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Original Research Article

Oxidative Stress and Paraoxonase (PON - 1) Status in Diabetic Nephropathy

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ABSTRACT

Background: Hyperglycemia in diabetes can increase oxidative stress through several well known pathways. Oxidative stress is hypothesized to play a role in the development of diabetic complications like diabetic nephropathy. Thus keeping this in mind we investigated the process of lipid peroxidation (MDA) and activities of antioxidant enzymes (PON-1) in patients with DM with or without nephropathy.
Objectives: 1.To measure the ratio of microalbumin/creatinine ratio in urine to identify nephropathy patients. 2. To estimate malondialdehyde (MDA) levels as an indicator of lipid peroxidation and paraoxonase-1(PON-1) as an antioxidant enzyme in diabetic nephropathy patients.3.To correlate MDA and PON-1 enzyme levels in pathophysiology of nephropathy.
Materials And Methods: The prospective case control study included total one forty nine (149) participants which were divided into groups as control; non diabetic (n=40), healthy, age and sex matched. DM without nephropathy (n=69), DM with nephropathy (n=40).The diabetic nephropathy was diagnosed based on urinary microalbumin/creatinine ratio. The estimation of serum MDA was done by method of Wilbur K. M et al (1949) and PON-1 spectrophotometrically by the method of Eckerson et al (1983) using paranitro-phenylacetate as substrate. The study protocol was approved by institutional ethics committee. The statistical analysis was done by using SPSS software version 19. The data obtained was analyzed by using student "t" test. The p –value of 0.001 was considered to be significant. Results: We found statistically significant rise in the levels of serum MDA (p=<0.001), in diabetic nephropathy as compared to diabetic group which was higher as compared with controls. We also found the statistically significant (p=<0.001), fall in the levels of serum PON-1 in diabetic nephropathy and diabetic cases as compared with controls. Conclusion: High serum MDA levels and low serum PON-1 levels are associated with pathophysiology of diabetic nephropathy.

Key Words: Malondialdehyde, Paraoxonase-1, Diabetic nephropathy.

INTRODUCTION

Diabetes is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin production and or insulin action and impaired function in the metabolism of carbohydrates, lipids, and proteins which leads to long term health complications. (1)

In diabetic patients long term damage, dysfunction and failure of different organs especially the eye (diabetic retinopathy), kidneys (nephropathy), nerves (neuropathy), heart (myocardial infarction) and blood vessels (atherosclerosis) are related to the uncontrolled hyperglycemia. (2)

Vascular complications are the major determinants of morbidity and mortality in patients with diabetes mellitus. The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have identified hyperglycemia as a major independent risk factor for the manifestation of micro vascular and cardiovascular complications in both Type 1 and Type 2 diabetes mellitus. [(3)] Hyperglycemia is clearly recognized as the primary culprit in the pathogenesis of diabetic complications. [(4)]

Hyperglycemia leads to non enzymatic glycation of intracellular and extracellular proteins with the formation of advanced glycation end products (AGEs), a heterogeneous group of compounds that have been implicated in the pathogenesis of many complications of diabetes. [(5)]

Oxidative stress is an imbalance between the systemic manifestations of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cells including proteins, lipids, DNA. [(6)] Oxidative stress is regarded as a common and major factor that couples hyperglycemia with vascular complications via two mechanisms i.e. first the metabolic modifications of target tissue molecules and second the alterations in the renal hemodynamic. There is association of hyperglycemia and oxidative stress and both affects the mechanism which alters renal hemostasis and ultimately contribute to the development of diabetic nephropathy. [(7)]

Free radicals damage lipids by initiating a process called lipid peroxidation. This process starts when one hydrogen atom is removed from a methylene group in a lipid. These include hydroxyl, hydroperoxyl, alkoxyl and peroxyl radicals. Polyunsaturated fatty acids are the usual targets of these which contain 2 or more double bonds.

