CHAPTER 1

Evaluation of oxidative stress

in diabetes mellitus
INTRODUCTION

Diabetes is associated with altered glucose and lipid metabolism and is characterized by hyperglycemia. Insulin resistance is a hallmark of type 2 diabetes that precedes and predicts the disease for several years (Juhan-Vague, 1993). In IR state, normal pancreatic β-cells can compensate for insulin insensitivity by up-regulating insulin secretion; however, insufficient secretion by β-cells can induce the onset of abnormalities in glucose metabolism, i.e. IGR (impaired glucose regulation). Once hyperglycemia becomes evident, the function of pancreatic β-cells deteriorates progressively due to ‘glucose toxicity’ (glucotoxicity), which leads to severe impairment of glucose-stimulated insulin secretion, apparent degranulation of β-cells and decreased β-cell number, resulting in the aggravation of IR (Fonseca, 2009). This vicious circle finally results in the clinical manifestation of diabetes.

The underlying mechanisms of the pathogenesis of type 2 diabetes still remains to be determined; however, oxidative stress has been shown to be responsible, at least in part, for the progression of type 2 diabetes by affecting insulin sensitivity and β-cell function. These effects lead to hyperglycemia which in turn increases the production of ROS, establishing a cyclic relationship between diabetes and OS, therefore triggering deleterious cellular processes. Recent cellular and ex vivo tissue models have indicated a causal role for OS in the development of IR (Houstis et al., 2006; Hoehn et al., 2009). In humans, an association between OS and IR has been observed in individuals with impaired fasting glucose (Meigs et al., 2007), but a relationship between them has not been unambiguously established.

Several methods have been developed to assess IR, insulin secretion, and sensitivity: some of them, such as the homeostasis model assessment (HOMA) for IR (HOMA-IR) and for insulin secretion (HOMA-β) and the quantitative insulin sensitivity check index (QUICKI) are based on fasting levels of glucose and insulin; others, such as the pancreatic insulin response to glucose (IRG) and the insulin sensitivity index (ISI) are derived from the glycemic and insulinenmic responses to the oral glucose tolerance test (OGTT). The easiest and thus the most common way of assessing β-cell function is homeostatic responsivity index, HOMA-β. It is derived from fasting plasma glucose (FPG) and fasting insulin levels since the relationship between glucose and insulin in the basal state reflects the balance between hepatic
glucose output and insulin secretion, which is maintained by a feedback loop between liver and β-cells (Turner et al., 1979).

Measuring biomarkers of OS is an essential step towards a better understanding of the pathogenesis of diabetes and developing treatment for controlling diabetes. There are several approaches that may be adopted, including measurement of the depletion of antioxidant defense, changes in the activities of antioxidant enzymes, free radical production and presence of protein, lipid, and DNA free radical adducts. Most commonly used antioxidant enzymes include CAT, SOD, GPx and GR, which act as primary free radical scavengers. Dietary antioxidant like vitamin C and endogenous antioxidants namely, GSH, bilirubin and uric acid are amongst the most commonly studied antioxidant molecules. In addition to this, end products of free radical mediated damage to cellular biomolecules are reliable and relatively stable indicators of oxidative stress. Damage caused to proteins, lipids and DNA can be assessed by quantitating protein oxidation and/or carbonylation products, lipid peroxides and hydroperoxides, and modified DNA bases such as 8-OHdG. Involvement of OS in diabetic patients has been shown by several studies where impaired level of antioxidant enzymes and molecules and a consequently increased levels of oxidative damage markers in diabetic patients, with and without secondary complications has been demonstrated. Several such studies where the extent of OS in diabetic patients has been studied are summarized below:

Table 1. An overview of studies reporting various oxidative stress parameters (OSP) in diabetic patients. (Note: † and ‡ arrows indicate an increase and decrease in the corresponding OSP, - indicates no change in the studied parameters, 8-iso-PGF2α: 8-iso-Prostaglandin F2α, AGEs: Advanced glycation end-products, AOPP: Anti-oxidation protein products, CAT: Catalase, CHD: Coronary heart diseases, DC: diabetic complications, GPx: Glutathione peroxidase, GR: glutathione reductase, GSH: Glutathione, LOOH: lipid hydroperoxides, PCG: Protein carbonyl group, SOD: superoxide dismutase, T1D: type 1 diabetes, T2D: Type 2 diabetes, TBARs: Thio-barbituric acid reacting substances, T-SH: total thiol group)

