and stroke, as evidenced by the death of 50% of people with diabetes due to cardiovascular disease in a multinational study.¹ Diabetic neuropathy in the feet combined with reduced blood flow increases the chance of foot ulcers, infection and eventual need for limb amputation. Diabetic retinopathy, an important cause of blindness attributing to 1% of global blindness, occurs due to long-term accumulated damage to the small blood vessels in the retina.² Diabetes is among the leading causes of kidney failure.³ The overall risk of dying among people with diabetes is at least double the risk of their peers without diabetes.⁴ In 2012, an estimated 1.5 million deaths were directly caused by diabetes.⁵ More than 80% of diabetes related deaths occur in low- and middle-income countries.⁵ WHO projects that diabetes will be the 7th leading cause of death in 2030.⁶ In 2010, India was considered as the “diabetes capital” of the world, with the largest number of people suffering from diabetes in the world (50.8 million), followed by China (43.2 million) and the United States (26.8 million).⁷ In 2011, the prevalence of type 2 diabetes was high in the relatively prosperous southern cities of India.⁷

Type 2 diabetes mellitus causes deregulation of carbohydrate, protein and fat metabolism with excess production of free radicals. The main lipid abnormalities in type 2 diabetes are hyper-triglyceridemia, hypercholesterolemia and low HDL. Low plasma HDL level is a powerful risk factor of coronary heart disease. Each 1 mg/dl increase in HDL cholesterol is estimated to decrease cardio-vascular events by 2% in men & 3% in

women. Apart from its role in reverse cholesterol transport, HDL has anti-inflammatory & antioxidant properties.⁸ Elevated triglyceride content in muscle, liver, and beta cell, leads to the development of peripheral insulin resistance and impaired insulin secretion causing hyperglycaemia in type 2 diabetes. Insulin resistance further contributes to the development of diabetic dyslipidemia by favouring the hepatic production of atherogenic lipoproteins and by suppressing the uptake of circulating lipids in peripheral tissues. Hyperglycaemia overloads glucose metabolic pathways resulting in excess free radical production and depletion of antioxidant defences such as glutathione, superoxide dismutase, catalase, ascorbic acid, ceruloplasmin⁹ and uric acid¹⁰ causing oxidative stress.¹¹ In uncontrolled diabetes due to the absence of sufficient electron acceptor substrates, free electrons are directly transferred to transition metals, such as iron and copper, which catalyze the free radical formation, as in the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + OH^-$). Chronic hyperglycaemia facilitates free radical mediated auto-oxidation of glucose,¹² non enzymatic protein glycosylation and lipid peroxidation.¹³ Lipid peroxidation can also produce singlet oxygen, hydroperoxides, lipid epoxides, and many damaging aldehydes particularly malondialdehyde and 4-hydroxy nonenal, which are rather long-lived and drift far from membranes, damaging a wide variety of proteins, lipids and nucleic acids. Such damaged molecules are called Advanced Lipid peroxidation End-products (ALE).¹⁴