When one hydrogen atom removed, it leaves lipid itself as free radical which on further combination with molecular oxygen gives peroxyl radical which reacts with another fatty acid to give peroxide molecule. Thus it starts with chain reaction generating many peroxides. The decomposition of lipid peroxides in presence of transition metals like Fe, Cu, gives cytotoxic compounds includes MDA - malondialdehyde and hydroxynonenal (NNE) which can cause chemical modification of membrane phospholipids, proteins and DNA. [(8)]

ANTIOXIDANT: Antioxidant is defined as any substance that prevents the process of oxidation or repairs the damage caused by action of oxidants in a living organism. *I. Enzymatic Antioxidants:* [(9)] Superoxide Dismutase(SOD), Catalase (CAT), Glutathione Peroxidase (GPx),Glutathione Reductase (GR), Paraoxonase-1(PON-1), etc. II. Non-Enzymatic Antioxidants (4): Vitamin E, Vitamin C, β- carotene, etc.

Can also be classified as, A) Preventive antioxidants: inhibit the initial production of free radicals e.g. catalase, EDTA, glutathione peroxidase, B) Chain breaking antioxidants: inhibit the propagative phase e.g. uric acid, SOD, Vitamin E. [(9)]

Paraoxonase-1(PON-1)-(E.C.3.1.8.1)

Paraoxonase is a polymorphic enzyme located on chromosome 7 having 3 gene families. PON 1, PON 2, PON3. It is one of the enzymatic antioxidants. Paraoxanase 1 (PON1) is a 355 amino acid glycoprotein with a molecular weight of 43Kda, synthesized in liver and transported in plasma by binding to High Density Lipoprotein (HDL). [(10,11)]

It is calcium dependent esterase associated with circulating HDL having three activities namely paraoxonase, arylesterase and diazoxonase with

lipophilic anti-oxidant characteristics. [12] It hydrolyses phospholipids, hydroperoxides and cholesterol ester to respective hydroxides and also degrades hydrogen peroxide (peroxidase activity). It has anti inflammatory and anti atherogenic properties. [13]

In DM, glycation has major impact on PON1 activity contributing typical inflammatory process leading to its manifestations and complications. PON1 also protects plasma membrane from free radical injury. [14] Furthermore, PON1 degrades bioactive phospholipids, such as platelet activating factor, thereby preventing intravascular coagulation. [15] Due to its central role in hydrolyzing xenobiotics in addition to its implications in atherosclerosis, the product of the PON1 gene i.e. PON1 has been studied extensively compared to other members of the family. However, PON2 and PON3 are also protective against oxidative stress. [14]

Calcium has two roles in the enzymatic reaction of PON1. First, it maintains the active site, by participating directly in the catalytic reaction or by maintaining the appropriate conformation of the active site. Secondly, calcium facilitates the removal of diethyl phosphate from the active site, rendering the phosphorus susceptible to nucleophilic attack. [16]

The structure of PON1 implies that HDL anchoring can modify its active site. Apolipoprotein-A1 (apoA1) contained in HDL may stabilize PON1, and stimulates its hydrolytic activities and its lactonase activity in particular to provide an optimal environment for the interaction of PON1 with its natural substrates. [17]

Substrates of PON1:

PON1 has two active sites, a calcium-dependent site which hydrolyses organophosphates and related substrates and a residue but not calcium dependent which hydrolyses oxidized lipids. PON1 hydrolyzes the active metabolites of several organophosphate insecticides like paraoxon, chlorpyrifos oxon and diazoxon and nerve agents such as soman and sarin. In addition, PON1 hydrolyzes a number of drugs or pro-drugs. [18]