<table>
<thead>
<tr>
<th>References</th>
<th>Characteristics of diabetic patients</th>
<th>OSP in comparison to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aydin et al., 2001</td>
<td>T2D patients (n=44) with poor glycemic control (PGC) and followed after treatment (AT)</td>
<td>PGC: CAT, GPx ‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SOD, TBARs †</td>
</tr>
<tr>
<td>Reference</td>
<td>Study Details</td>
<td>AT:</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>-----</td>
</tr>
<tr>
<td>Kesavulu et al., 2001</td>
<td>T2D patients with (n= 25) and without (n=28) CHD</td>
<td><strong>CAT, GPx ↓</strong>&lt;br&gt;<strong>SOD, TBARs ↑</strong></td>
</tr>
<tr>
<td>Vessby et al., 2002</td>
<td>T1D patients (n=38)</td>
<td>Vitamin E ↑&lt;br&gt;Urate ↓&lt;br&gt;8-iso-PGF2α and MDA -</td>
</tr>
<tr>
<td>Bhatia et al., 2003</td>
<td>T2D patients with (n=30) and without (n=30) nephropathy</td>
<td><strong>DC (-):</strong>&lt;br&gt;Uric acid, MDA↑&lt;br&gt;GSH, SOD, CAT↓&lt;br&gt;<strong>DC(+):</strong>&lt;br&gt;MDA ↑&lt;br&gt;Uric acid, GSH, SOD and CAT↓</td>
</tr>
<tr>
<td>Martin-Gallan et al., 2003</td>
<td>T1D patients with (n= 26) and without (n=28) microangiopathy</td>
<td><strong>DC(-):</strong>&lt;br&gt;GPx, GSH ↑&lt;br&gt;Vitamin E, SOD, MDA ↑&lt;br&gt;LOOH, PCG -&lt;br&gt;<strong>DC(+):</strong>&lt;br&gt;GPx, GSH, Vitamin E ↓&lt;br&gt;SOD, MDA, LOOH, PCG ↑</td>
</tr>
<tr>
<td>Abou-Seif and Youssef, 2004</td>
<td>T1D patients (n=40) and T2D patients (n=55)</td>
<td>CAT, SOD, GSH ↑&lt;br&gt;AGEs, AOPP, MDA and NO ↓</td>
</tr>
<tr>
<td>Pasaoglu et al., 2004</td>
<td>Newly diagnosed T2D patients (ND) (n= 20) and T2D patients receiving oral anti-diabetic drugs (OAD) (n= 20)</td>
<td><strong>ND and OAD:</strong>&lt;br&gt;GSH, Total-SH↓&lt;br&gt;MDA ↑</td>
</tr>
<tr>
<td>Martinez-Sanchez et al., 2005</td>
<td>T2D patients (n=101) with microangiopathic complications</td>
<td>CAT, SOD, GPx, MDA, AOPP↑&lt;br&gt;GSH ↓</td>
</tr>
<tr>
<td>Ramakrishna and Jailkhani, 2007</td>
<td>T1D (n=15)</td>
<td>GPx, SOD, CAT, Uric acid and Vitamins A, C &amp; E ↓&lt;br&gt;Lipid peroxidation, PCG and Nitric oxide ↑</td>
</tr>
</tbody>
</table>
Thus, it can be summarised that there exist many differences in the pattern of OS in diabetic patients which are possibly related to the duration of disease, the degree of glycemic control, the presence of chronic complications and comorbidities (such as obesity), besides genetic factors and lifestyle. Nevertheless, these studies unambiguously demonstrate the impairment of antioxidant defense in diabetic patients along with an increase in the concentration of oxidative damage markers. However, it still remains to be elucidated whether oxidative stress acts as a primary cause, or is a consequence of the onset of diabetes. In this context, oxidative damage to DNA in the pre-diabetic state has been reported by Song et al., (2007) and Al-Aubaidy et al., (2011) which suggests involvement of OS in pre-diabetic state.

In patients with frank hyperglycemia, glycemic control remains the major therapeutic objective for prevention of target organ damage and diabetic complications (Koro et al., 2004). Since hyperglycemia is the main factor contributing to OS in diabetes, controlling glucose levels in diabetic patients would alleviate extent
of oxidative damage. Modak et al., (2013) demonstrated that excess free radicals generated as a result of persistent hyperglycemic conditions caused damage to the cellular biomolecules in islets and this extent of damage further increased in animals with uncontrolled diabetes, whereas this damage was augmented and antioxidant defense status was restored in islets of insulin treated diabetic animals. Several large clinical trials have also demonstrated that blood glucose control correlates with a reduction in the microvascular complications of diabetes (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998).