In the presence of hyperglycaemia, non-enzymatic reactions occur between intracellular glucose-derived dicarbonyl precursors like glyoxal, methylglyoxal, and 3-deoxyglucosone and the amino groups of intracellular and extracellular proteins forming Advanced Glycation End products (AGEs). AGEs bind to their cognate cell-surface receptor, RAGE, resulting in the generation of intracellular oxygen free radicals and the activation of gene expression resulting in cellular damage. The detrimental effects of the AGE/RAGE signalling axis within the vascular compartment include generation of reactive oxygen species in endothelial cells, release of pro-inflammatory cytokines and growth factors from intimal macrophages, increased pro-coagulant activity on endothelial cells and macrophages and enhanced proliferation of vascular smooth muscle cells and synthesis of extracellular matrix. AGEs can directly cross-link extracellular matrix proteins like type I collagen in large vessels, decreasing their elasticity, predisposing these vessels to shear stress and endothelial injury leading to atherosclerosis.¹⁵ AGE-modified matrix components also trap non-glycated plasma or interstitial proteins like LDL, retard their efflux from the vessel wall and enhance the deposition of cholesterol in the intima, thus accelerating atherogenesis. AGE-induced cross-linking of type IV collagen in basement membrane decreases endothelial cell adhesion and increases extravasation of fluid. Proteins cross-linked by AGEs are resistant to proteolytic digestion, thus decreasing protein degradation while enhancing protein deposition. In capillaries, including those of renal glomeruli, plasma proteins such as albumin bind to the glycated basement membrane, accounting in part for the basement membrane thickening that is characteristic of diabetic microangiopathy. Retinal expression of VEGF, a mediator of the late complications of diabetes is increased by AGE-RAGE interaction.¹⁶ Chronic hyperglycaemia causes increased Protein Kinase C (PKC) activity and is associated with many processes involved in the pathology of diabetic complications including the regulation of vascular permeability, blood flow and neovascularisation. Persistent hyperglycaemia leads to an increase in intracellular glucose in some tissues that do not require insulin for glucose transport e.g., nerves, lenses, kidneys, blood vessels. This excess glucose is metabolized by the enzyme aldose reductase to sorbitol, a polyol which increases cellular susceptibility to oxidative stress causing cell death. Furthermore, stress-sensitive signalling pathways including p38 Mitogen Activated Protein kinase (MAPK) and, c-Jun N-terminal kinase (JNK) are strongly activated by sorbitol. This pathway is significant in the development of complications like neuropathy, nephropathy, retinopathy and the cataract formation in diabetics. Thus, hyperglycaemia mediated rise in ROS production activates well characterized biochemical pathways like AGE/RAGE pathway, PKC pathway, polyol pathway, hexokinase pathway in addition to the stress sensitive signalling pathways such as Nuclear factor - κ B (NF- κ B), serine/threonine kinase(s), JNK, MAPK, that play a significant role in the development of diabetic vascular complications.

II. Materials And Methods

This cross-sectional study was conducted in the Department of Biochemistry of Siddhartha medical college, Vijayawada. This study was approved by the Institutional Ethical Committee. All the participants in the study were randomly selected from the patients attending the Diabetic clinic in the Government general hospital, Vijayawada for treatment. After explaining the purpose of this study, an informed written consent was obtained from each participant in their mother tongue. The subjects belonging to the age group of 35-60 years were included in this study. This study included 50 apparently healthy individuals accompanying the patients as controls, 50 Type 2 diabetics without any complications attending the diabetic clinic for routine check-up and 50 Type 2 diabetics with long term vascular complications admitted in the Government general hospital, Vijayawada for treatment. Patients with Hemoglobinopathies, Anemia, renal or hepatic or thyroid disorders, febrile illness, diabetic keto-acidosis, and any acute or chronic inflammatory diseases were excluded from the

study. Patients with Type 1 diabetes mellitus were also excluded. Pregnant women, Smokers and Chronic alcoholics were also excluded from the study.

After taking complete history, a thorough clinical examination and appropriate laboratory investigations were performed for all the study subjects. The study subjects were divided into three groups with each group containing 50 subjects. Group 1 included age and sex matched healthy controls, group 2 included type 2 diabetics without any identifiable complications and group 3 included type 2 diabetics with vascular complications such as cardiovascular, cerebro-vascular, peripheral vascular and micro-vascular complications like nephropathy, retinopathy and neuropathy. Cardiovascular complications were confirmed by ECG findings of post myocardial infarction. Cerebro-vascular complications like ischemic stroke were confirmed by CT scan findings suggestive of cerebral infarction. The clinical diagnosis of diabetic foot ulcer confirms diabetic neuropathy combined with reduced blood flow in the feet due to peripheral vascular disease. Diabetic nephropathy was confirmed by proteinuria and other renal function tests. Diabetic retinopathy was confirmed by the examination of fundus with ophthalmoscope. Group 3 consisted of 7 cases of stroke, 11 cases of myocardial infarction, 18 cases of diabetic foot ulcer, 9 cases of diabetic nephropathy and 5 cases of diabetic retinopathy.