The hydrolytic activities of PON1 are defined under three broad categories: lactonase, arylesterase and phosphotriesterase. The oxidized lipids, homocysteine and a range of lactones including thiolactones act as possible substrates. PON1 also exhibits homocysteine thiolactonase or HTLase activity that detoxifies homocysteine thiolactone which may cause protein damage by homocysteinylation of the lysine residues and thus lead to atherosclerosis. PON1 destroys hydrogen peroxide, suggesting that it has peroxidase activity. [15] PON1 efficiently metabolizes the enzymatic and nonenzymatic oxidation products of arachidonic and docosahexaenoic acid respectively, suggesting that both are possible endogenous substrates of PON1. [18]

Biochemical Functions of PON1:

1) PON1 has been associated with the development of atherosclerosis. [19] 2) The hydrolytic active site of PON1 was shown to mediate two major anti-atherogenic functions i.e. protection of LDL from oxidation and stimulation of HDL-mediated macrophage cholesterol efflux. [17]

3) Serum PON1 thus metabolizes oxidized lipids. PON1 is also associated with phospholipids.

4) Phospholipid complexes promote the release of PON1, and maintain enzyme activity by stabilizing the enzyme after its release. PON1 hydrolyses arylesters and organophosphate compounds. [11] PON1 was shown to inhibit cholesterol influx by Ox-LDL into macrophages by different mechanisms:

1) Hydrolysis of macrophage oxidized lipids,

2) Reducing macrophage-mediated formation of Ox-LDL,

3) Increasing the breakdown of oxidized lipids in Ox-LDL, and

4) Decreasing macrophage uptake of Ox-LDL.

5) PON1 also enhances macrophage cholesterol efflux. [17]

Enzymatic and nonenzymatic systems of antioxidative protection are included in scavenging free radicals and their metabolic products and in maintaining normal cellular physiology. Increased level of free radicals and impairment of antioxidant status are the processes underlying pathophysiologic mechanisms in a variety of diseases like atherosclerosis, diabetes mellitus, etc. [20]

Thus to study the oxidative stress status in patients with diabetic nephropathy as a micro vascular complication of diabetes mellitus, we have planned to study the relationship between one of the lipid peroxidation marker malondialdehyde (MDA) and the antioxidant paraoxonase (PON-1) in patients with diabetic nephropathy.

AIM AND OBJECTIVES:

AIM: To evaluate the relationship between the oxidative stress and PON -1 in diabetic nephropathy. OBJECTIVES:1.To estimate the MDA levels as an lipid peroxidation residue in diabetic nephropathy.2.To estimate PON-1 levels as an antioxidant in diabetic naphropathy.3.To measure the microalbumin to creatinine ratio to identify nephropathic patients.4.To correlate MDA and PON-1 levels in diabetic nephropathy.

MATERIALS AND METHODS

• **Study Design:** The study was a prospective case control study, conducted in the department of biochemistry; of university medical college with a tertiary care hospital. Between May 2014 to October 2014.

• **Ethics:** Institutional ethical committee approval was obtained for the study.

• **Selection of patients:** The study group was selected from the patients attending the general medicine O.P.D. of the tertiary care hospital. The study was comprised of total one forty nine (149) participants. They were divided into the study group and control group as follows

• **Group A:** Control; Non diabetic:
Forty (40) age and gender matched normal; healthy non diabetic individuals were enrolled in the study as controls.

• **Group B:** DM without nephropathy:
Sixty nine (69) clinically diagnosed type 2 diabetic patients without nephropathy.

• **Group C:** DM with nephropathy:
Based on clinical examination and urinary microalbumin to creatinine ratio, forty (40) clinically diagnosed type 2 diabetic patients with nephropathy were enrolled in the study. Nephropathy was diagnosed according to microalbumin to creatinine ratio, calculated from first spot morning urine collections. (pathological if > 2.5 mg / mmol in men and > 3 mg / mmol in women)

• **Inclusion Criteria for Controls:**
Healthy, nondiabetic, age (age group above 40 years) and gender matched.

• **Inclusion Criteria for Cases:**
The study group included clinically diagnosed type 2 diabetic non nephropathic and nephropathic patients of age group above 40 years.