With this background, the present work was undertaken to comprehensively evaluate the OSP in terms of antioxidant defense status as well as oxidative damage markers in newly diagnosed diabetic patients. Additionally, the effect of glycemic control on OS state of diabetic patients was assessed by serially measuring the OSP from the time of diagnosis to four and eight weeks after anti-diabetic treatment. These measurements were compared with serial measurements in healthy non-diabetic subjects. Additionally, in order to facilitate easy understanding of an overall OS state, oxidative stress score (OSS) was computed by taking into consideration twelve different OSP which included CAT, SOD, GPx, GR among antioxidant enzymes; bilirubin, uric acid, Vitamin C and GSH among antioxidant molecules; and protein sulphhydryl, protein carbonyl, TBARS and 8-OHdG among oxidative damage markers. Further, the inter-relationships between oxidative stress status, IR and pancreatic β-cell secretory function was explored in an attempt to investigate the role of OS in the type 2 diabetes, which may have important implications for the prevention of type 2 diabetes in newly diagnosed diabetic patients.
Evaluation of oxidative stress parameters (OSP) in study participants
(Recruited at Diabetes Research Unit, KEM Hospital)

Non-diabetic subjects (n=50)
- Baseline
- 4 weeks
- 8 weeks

Diabetic patients (n=54)
- Baseline
- 4 weeks
- 8 weeks
  - Anti-diabetic treatment

Anthropometric parameters
- BMI
- Waist: hip ratio

Biochemical parameters
- Fasting plasma glucose
- HbA1C
- Insulin
- Lipid profile
- SGPT, SGOT
- Electrocardiogram (ECG)

Antioxidant enzymes
- Catalase
- Glutathione Peroxidase
- Superoxide dismutase
- Glutathione reductase

Antioxidant molecules
- GSH
- Vitamin C
- Uric acid
- Bilirubin

Oxidative damage markers
- Protein carbonyls
- Protein sulphhydryls
- TBARs
- 8-OHdG
MATERIALS AND METHODS

1. Chemicals

All chemicals used in this study were purchased from either Sigma Chemical Co. (U.S.A.), Sisco Research Laboratories (India), Roche (Germany), Merck (Germany), Hi-media (India), Invitrogen (USA), or Bangalore Genei (India).

2. Glassware and plasticware

Glassware was obtained either from Borosil (India) or Qualigens (India) and plasticware was from either Tarsons (India) or Laxbro (India). All plasticware and glassware was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

3. Biologicals

Newly diagnosed type 2 diabetic patients (n = 54) attending the Diabetes Unit, KEM Hospital, Pune with fasting plasma glucose (FPG) concentration ≥ 7.0 mmol/L (126 mg/dL) and healthy non-diabetic subjects (n = 50) with FPG concentration < 7.0 mmol/L (126 mg/dL) were recruited. Non-diabetic subjects were healthy volunteers from various academic institutions in Pune.

Following groups of subjects were excluded: pregnant women, individuals with excessive alcohol intake, chronic smokers, those receiving antioxidants, those with clinical infection, recent clinical evidence of liver cell failure (rise in ALT upto 100IU/L was accepted), those associated with renal failure (creatinine > 1.5 mg%) and with inflammatory or malignant disease, history of a recent (<6 months) cardiovascular event or with symptomatic heart disease (NYHA Class III, IV).

4. Methodology

4.1 Sample collection

Fasting blood samples were collected at baseline and four and eight weeks later from diabetic patients and non-diabetic subjects. Diabetic patients were advised on diet, physical activity and were put on anti-diabetic drugs to control hyperglycemia as necessary. Patients were also advised not to take any oral antioxidant supplements.

The study protocol was approved by the Institutional Ethical Committee, KEM Hospital and Research Centre, Pune, and informed consent was obtained from
all the individuals after the purpose and nature of the study had been explained. A proforma of detailed consent form is attached in Annexure I. Medical history was noted and a standardized physical examination including body weight, height, waist: hip ratio, blood pressure and electrocardiogram (ECG) was carried out for each subject.

4.2 Sample preparation
1. Fasting and post prandial blood samples (10 ml) were freshly withdrawn from both diabetic patients and non-diabetic subjects.
2. Each sample was centrifuged at 4000 rpm for 10 mins to separate the plasma.
3. The buffy coat was removed and the erythrocytes were washed three times in cold saline (9.0 g/L NaCl), and hemolysed by adding ice-cold ultrapure water (MilliQ plus reagent grade; Millipore, Bedford, MA) to yield a 50% hemolysate. Aliquots of hemolysate were stored at -80°C until analysis.
4. Plasma glucose was measured by GOD PAP (glucose oxidase Peroxidase) method and total cholesterol, triglyceride, HDL cholesterol and creatinine concentrations were determined on an automated analyser (Hitachi 902, Japan).
5. HbA1C was measured by using an HPLC cation exchange column on D10 HbA1C analyser (Bio-Rad Laboratories, Hercules, CA).
6. Plasma insulin was measured using insulin kit (Mercodia, Uppsala, Sweden).
7. Insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were calculated from the fasting insulin and glucose concentrations using online calculator for specific insulin assay (Homeostatic Model Assessment) (HOMA calculator).