Under aseptic precautions, 8ml of blood was drawn from the median cubital vein of the study subjects after 12 hours of over-night fasting. The sample was collected in two tubes, one plain and the other containing anticoagulant mixture of potassium oxalate and sodium fluoride in the ratio of 2:1. Whole blood was used to estimate serum Malondialdehyde by Thiobarbituric acid method¹⁷ and HbA1C by Ion exchange resin method.¹⁸ Fasting plasma glucose levels were estimated by Glucose Oxidase-Peroxidase method. Serum was used to estimate Cholesterol by Cholesterol Oxidase-Peroxidase method, Triglycerides by Glycerol Phosphate Oxidase method, HDL-C by Phosphotungstic acid method, Ceruloplasmin by Ravin's method¹⁹ and Uric acid by Modified Trinder Peroxidase method.²⁰ VLDL-C and LDL-C were calculated by Friedwald's formula. Results were analyzed by descriptive statistical analysis using Excel and Medcal statistical software. Results were expressed in terms of Mean \pm standard deviation for each variable. Comparisons between variables were done using Analysis of variance (ANOVA) and $p < 0.001$ was considered as statistically significant. Pearson's correlation was used to correlate between the variables and $p < 0.05$ was considered as statistically significant.

III. Results

Fasting plasma glucose levels were significantly elevated in type 2 diabetics with vascular complications than in healthy controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). The mean HbA_{1c} levels, a measure of average glycaemic status over previous 8-12 weeks, were significantly elevated in type 2 diabetics with vascular complications than in healthy controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). This study showed significant increase in the mean levels of serum cholesterol, serum triglycerides, VLDL-C and LDL-C in type 2 diabetics with vascular complications when compared to control group ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). While mean HDL-C levels showed significant decrease in the cases when compared to healthy controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). These findings were suggestive of the significant dyslipidemia in type 2 diabetics with vascular complications compared to healthy controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). The levels of malondialdehyde, ceruloplasmin and uric acid were significantly elevated in type 2 diabetics with vascular complications than in controls ($p < 0.001$) and type 2 diabetics without vascular complications ($p < 0.001$). The results obtained were summarised in the table-1. There was statistically significant positive correlation between malondialdehyde, ceruloplasmin and uric acid with each other ($p < 0.001$) and with fasting glucose, HbA_{1c}, total cholesterol, triglycerides, LDL, VLDL ($p < 0.001$) in type 2 diabetics with vascular complications. These parameters had statistically significant negative correlation with HDL ($p < 0.001$) in type 2 diabetics with vascular complications. These results were summarised in the table-2.

Table 1: Comparison of different parameters among study groups

S. No.	Parameter	Controls (N=50) (Mean \pm S.D)	Type 2 diabetics without complications (N=50) (Mean \pm S.D)	Type 2 diabetics with vascular complications (N = 50) (Mean \pm S.D)
1.	Age (in years)	46.00 \pm 5.31	48.00 \pm 7.28	49.00 \pm 6.49
2.	Fasting plasma sugar (mg/dL)	87.08 \pm 15.68	152.86 \pm 23.80	174.64 \pm 38.68*
3.	HbA _{1c} (% of Hb)	5.84 \pm 0.28	8.18 \pm 0.96	12.34 \pm 3.21*
4.	Serum total cholesterol (mg/dL)	162.94 \pm 37.43	240.94 \pm 31.91	298.37 \pm 56.19*
5.	Serum triglycerides (mg/dL)	127.08 \pm 44.24	208.12 \pm 40.07	264.86 \pm 61.91*
6.	High density lipoprotein (mg/dL)	38.50 \pm 4.05	32.50 \pm 3.26	26.49 \pm 4.23*
7.	Very low density lipoprotein (mg/dL)	25.38 \pm 8.92	41.66 \pm 8.06	52.97 \pm 12.38*
8.	Low density lipoprotein (mg/dL)	99.36 \pm 34.49	167.42 \pm 29.22	218.91 \pm 39.58*
9.	Malondialdehyde (nmol/mL)	2.31 \pm 0.61	4.54 \pm 0.79	7.83 \pm 3.52*
10.	Ceruloplasmin (mg/dL)	45.50 \pm 8.11	70.20 \pm 8.47	96.31 \pm 11.33*
11.	Uric acid (mg/dL)	3.53 \pm 0.38	4.43 \pm 0.53	6.79 \pm 1.94*