• **Exclusion Criteria for cases and controls:**

1. Cardiovascular diseases, renal diseases, Liver diseases, malignancy
2. Participants taking following drugs were excluded from the study ACE inhibitors, CCB, Diuretics, B- blockers
3. Alcoholics and smokers.

Collection of Blood Sample: About 2 ml of fasting blood sample was collected by venous puncture with all aseptic precautions in a plain and fluoride vacutainer. It was allowed to clot for one hour for separation of serum. The serum and plasma was separated by centrifugation at 2500 rpm for 5 minutes at room temperature. Serum and plasma were ensured to be free from hemolysis and turbidity. Separated serum and plasma samples were subjected towards estimation of following parameters,

1. Blood Glucose: Automated Biochemistry analyzer: Glucose –Oxidase Peroxidase method (GOD-POD)
2. Serum Lipid profile:
i)Serum Total Cholesterol (CHO):Cholesterol Oxidase- peroxidase (CHOD-PAP) method
ii) Serum Triglycerides (TG): Glycerol phosphate oxidase method
iii) Serum High Density Lipoprotein Cholesterol (HDL): direct Enzymatic method
iv)Serum Low Density Lipoprotein Cholesterol (LDL) and
v) Serum Very Low Density Lipoprotein Cholesterol (VLDL) Calculated by Friedewald's equation
3. Glycosylated Haemoglobin: Boronate Affinity Assay
Spot morning urine sample was collected from diabetic group B and C. They were classified as diabetic nephropathic and diabetic nonnephropathic based on urinary microalbumin / creatinine ratio.

OBSERVATION & RESULTS

Table no.1: Biochemical characteristics in control and type 2 diabetic and diabetic nephropathy patients

Variables	Controls (Group A, *n*=40)	Type 2 diabetic patients(Group B, *n*=69)	Type 2 diabetic pts. With nephropathy (Group C, *n* =40)	p value
BGL (F) mg/dl	94.68 ± 13.822	129.77 ± 55.989	136.10 ± 49.223	<0.001
BGL(PP) mg/dl	123.50 ± 14.31	168.09 ± 82.251	190.23 ± 73.895	<0.001
HbA_1C %	5.14 ± 0.53	7.13 ± 1.87	7.36 ± 1.77	<0.001
Micro.alb/crt (ACR) mg/mmol	5.14 ± 0.53	21.30±7.690	47.55 ± 3.34	<0.001
Total Cholesterol mg/dl	162.0 ± 33.2	171.7±45.4	169.4±47.59	0.0524
TG mg/dl	106.08 ± 54.87	142.14 ± 71.02	154.20 ± 88.45	<0.001
HDL –C mg/dl	43.2 ± 9.4	41.7±9.1	41.2 ±9.6	0.601
LDL-C mg/dl	96.06 ± 33.5	92.99±41.53	85.52±43.25	0.475
VLDL-C mg/dl	23.28 ±16.03	40.7 ±44.2	42.9±35.3	<0.001
MDA nmol/ml	2.22 ± 1.16	4.00±2.28	4.15±2.34	<0.001
PON-1 IU/L	341.5 ±161.8	252±167.77	230.17±166.01	< 0.001