4.2 Protein estimation by Folin-Lowry method (Lowry et al., 1951)

Principle
Lowry protein assay for determining protein concentration in a sample depends on the reactivity of the peptide nitrogen(s) with the copper (II) ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdenum blue by the copper-catalyzed oxidation of aromatic acids.

Materials
1. Lowry A (2% Na₂CO₃): 2 gm Na₂CO₃ was dissolved in 0.1 N NaOH and volume was made up to 100 ml.
II. 0.1 N NaOH: 4 gm NaOH was dissolved in distilled water and volume was made up to 1000 ml.

III. Lowry B (0.5% CuSO₄): 0.5 gm CuSO₄ ⋅ 5H₂O was dissolved in 100 ml of 1% sodium citrate.

IV. 1% Sodium citrate: 1 gm of sodium citrate was dissolved in 100 ml of distilled water.

V. Lowry C: 1 ml Lowry B was mixed with 49 ml Lowry A.

VI. Lowry D: Folin-Ciocalteau reagent was diluted 1:3 with distilled water.

Methods
1. To 0.1 ml of sample, 1 ml of reagent C was added and incubated at room temperature for 10 mins. Bovine serum albumin (BSA) was used as a standard in the range of 10-100 μg/ml.
2. To this, 0.1 ml Folin-Ciocalteau reagent was added and incubated at room temperature for 30 mins in dark. The absorbance was read at 660 nm.

Calculations
The standard graph of absorbance at 660 nm versus BSA concentration in μg of protein was plotted and protein concentration of sample was extrapolated on the standard graph.

4.3 Evaluation of oxidative stress parameters (OSP)
4.3.1 Antioxidant enzymes: Antioxidant enzymes namely, CAT, GPx, GR and SOD were measured from the hemolysate by diluting the hemolysate 10 times with saline.

4.3.1.1 CAT activity (EC – Number 1.11.1.6) (Aebi, 1984)
Principle
CAT is a heme-containing enzyme, which catalyzes dismutation of hydrogen peroxide into water and oxygen.

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2 \]

Decomposition of H₂O₂ by CAT was measured spectrophotometrically at 240 nm.
Materials

I. 0.1 M Potassium phosphate buffer: 6.8 gm KH₂PO₄ was dissolved in distilled water and final volume was made up to 50 ml. 8.75 gm K₂HPO₄ was dissolved in distilled water and final volume was made up to 50 ml. These solutions were mixed in equal volumes to get 1 M potassium phosphate buffer which was diluted 10 times to get final concentration of 0.1 M.

II. 30% H₂O₂: H₂O₂ was diluted 10 times and used in assay mixture.

Method

1. The reaction was set as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Phosphate buffer</td>
<td>1 ml</td>
</tr>
<tr>
<td>Hemolysate</td>
<td>1 µl</td>
</tr>
<tr>
<td>The contents of cuvettes were mixed thoroughly.</td>
<td></td>
</tr>
<tr>
<td>30% H₂O₂</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

2. The absorbance was read at 240 nm, continuously for 2 mins at an interval of 30 sec using auto rate assay mode on spectrophotometer.

Calculations:

\[ b = \frac{\Delta A \times V \times 1000}{\Delta t \times \varepsilon_0 \times d \times v} \times \text{dilution factor } \mu \text{M/L} = \text{U/ml/min} \]

where,

\[ \frac{\Delta A}{\Delta t} = \text{slope where } \Delta A \text{ is the difference in the absorbance and } \Delta t \text{ is the difference in time points} \]

\[ V = \text{total volume of reaction} \]

\[ v = \text{volume of sample} \]

\[ d = \text{path length} \]

\[ \varepsilon_0 = 0.0039 \text{ lit mM}^{-1} \text{ mm}^{-1} \]

\[ b = \text{enzyme activity} \]
4.3.1.2 GPx activity (EC – Number 1.11.1.9) (Pagila and Valentine, 1967)

**Principle**

GPx removes H₂O₂ by coupling its reduction to H₂O with oxidation of reduced glutathione, GSH.

\[
\text{GPx} \\
\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow \text{GSSG} + 2 \text{H}_2\text{O} \\
\text{GR} \\
\text{GSSG} + 2\text{NADPH} \rightarrow \text{NADP} + 2\text{GSH}
\]

**Materials**

I. **0.1 M Potassium phosphate buffer**: This buffer was prepared by the same method as described for CAT assay.

II. **20 mM EDTA**: 0.744 gm EDTA was dissolved in distilled water and final volume was made up to 100 ml with distilled water.

