## ORIGINAL ARTICLE

# To study the Oxidative Stress and Antioxidants status in Diabetes Mellitus without Complication

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## ABSTRACT

**Objective:** To study the oxidative stress and Antioxidants status in Diabetes mellitus without complication

**Methods:** Twenty Two patients of type II diabetes mellitus without complication and Fourteen healthy, non-diabetic, age and sex matched individuals were selected as the controls. Oxidative stress markers (NO, LPO) and antioxidant markers (GSH, SOD) measured by appropriate methods in both the groups.

**Results:** In type II diabetes mellitus without complication Free Radicals measurement were LPO MDA (23.95±0.85 μmol/L) and NO (702.1±19.1 nmol/L) and in healthy, non-diabetic, age and sex matched individuals Free Radicals measurement were LPO MDA (17.05±2.10) and NO (527.7±16.0) with p value 0.007 and <0.001 respectively which is statistically significant. Where as In type II diabetes mellitus without complication antioxidant measurement GSH (78.3±2.77μmol/L)and SOD (891.6±52.3 U/L) and in healthy, non-diabetic, age and sex matched individuals antioxidant measurement were GSH (96.2±1.69) and SOD (1323.2 ± 105) with p value <0.001 and 0.002 respectively which is statistically significant.

**Conclusion:** The current study suggests that the oxidative stress may have role in the pathogenesis of diabetes, as indicated by the increased levels of lipid peroxidation product malondialdehyde in patients than age and sex matched healthy controls. The decreased levels of the Glutathione peroxidase and Superoxide dismutase in the antioxidant enzyme mechanisms indicates that the anti-oxidative enzyme system gets impaired more diabetic patients.

**Key words:** Oxidative Stress, Antioxidants, Diabetes Mellitus, Complications

## INTRODUCTION:

Diabetes mellitus (DM) is one of the most common non-communicable diseases globally. The global prevalence of diabetes mellitus was 366 million in 2001 and it is estimated to increase 522 million by 2030. Eighty per cent of people with diabetes live in low-and middle income countries. According to the number of patients, India (61.3 millions) rank second in world, and China (90 millions) is first in 2011.[1]

Diabetes is a leading cause for cardiovascular disease, stroke, blindness, amputations and end stage renal disease in the world (IDF 2005). The most common form of diabetes mellitus is type-2 and constitutes about 60-95% of all diabetes cases (ADA, 2011). Diabetes is usually associated with the increased production of free radicals or impaired antioxidant defences has been shown that oxidative stress has an adverse effect on glucose metabolism.[2] Development of the disabling chronic complications of diabetes mellitus (DM) has also been attributed to oxidative stress The formation of oxygen free radicals is a normal consequence of endogenous essential biochemical reactions. Neutrophil use ROS, generated by enzyme NADPH oxidase as defence mechanism against microbes. There are also exogenous sources of free radicals, such as cigarette smoke, air pollutants and radiation. [3]

Reactive oxygen species lead to oxidative damage to protein lipid and DNA, which leads to cell dysfunction or cell death. These endogenously or exogenously produced ROS are neutralized by antioxidant system. Antioxidant systems are mainly two types enzymatic and nonenzymatic. The enzymatic antioxidants include several enzymes,such as glutathione peroxidase, catalase and superoxide dismutase. The non-enzymatic micronutrient system involves small molecular weight molecules, e.g. glutathione and vitamins, e.g. vitamin E (tocopherol), vitamin C (ascorbic acid) and pre-vitamin A (β-carotene).[4]

Increasing evidence in both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus Hyperglycaemia is the hallmark of diabetes mellitus. There is also evidence of impaired antioxi-

dant defence system, such as reduced levels of endogenous antioxidants, reduced antioxidant enzyme activities in diabetes. [5]

The data pertaining to oxidative stress and Antioxidants in type II diabetes are scanty from our country and especially from this region. Present study is aimed to look into the status of oxidative stress markers and Antioxidants levels related to the antioxidative system, in the patients of the type II diabetes, and its comparisons with the healthy non-diabetic controls.

The present study was conducted in the Department of General Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi in collaboration with Department of Pharmacology during the period of June 2013 to June 2014.

## METHODOLOGY

**Selection of cases:** 22 patients of type II diabetes without complication, of age more than 25 years, were selected from the Department of Medicine, IMS, BHU, Varanasi.

**Selection of Control:** 14 age and sex matched healthy non-diabetic & Normotensive individuals were selected as the controls, whose blood samples were drawn with their consent for comparison with the blood samples of the cases . Detailed history and clinical examination (including fundoscopy by ophthalmologist) was done in all selected cases and controls. Then the venous blood samples about 10 ml were collected in clean and dry plain vials without any anticoagulant. The blood was allowed to clot at room temperature. The sera was removed and stored at -20$^0$C in a sterile plain glass vial until analyzed in Department of Pharmacology, IMS BHU.