*statistically significant, $p < 0.001$

Table 2: Correlation of oxidative stress markers with other parameters

Parameter	Malondialdehyde (r value)*	Ceruloplasmin (r value)*	Uric acid (r value)*	Statistical significance
Fasting glucose	0.897	0.832	0.810	$p < 0.001$
HbA _{1c}	0.886	0.846	0.804	$p < 0.001$
Total cholesterol	0.862	0.839	0.811	$p < 0.001$
Triglycerides	0.821	0.784	0.795	$p < 0.001$
HDL-c	- 0.782	-0.765	-0.746	$p < 0.001$
VLDL-c	0.820	0.781	0.797	$p < 0.001$
LDL-c	0.852	0.864	0.786	$p < 0.001$
Malondialdehyde	-	0.973	0.798	$p < 0.001$
Ceruloplasmin	0.973	-	0.827	$p < 0.001$
Uric acid	0.798	0.827	-	$p < 0.001$

*r value = Pearson correlation coefficient, $p < 0.001$ is considered statistically significant

IV. Discussion

In this study fasting plasma glucose levels and HbA_{1c} were significantly increased along with marked dyslipidemia in type 2 diabetics with vascular complications. Elevated HbA_{1c} indicates poor metabolic control in type 2 diabetics with a substantially increased risk of incident cardiovascular events and deaths.^{21, 22} The present study showed a significant increase in mean MDA levels in type 2 diabetics with vascular complications when compared to controls indicating increased lipid peroxidation in cases when compared to controls. In this study, type 2 diabetics with vascular complications showed increased values of the acute phase protein, Ceruloplasmin indicating the presence of inflammation when compared to control groups. Ceruloplasmin (CP) acts as a ferroxidase and decreases the availability of the iron in free radical generating reactions.²³ An increase in the level of CP provides protective action against free radical injury.²⁴ Alternatively, an increase in serum CP in type 2 diabetes could generate excess oxidized LDL, which causes atherosclerosis.²⁵ It could also cause vascular injury by generating free radicals, such as hydrogen peroxide, in the course of oxidation of serum homocysteine.²⁶ This study showed significantly increased mean levels of serum Uric acid in cases when compared to controls. In humans, uric acid is the main plasma antioxidant followed by vitamin C. Uric acid stabilizes vitamin C in plasma and protects it from oxidation. Uric acid in the blood can scavenge superoxide radicals, hydroxyl radicals, singlet oxygen and can chelate transition metals.²⁷ Uric acid can also block the reaction of super oxide anion with nitric oxide forming peroxynitrite which is a particularly toxic product that can injure cells by nitrosylating the tyrosine residues (nitro tyrosine formation) of proteins.²⁸

This study showed significant positive correlation of MDA, Ceruloplasmin and Uric acid with HbA_{1c} indicating that poor glycaemic control is associated with increased lipid peroxidation, inflammation and oxidative stress in type 2 diabetic patients. In this study, there was significant positive correlation of MDA, Ceruloplasmin and Uric acid with Total Cholesterol, Triglycerides, VLDL-C and LDL-C and negative correlation with HDL-C indicating the association of dyslipidemia with elevated lipid peroxidation, inflammation and oxidative stress. This study also showed significant positive correlation between MDA, Ceruloplasmin and Uric acid indicating association of lipid per-oxidation, low grade inflammation and oxidative stress with each other. This association has been known to promote atherogenesis, endothelial dysfunction and vascular injury of both macro and micro-vasculature, leading to the development of diabetic vascular complications. A similar finding was observed in other studies conducted by M. M. Kesavulu et al.,²⁹ Turk et al.,³⁰ Pasaoglu H et al.,³¹ Komosinska et al.,³² Ozdemir et al.,³³ B.Virgolici et al.,³⁴ Sarkar et al.,³⁵ and Natheer H Al-Rawi et al.³⁶

V. Conclusion

In this study, the type 2 diabetics with vascular complications had poor metabolic control, severe dyslipidemia, increased lipid peroxidation, inflammation and oxidative stress when compared to type 2 diabetics without complications and healthy control group. These observations suggest that strict glycaemic control and supportive therapy aimed at the reduction of dyslipidemia and oxidative stress may prevent the development and progression of vascular complications, responsible for the increased mortality and morbidity associated with type 2 diabetes mellitus.

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