III. **20 mM Sodium azide (NaN₃)**: 0.026 gm sodium azide was dissolved in distilled water and final volume was made up to 20 ml with distilled water.

IV. **2 mM NADPH**: 3.3 mg NADPH was dissolved in 1% NaHCO₃ (W/V) and final volume was made up to 2 ml.

V. **10 mM Glutathione reduced (GSH)**: 12.28 mg GSH was dissolved in distilled water and final volume was made up to 4 ml with distilled water.

VI. **2.5 mM H₂O₂**: 2.84 µl of 30% H₂O₂ was added to 10 ml distilled water.

VII. **GR**: 4 µl from the original stock of GR (2.7 mg protein/ml) was used.

**Method**

1. The reaction was set as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M PO₄</td>
<td>50 mM</td>
<td>500 µl</td>
</tr>
<tr>
<td>20 mM NaN₃</td>
<td>1 mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>GR</td>
<td>1 EU</td>
<td>4 µl</td>
</tr>
<tr>
<td>10 mM GSH</td>
<td>1 mM</td>
<td>100 µl</td>
</tr>
<tr>
<td>2.5 mM H₂O₂</td>
<td>0.25 mM</td>
<td>100 µl</td>
</tr>
<tr>
<td>20 mM EDTA</td>
<td>1 mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>---</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The contents of cuvettes were mixed thoroughly and incubated for 5 mins.

| 2 mM NADPH | 0.2 mM | 16 µl |

---
2. Contents were mixed thoroughly and the absorbance was read at 339 nm for 5 mins at an interval of 30 sec.

Calculations
Amount of GPx was measured according to the equation is as follows:
\[
b = \frac{\Delta A \times V \times 1000}{\Delta t \times \varepsilon_0 \times d \times v} \times \text{dilution factor} = \text{U/ml}
\]

Where,
\[
\frac{\Delta A}{\Delta t} = \text{slope}
\]
\[V = \text{Total volume of reaction; } \varepsilon_0 = 6.3 \times 10^2 \text{lit mM}^{-1} \text{mm}^{-1}
\]
\[v = \text{volume of sample}
\]
\[d = \text{path length}
\]
One unit of enzyme is defined as amount of enzyme required to convert 1 µmole substrate to product in one minute.

4.3.1.3 GR activity (EC – Number 1.8.1.10) (Goldberg and Spooner, 1983)
Principle
GR reduces glutathione disulfide (GSSG) to the sulphydryls form GSH, which is an important cellular antioxidant. The reaction catalyzed is as follows:
\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+
\]
Though GR reaction is reversible, the reaction forming GSH is strongly favoured. Catalytic activity of GR is measured by measuring the decrease in absorbance due to the oxidation of NADPH.

Materials
I. 0.12 M KH₂PO₄ buffer: 1.633 gm KH₂PO₄ was dissolved in distilled water. pH was adjusted to 7.2 with NaOH. The final volume was made up to 100 ml.
II. 15 mM EDTA: 0.56 gm EDTA was dissolved in distilled water and final volume was made up to 100 ml.
III. 9.6 mM Nicotinamide adenine dinucleotide phosphate reduced (NADPH): 8 mg NADPH was dissolved in 1% NaHCO₃ (W/V) and final volume was made up to 1 ml.
IV. 65.3 mM Glutathione oxidized (GSSG): 40 mg GSSG was dissolved in distilled water and final volume was made up to 1 ml.

**Method**

1. The reaction was set as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12 M KH$_2$PO$_4$</td>
<td>100 mM</td>
<td>880 µl</td>
</tr>
<tr>
<td>15 mM EDTA</td>
<td>0.5 mM</td>
<td>33 µl</td>
</tr>
<tr>
<td>65.3 mM GSSG</td>
<td>2.2 mM</td>
<td>33 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>--------</td>
<td>20 µl (1:10)</td>
</tr>
</tbody>
</table>

The contents of cuvettes were mixed thoroughly and incubated for 5 mins

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6 mM NADPH</td>
<td>0.17 mM</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

2. Contents were mixed thoroughly and the absorbance was read at 339 nm for 5 mins at an interval of 30 sec.

**Calculations**

Activity of GR was measured according to the following equation:

$$b = \frac{\Delta A \times V \times 1000}{\Delta t \times \varepsilon_0 \times d \times v} \frac{\mu M/min/L}{U/ml}$$