Before final estimation of Free radical markers and Antioxidants levels, all the cases and controls underwent following investigations :Blood: complete blood counts (CBC),Renal function test (RFT),Liver function test (LFT),Plasma glucose fasting and post prandial.HbA1c.Urine : Urine R/M,24 hour urinary protein. Electrocardiogram (ECG),Treadmill test (TMT) and 2D echocardiogram (optional, clinically suspected coronary artery disease patients only)

### Criteria for Selection

### Criteria for diagnosis of diabetes (Adapted from American Diabetes Association 2011)

Fasting Plasma Glucose (FPG) ≥ 126 mg/dl (7.0 mmol/l) (Fasting is defined as no caloric intake for at least 8 h.) or 2-h plasma glucose ≥ 200 mg/dl (11.1mmol/l) during an Oral Glucose Tolerance Test (OGTT). The test should be performed by, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

### Collection of Blood Samples:

Blood samples were collected especially from the antecubital vein of the above stated subjects. Venous blood sample about 10 ml were collected in clean and dry plain vials without any anticoagulant. The blood was allowed to clot at room temperature and then subjected to 2000 rpm centrifugation for 10-15 minutes. The sera, thus removed, were stored at -20 $^0$C in a sterile plain glass vial until analyzed. Serum was used to pipette out at the time of analysis after thawing.

Precautions were taken to ensure that there was no hemolysis. Use of tourniquet was avoided. The blood was drawn slowly and steadily into the syringe and expelled the sample way after removing the needle and tip touching the side of the container. These collected sera samples subjected to the estimation of the following parameters in the Department of Pharmacology, IMS, BHU, Varanasi.

### Estimation of protein

The protein content of the granulation tissue was estimated using the method of Lowry *et al*., (1951). To 0.1 ml homogenate (equivalent to 10 mg tissue) was added 0.9 ml Absolute alcohol. It was centrifuged at 3000 rpm for 5 minutes. The precipitate so obtained was dissolved in 1 ml of 0.1N NaOH. Out of this 1 ml solution, 0.4 ml was taken into another test tube and to this added 4 ml of alkaline reagent and kept for 10 minutes. Then 0.4 ml of the phenol reagent was added and again 10 minutes were allowed for color development. Readings were taken, against the blank prepared with water, at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and expressed in terms of mg/g wet tissue.

### Estimation of free radical parameters

**Lipid peroxidation (LPO):** LPO level is estimated in terms of malondialdehyde (MDA). To 0.2 ml of 100 mg/ml tissue homogenate was added 0.1 ml of 8.1 % SDS, 0.75 ml of 20 % acetic acid solution (pH 3.5) and 0.75 ml of 0.8 % aqueous solution of TBA in stoppered tubes. The mixture was made up to 2 ml with distilled water, and then heated in an oil bath at 95°C for 60 minutes. After cooling with tap water, 0.5 ml of distilled water and 2.5 ml of mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 3000 rpm for 10 min the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 ml of distilled water in place of sample. 1, 1, 3, 3- tetra-methoxypropane was used as external standard and the level of LPO was expressed as nmol MDA/g wet tissue.

**Nitric oxide (NO):** As nitrite and nitrate are formed as end products of the reactive nitrogen in-

termediates, the measurement of nitrite by using the Griessreagent is generally employed as a marker for formation of NO. 0.4 ml of granulation tissue homogenate (100 mg/ml) is mixed with 0.4 ml of absolute alcohol and then centrifuged at 4°C at 14000 rpm for 1 hr. 0.5ml of supernatant was taken, mixed with 0.5 ml of vanadium (III) chloride and 0.5 ml of freshly prepared Griess reagent, and incubated at 37°C for 30 min. The absorbance was measured at 540 nm spectro-photometrically, against blank prepared by using distilled water. Nitrite content was determined from standard curve prepared by using sodium nitrite and expressed as nmol/g wet tissue.

**Reduced Glutathione (GSH):** 1 ml of tissue homogenate (100 mg/ml) was mixed in 15 ml test tube with 0.8 ml of distilled water and 0.2 ml of 50 % TCA. The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at 3000 rpm for 10 min. 0.6 ml of supernatant was mixed with 0.8 ml of 0.4M Tris buffer (pH 8.9) and 20 µl of 0.1M DTNB in absolute methanol, and the sample was shaken. The absorbance was read within 5 min of the addition of 40 µl DTNB at 412 nm against a reagent blank with no homogenate. The results were expressed as nmol/g wet tissue and were calculated from the standard curve prepared by using standard glutathione.