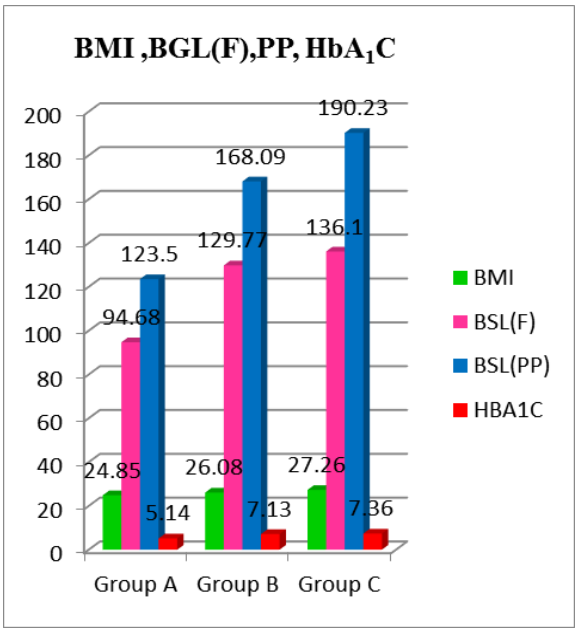

Diagram 1: Diagram showing the comparison between BMI,BGL(F),PP and HbA1C among three groups

After comparing the biochemical characteristics among cases and controls we found that there was statistically significant difference in values of BGL(F,PP), HbA1C, TG, VLDL-C, MDA, PON-1,Microalbumin/crt.ratio(ACR) with p –value <0.001 whereas there was no statistically significant difference in values of HDL-C, LDL-C, total cholesterol.

However, the rise in BGL (F,PP), TG, HbA1C, VLDL-C, MDA, PON-1, Microalbumin/crt .ratio(ACR) was seen more in diabetic nephropathy group as compared to diabetic group.

There was a statistically significant increase in BMI in diabetic and diabetic nephropathy patients group as compared to normal controls with p value 0.008.

There was statistically significant increase in the BGL (F),PP and HbA1C levels among diabetic and diabetic nephropathy group as compared to normal group with p value < 0.001.

p value < 0.001 was considered as statistically significant.

"Z" test was used to find difference between means of all groups. Anova test was applied for comparing BMI, BGL (F), PP and HbA1C in all the groups.

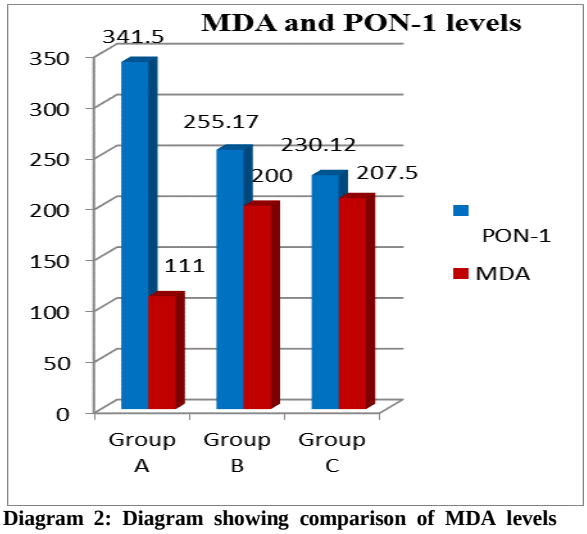

Diagram 2: Diagram showing comparison of MDA levels and Paraoxonase-1 (PON-1) levels in all groups

There was statistically significant decrease in the activity of PON1 in diabetic and diabetic nephropathy group as compared to control group whereas there was statistically significant increase in the levels of MDA in DM and DM with nephropathy group as compared to normal group.

There was an increase in cholesterol levels in diabetic and diabetic nephropathy patients group as compared to normal controls but that was not statistically significant with p value 0.524. There was a statistically significant increase in triglyceride levels in diabetic and diabetic nephropathy patients group as compared to normal controls with p value 0.009.