Where,

$$\frac{\Delta A}{\Delta t} = \text{slope}$$

$V = \text{Total volume of reaction;}$

$\varepsilon_0 = 6.3 \times 10^2 \text{lit mM}^{-1} \text{ mm}^{-1}$

$v = \text{volume of sample;}$

$d = \text{path length}$

One unit of enzyme is defined as amount of enzyme required to convert 1 µmole substrate to product in one minute.
4.3.1.4 Superoxide dismutase activity (EC – Number 1.15.1.1) (Beauchamp and Fridovich, 1971)

Principle

Superoxide dismutase catalyses the breakdown of the superoxide anion into oxygen and hydrogen peroxide. Photochemically reduced flavins generate oxygen free radicals upon re-oxidation in air. The oxygen free radicals reduce nitroblue tetrazolium (NBT) to blue coloured formazan.

\[
\text{Riboflavin} \xrightarrow{\text{Light}} \text{oxygen free radicals (ROS)}
\]

Oxygen free radicals + L-methionine + NBT \xrightarrow{} Formazan

Materials

I. 0.1 M Potassium phosphate buffer: This buffer was prepared by the same method as described for CAT activity assay.

II. 5 X L-Methionine: 969.8 mg L- methionine was dissolved in distilled water and final volume was made up to 100 ml with distilled water.

III. 10 X Nitroblue tetrazolium (NBT): 61.32 mg NBT was dissolved in distilled water and final volume was made up to 100 ml with distilled water.

IV. 10 X Riboflavin: 7.52 mg riboflavin was dissolved in distilled water and final volume was made up to 100 ml with distilled water.

V. 1 mM Ethylenediamine tetra-acetic acid (EDTA): 3.72 mg EDTA was dissolved in distilled water and final volume was made up to 10 ml with distilled water.

Method

1. The reaction was set as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M phosphate buffer</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>65 mM L- Methionine</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>750 μM NBT</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>2 mM Riboflavin</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>0.01 M EDTA</td>
<td>0.001 ml</td>
<td>0.001 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>--------</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.560 ml</td>
<td>0.460 ml</td>
</tr>
</tbody>
</table>
2. One set of tubes was illuminated under the light source for 30 min at a distance of 30 cm and identical set of tubes was kept in dark for 30 min. The controls were the reaction mixture without enzyme extract in both the sets.

**Calculations**

\[
\frac{C - E}{C/2} \times \frac{\text{Total volume of assay mixture}}{\text{Amount of enzyme extract}}
\]

C = difference in the absorbance at 560 nm of the control tubes from light and dark
E = difference in the absorbance at 560 nm of the enzyme tubes from light and dark

**4.4.2 Antioxidant molecules**

**4.4.2.1 Reduced glutathione (GSH)** (Teixeira and Meneghini, 1996)

Glutathione was estimated using kit from Sigma chemicals, USA.

**Principle**

GSH was calculated by employing a kinetic assay in which catalytic amounts (nmoles) of GSH causes a continuous reduction of 5,5’–dithiobis (2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by GR and NADPH.

\[
2\text{GSH} + \text{DTNB} \rightarrow \text{GSSG} + 2\text{TNB}
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+
\]

The reaction rate is proportional to the concentration of glutathione. The yellow product 5-thio-2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The standard curve of reduced glutathione was used to determine the amount of glutathione in erythrocytes.

**Method**

1. Erythrocyte fraction was deproteinised with thrice the volume of 5% Sulfosalicylic acid (SSA).
2. Samples were vortexed thoroughly and incubated for 10 mins at 4°C and centrifuged at 10,000 rpm for 10 minutes.
3. Supernatant was diluted ten times and 10 µl was aliquoted for GSH estimation.
4. The reaction was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Reagent blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>---</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>5% SSA</td>
<td>10 µl</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Working mixture</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
</tr>
</tbody>
</table>

5. The reaction mixture was mixed and incubated for 5 mins at room temperature.

6. Reaction was started with the addition of 50 µl NADPH and absorbance was recorded at 412 nm at 2 mins intervals for 10 mins using Multiskan ELISA reader (Thermo Scientific, Germany). Results were expressed as nmoles GSH/ml.

**Calculations**

\[
n\text{moles GSH/ml of sample} = \frac{A_{412}/\text{min (Sample)} \times \text{dil}}{A_{412}/\text{min (standard)} \times \text{vol}}
\]

where,

- \( A_{412}/\text{min (Sample)} \) = slope generated by sample (after subtracting the values generated by the blank reaction)
- \( A_{412}/\text{min (standard)} \) = slope calculated from standard curve for 1 n mole of GSH
- \( \text{dil} \) = dilution factor of original sample
- \( \text{vol} \) = volume of the sample in the reaction mixture in ml

4.4.2.2 Vitamin C (Ascorbic acid) (Omaye et al., 1979)

**Principle**

Vitamin C was oxidized by copper to form dehydroascorbic acid which reacts with DNPH to form 2, 4- dinitrophenyl hydrazone. This undergoes further rearrangement to form a product with absorption maxima at 520 nm. Thiourea provides the reducing medium to prevent interference from non-ascorbic acid chromogens.