**Superoxide dismutase (SOD):** The inhibition of reduction of nitro-blue tetrazolium (NBT) to blue coloredformozan in presence of phenazinemetha sulphate (PMS) and Nicotinamide adenine dinucleotide (NADH) was measured at 560 nm using n-butanol as blank. To 0.2 ml of tissue homogenate was added 0.6 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3), 50 µl of 186 µM of PMS, 150 µl of 300 µM NBT and 0.4 ml of distilled water to make up the volume up to 1.5 ml including with 0.1 ml of 780 µM NADH. Reaction was started by the addition of NADH. After incubation at 30°C for 60 sec, the reaction was stopped by the addition of 0.5 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 2 ml of n-Butanol. The mixture was allowed to stand for 10 min, centrifuged at 3000 rpm for 10 min and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm in spectrophotometer against n-Butanol, a system devoid of enzyme served as control. One unit of enzyme activity is defined as enzyme concentration required inhibiting the optical density at 560 nm of chromogen protection by 50% in one min under the assay conditions, and the results have been expressed as units (IU) of SOD activity/g wet tissue.

## RESULTS

The present study was conducted in the Departments of Medicine and Pharmacology, Institute of Medical Sciences, Banaras Hindu University. The study consisted of 36 patients of i) Diabetes without Complications (DWOC group, n=22), and 14 healthy Non diabetic and Non-hypertensive (Control) age and sex matched controls.

### Clinical characteristics of study population

**Sex distribution:** Male numbers were slightly higher in control 64.3% as compared to 54.5% in (DWOC). Female numbers were slightly higher in 45.5% in (DWOC) as compared to 35.7 % in control group. (Table 1).

**Age distribution:** The percent in different age group varied from 7.14% (>70 yrs) to 35.7% (41-50 yrs) in Control subject, 4.6 (30-40 yrs) to 31.8 (51-70 yrs) in DWOC.

**Family history:** In DWOC and Control groups had 7 (31.8%) and 6 (42.83%) family history of type-2 diabetes in first degree relatives respectively. (Table 1).

In DWOC group 4 (18.2%), 10 (45.4%), and 8 (36.4%) patients were obese, overweight and normal weight respectively most of the controls were of normal weight 8 (66.7%), while 4 (33.3%) were overweight (Table 1).

In Diabetes without complication and Control patient don't have significant proteinuria.

### Lab characteristics of study population

Mean serum level of Superoxide dismutase was significantly lower in HWOC group. The difference between both group was statistically significant ($P$ = 0.006).

Mean serum MDA level in age/sex matched healthy controls were 17.05±2.10µmol/L, while in DWOC group it was 23.95±0.85 µmol/L. The mean serum MDA level in DWOC was elevated as compared to mean MDA levels in control group. The difference between both group was statistically significant ($P$=0.007).

Mean serum level of Nitric Oxide (NO) was 527.7±16.0 nmol/L in control group and 702.1±19.1nmol/L in DWOC group. The mean NO levels in DWOC were elevated as compared to mean NO levels in controls. The difference between both group was statistically significant ($P$<0.001).

Mean serum level of Glutathione was significantly lower in the Diabetes with complications group. The difference between both group was statistically significant ($P$<0.001). Mean serum level of Superoxide Dismutase was significantly lower in the Diabetes with complications group. The difference between both group was statistically significant ($P$ = 0.002).

**Table 1: Clinical characteristics of study population**

| Characteristics | Diabetes without Complication (DWOC) N=22 (%) | Normotensive Non Diabetic (Control) N=14 (%) |
|---|---|---|
| **Sex** | | |
| Male | 12 (54.5) | 09 (64.3) |
| Female | 10 (45.5) | 05 (35.7) |
| **Age (years)** | | |
| 30-40 | 2 (9.1) | 4 (28.5) |
| 41-50 | 5 (22.7) | 5 (35.7) |
| 51-60 | 7 (31.8) | 2 (14.3) |
| 61-70 | 7 (31.8) | 2 (14.3) |
| >70 | 1 (4.6) | 1 (7.140 |
| **Family history of type-2 diabetes** | | |
| Present | 7 (31.8) | 6 (42.8) |
| Absent | 15 (68.2) | 8 (57.2) |
| **BMI (Kg/m²)** | | |
| <25 (Normal) | 8 (36.4) | 8 (66.7) |
| 25-30 (Overweight) | 10 (45.4) | 4 (33.3) |
| > 30 (Obese) | 4 (18.2) | 0 (0) |
| **Proteinuria (mg/day)** | | |
| <30 | 22 (100 | 14 (100 |
| 30-299 | - | - |
| > 300 | - | - |