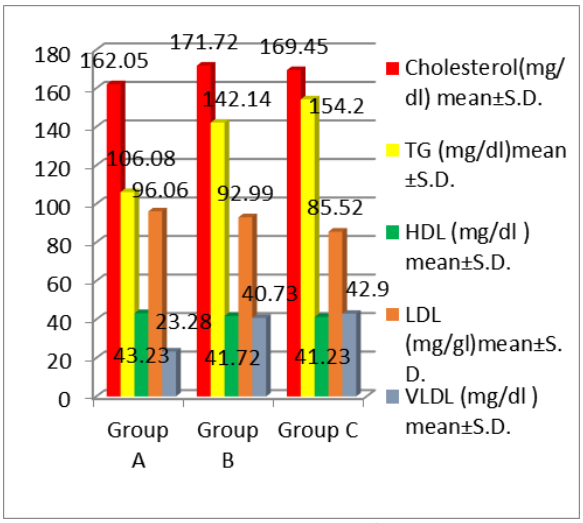

Diagram 3: Diagram showing lipid profile among groups

DISCUSSION

The present study was designed to evaluate the relationship between oxidative stress and antioxidant status in patients with type 2 diabetes and diabetic nephropathy. Diabetic nephropathy is one of the most important micro vascular complication of DM and a major cause of end stage renal disease. Many pathways have been involved in the pathogenesis of diabetic nephropathy including oxidative stress, activation of protein kinase C, increased production of AGE, polyol - hexosamine pathway flux. The extreme production of ROS has been suggested as a common result leading to intensified oxidative damage at the level of lipid peroxidation and peak in diabetic nephropathy in association with diabetes. Thus any treatment that can stabilize oxygen metabolism and regulate oxidative stress can attenuate and delay the development of diabetic nephropathy. In diabetic nephropathy, excessive free radical generation has been shown to decrease the activities of antioxidant enzymes. [(21)]

SUMMARY AND CONCLUSION

The objectives of the present study were to evaluate oxidative stress in patients of diabetic nephropathy by measuring MDA and PON-1 levels.

The study included clinically diagnosed hundred and nine (109) cases of type 2 diabetes mellitus. These were divided into two subgroups i.e. diabetic patients without nephropathy (69) and diabetics with nephropathy (40). These cases were compared with 40 normal, healthy, age and sex matched controls.

In the group of diabetic patients, serum MDA levels were (4.00 ±2.28).In the group of diabetic nephropathy, serum MDA levels were (4.15 ±2.34).In control group the serum MDA levels were (2.22 ±1.16).

This indicates that there is increased lipid peroxidation in diabetics

and in diabetics with nephropathy patients. The result also showed significant fall in serum PON-1 activity in diabetic group (255.17 ± 166.01) as well as in diabetic nephropathy group (230.12 ± 167.85) as compared with control group (341.50 ±161.85).

This indicates that PON-1 has antioxidant activity and the defective antioxidant defense mechanism in diabetic nephropathy patients leading to increased lipid peroxidation.

Our study findings reveal increased production of reactive oxygen species enhancing lipid peroxidation with concomitant failure of antioxidant defense mechanism. There is disturbance in the oxidant-antioxidant status in type 2 diabetes. Alteration in antioxidant barrier and increase in the process of lipid peroxidation may enhance the progression of atherogenesis in diabetes.

The further scope for research in this area would be the study of oxidative stress and antioxidant status in relation with -

1. Role of PON-1 as an alternative antioxidant therapy seems to be promising in preventing the induction and progression of diabetic nephropathy along with ACE inhibitor and AT1 receptor blockers, which provide direct renoprotective effects. [(22)]
2. Role of PON-1 to ameliorate oxidative stress and delay the occurrence of diabetic nephropathy in diabetes mellitus or to halt the progression of diabetic nephropathy.
3. Role of PON-1 (along with microalbumin to creatinine ratio which is an early established marker) as an early marker of an reversible incipient stage of diabetic nephropathy so that intervention at this stage of disease would help to reverse the condition and prevent the patient from landing into the fatal end stage renal disease, thus reducing mortality and improving the survival in diabetic nephropathy individuals.

Limitation of our study:

1. As we have studied the PON-1 status in respect of its aryl esterase activity only, PON-1 status can be studied in detail with respect to its polymorphism and other activities and its association with diabetic nephropathy which will add further details to the present knowledge as PON-1 is considered as a candidate gene for susceptibility for diabetic nephropathy.

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