**Materials**

I. 10% Trichloro acetic acid (TCA): 10 ml of 100% TCA was diluted in 90 ml of distilled water.

II. 1X DNPH reagent: 2 gm DNPH, 0.25 g thiourea and 0.03 gm CuSO\(_4\), 5H\(_2\)O was dissolved in 100 ml of 9N H\(_2\)SO\(_4\).

III. 65% H\(_2\)SO\(_4\): 65 ml H\(_2\)SO\(_4\) was added to 35 ml distilled water.
IV. 2 mg/dl Vitamin C: 20 mg of ascorbic acid was dissolved in 100 ml distilled water. This was diluted 10 times to make working solution of 2 mg/dl.

**Method**

1. Plasma was deproteinised by adding 1.2 ml 10 % TCA to 0.3 ml plasma, mixed thoroughly and incubated for 10 mins.
2. This was then centrifuged at 8000 rpm for 10 mins. The supernatant obtained was used for the determination of Vitamin C and reaction mixture was set as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>-----</td>
<td>0.1 ml</td>
<td>-----</td>
</tr>
<tr>
<td>10 % TCA</td>
<td>0.5 ml</td>
<td>0.4 ml</td>
<td>-----</td>
</tr>
<tr>
<td>DNPH reagent</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

3. Samples were then incubated at 37°C for 3 hours.
4. After incubation, samples were kept on ice for 5 mins.
5. 0.8 ml of 65 % H$_2$SO$_4$ was added to the samples and incubated at 37°C for 30 mins. The absorbance was read at 520 nm. Results were expressed as mg/dl.

**Calculations**

Concentration of Ascorbic acid (mg/dl) =

\[
\frac{\text{(Absorbance of test)}}{\text{(Absorbance of standard)}} \times \text{Concentration of standard}
\]

**4.4.2.3 Uric acid**

**Principle**

Uric acid was estimated using Accurex kit (Accurex Biomedical Pvt. Ltd, India). Uricase converts uric acid in to allantoin and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with phenolic chromogen to form a red colored compound which was read at 510 nm.

\[
\text{Uricase} \quad \text{Uric acid} + \text{H}_2\text{O} \quad \rightarrow \quad \text{allantoin} + \text{H}_2\text{O}_2
\]

\[
\text{Peroxidase} \quad \text{H}_2\text{O}_2 + \text{phenolic chromogen} \quad \rightarrow \quad \text{red colored compound}
\]
Method

1. Plasma sample was used to quantitate uric acid concentration. Reaction was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>0.025 ml</td>
<td>-----</td>
</tr>
<tr>
<td>Reagent</td>
<td>1 ml</td>
<td>2 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

2. The reaction mixture was mixed well and incubated for 5 mins at 37°C.

3. Absorbance of samples against blank was read at 510 nm. The results were expressed as mg/dl.

Calculations

Uric acid (mg%) = \[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 6
\]

4.4.2.4 Bilirubin

Principle

Bilirubin was estimated using Accurex kit (Accurex Biomedical Pvt. Ltd, India). Bilirubin reacts with diazotised sulphanilic acid to produce azobilirubin. DMSO catalyzes the formation of azobilirubin from bilirubin. The pink color is proportional to bilirubin concentration measured at 546 nm.

Method

1. Plasma sample was used to quantitate bilirubin concentration. Reaction was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Plasma/ Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent T1</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Reagent T2</td>
<td>----</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.05 ML</td>
<td>0.06 ml</td>
</tr>
</tbody>
</table>

2. The reaction mixture was mixed well and incubated at room temperature for 5 mins.

3. Absorbance of test against the respective blank was taken at 546 nm. Results were expressed as mg%.
Calculations
Bilirubin (mg%) = (Absorbance of test-Absorbance of blank) X 20.2

4.4.3 Oxidative damage markers

4.4.3.1 Protein carbonyls (Levine et al., 1990)

Principle
Protein oxidation was evaluated by measuring protein carbonyl content in plasma proteins according to the method of Levine et al., (1990) using DNPH. DNPH reacts with carbonyl groups forming stable hydrazone products, which was read at 365 nm.

Materials
I. 0.21% DNPH: 0.21 mg DNPH was dissolved in 2 N HCl and volume was made up to 10 ml.
II. Ethanol: Ethyl acetate: Ethanol and ethyl acetate were mixed in the ratio of 1:1 (v:v).
III. 6 M Guanidine hydrochloride: 57.3 gm was dissolved in 20 mM potassium phosphate buffer and volume was made up to 100 ml.
IV. 20% TCA: To the 500 gm TCA powder, 227 ml of distilled water was added which gives 100% TCA. This solution was diluted 5 times to get 20% TCA.