**Table 2: Serum Protein (PR), Lipid peroxidation (MDA), Nitric Oxide (NO), Reduced Glutathione (GSH) and Superoxide Dismutase (SOD) levels in Diabetes without Complication (DWOC) and Control**

| | Diabetes without Complication (DWOC) (N=22) (%) | Normotensive Non Diabetic (Control) (N=14) (%) | P value |
|---|---|---|---|
| **Protein** | 7.24±0.55 | 7.20±0.61 | 0.923 |
| **Free radicals** | | | |
| LPO (MDA, µmol/L) | 23.95±0.85 | 17.05±2.10 | 0.007 |
| NO (nmol/L) | 702.1±19.1 | 527.7±16.0 | <0.001 |
| **Antioxidants** | | | |
| GSH (µmol/L) | 78.3±2.77 | 96.2±1.69 | <0.001 |
| SOD (U/L) | 891.6±52.3 | 1323.2 ± 105 | 0.002 |

## DISCUSSION

Present study was carried out in 22 patients of type-2 diabetes without complication, and 14 healthy normotensive non diabetic age and sex matched controls. The objective of study was to compare the levels of oxidative stress and antioxidants. Diabetes mellitus is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency Diabetes is associated with increased production of free radicals and impaired antioxidant defenses. [6]

In the present study, lipid per oxidation was measured indirectly by malondialdehyde estimation in serum. Free radicals and anti-oxidants were measured by the estimation of Nitric Oxide (NO), Glutathione and Superoxide Dismutase. Increasing evidences in both experimental and clinical studies suggest that the oxidative stress plays a major role in the pathogenesis of diabetes mellitus. Enhanced free radicals formations in diabetes mellitus occur due to glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. High levels of free radicals and the associated decline in the antioxidant defense mechanisms can lead to the damage of cellular organelles and enzymes. Oxidative stress thus can lead to the development of complications of diabetes mellitus. [7]

Malondialdehyde (MDA) is a highly toxic byproduct of the lipid per oxidation of unsaturated fatty acids by free radicals. Since it is the stable product that is why it is used as the marker of oxidative damage of unsaturated fatty acids. Malondialdehyde level was found to be elevated in the diabetic patients without complications (23.95 ±0.85 µmol/L) compared to the healthy controls (17.05±2.10 µmol/L) ($p<0.01$). Previous studies by Turk et al., (2002) and Kamal M et al., (2009) showed similar results. [8]

Nitric Oxide level was found to be elevated in diabetic patients without complications (702.1±19.1 nmol/L) when compared to the healthy controls (527.7±16 nmol/L) ($p < 0.001$). The above results was consistent with other previous studies by Maejima et al., ($p<0.001$).Although ROS, thus generated oxidizes a variety of substances, the most important mechanism in blood pressure regulation is ROS reaction with endothelium released NO and the ensuing inactivation of NO.[9]

Glutathione level was found to be reduced in the diabetic patients without complications (78.3±2.77 µmol/L) when compared to healthy controls (96.2±1.69 µmol/L). The difference was statistically significant ($p <0.001$). The above results were consistent with other previous studies by Medina et al., ($p<0.001$). Glutathione is important in the overall cellular redox balance. Because cellular glutathione concentration is 500 to 1000 fold higher than the other redox regulating proteins, changes in the ratio of reduced to oxidized glutathione are directly reflective of intracellular redox alterations (Schafer FQ et al., 2001).[10]

SOD level was found to be reduced in the diabetic patients without complications (891.6±52.3 U/L) when compared to healthy nondiabetic controls (1323.2 ±105.0 U/L) and the difference was statistically significant ($p<0.01$). The above results were not consistent with other previous studies by Medina et al., ($p<0.005$). Superoxide dismutase (SOD) maintains the cellular levels of $O_2$ within the physiological concentrations by converting $O_2$ to $H_2O_2$, a more stable ROS.[11]

## CONCLUSION

The current study suggests that the oxidative stress may have role in the pathogenesis of diabetes, as indicated by the increased levels of lipid peroxidation product malondialdehyde in patients than age and sex matched healthy controls. Enhanced level of malondialdehyde in uncomplicated diabetic patients indicates that the oxidative stress increased before the development of complication, and so it might had contributed in the development of the complications.The decreased levels of the Glutathione peroxidase and Superoxide dismutase in the antioxidant enzyme mechanisms indicates that the anti-oxidative enzyme system gets impaired more in diabetic patients. Yet it is difficult to come on definite conclusion whether the altered levels of oxidative stress and antioxidant enzyme are the cause or the effect of diabetes mellitus.Antioxidant supplementations may have clinical usefulness in the treatment of this complex disorder and in preventing complications, but final verdict and consensus can only be obtained after large randomized cohort studies.

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