Method
1. Plasma protein was precipitated by adding 0.1 ml of 20 % TCA to 0.1 ml plasma. Samples were vortexed thoroughly and centrifuged for 10 mins at 6000 rpm.
2. The supernatant was decanted and to the half of the tubes of each sample (duplicate), 0.5 ml of 2N HCl was added and to the other half 0.5 ml of DNPH in 2N HCl was added. This was thoroughly vortexed and the pellet was resuspended.
3. Samples were incubated at room temperature for 1 h. To all the tubes, 0.750 ml of 20 % TCA was added and vortexed thoroughly.
4. Samples were then centrifuged at 2,200 rpm for 15 mins.
5. The pellet was washed three times with ethanol: ethyl acetate mixture (1:1). After third wash supernatant was decanted and the pellet was kept for drying.
6. Each pellet was dissolved in 0.750 ml of 6 M guanidine hydrochloride by incubating at 37ºC for 4 hrs.
7. These samples were centrifuged for 20 mins at 6000 rpm and the absorbance of supernatant was measured at 365 nm.

4.4.3.2 Protein sulphydryl (Ellman, 1959)

Principle
This assay measures the sulphydryl groups in proteins using the Ellmans reagent, 5,5'-dithiobis (2-nitrobenzoic acid) DTNB. Thiols react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB), which ionizes to the NTB\(^2\) dianion in water at neutral and alkaline pH. This NTB\(^2\) ion has a yellow colour and can be quantified by measuring the absorbance of visible light at 412 nm.

Materials

I. 14% Perchloric acetic acid (PCA): 20 ml of 70% PCA was used and volume was made to 100 ml with distilled water.

II. 7% PCA: 10 ml of 70% PCA was used and volume was made to 100 ml with distilled water.

III. 10% Triton X 100: 10 ml of 100% Triton X 100 was diluted with distilled water and volume was made to 100 ml with distilled water.

IV. 2 mM DTNB: 7.9 mg was dissolved in 10 ml of 0.2 M potassium phosphate buffer.

Method

1. Proteins from plasma were precipitated by mixing 0.050 ml plasma with 0.3 ml distilled water and 0.5 ml of 14 % PCA and then centrifuged at 4500 rpm for 5 mins.

2. Pellet was suspended in 0.1 ml of 7% PCA and 900 \(\mu\)l distilled water and centrifuged at 4500 rpm for 5 mins.

3. Supernatant was discarded and the pellet was suspended in 0.2 ml of 10 % Triton X 100, 0.6 ml DW and 0.2 ml of 0.2 M potassium phosphate buffer (pH 7.4).

4. After vortexing, 0.11 ml of 2 mM DTNB was added, incubated for 5 mins in the dark and absorbance was read at 412 nm.

Calculations
The amount of sulphydryl groups was determined from molar extinction coefficient of sulphydryl groups, \(\varepsilon = 13,600 \text{ M}^{-1}\), using the difference in absorbance at
412 nm before and 5 min after the addition of DTNB corrected for the absorbance of DTNB.

4.4.3.3 Lipid peroxides (Heath and Packer, 1965)

Principle

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural by-products of lipid peroxidation. MDA can be quantified colorimetrically following its controlled reaction with thio-barbituric acid resulting in the formation of an adduct that can be read at 535 nm.

Materials

I. 8.1 % SDS: 8.1 gm SDS was dissolved in 100 ml distilled water.

II. 20 % glacial acetic acid: 20 ml acetic acid was mixed with 80 ml distilled water.

III. 20 % TCA: 20 ml TCA was mixed with 80 ml distilled water.

IV. 0.8 % TBA reagent: 0.8 gm TBA was dissolved in 100 ml of 20 % TCA. This solution was stirred on a magnetic stirrer for 1 hour and was prepared freshly for every experiment.

Method

1. Plasma was deproteinised by treating 0.1 ml plasma with 0.1 ml SDS, 0.750 ml acetic acid and 0.750 ml TBA reagent.

2. The samples were then boiled at 95°C for 60 mins and immediately kept on ice to stop the reaction.

3. After cooling, the solution was centrifuged at 10,000 rpm for 10 mins and the precipitate obtained was removed.

4. The absorbance of the pink supernatant representative of thiobarbituric acid reactive substances (TBARs) was determined at 535 nm.

Calculations

Concentration of TBARS was then calculated with the help of standard graph using 1,1,3,3-tetramethoxy propane (TMP), as nmoles of malondialdehyde equivalents.
4.4.3.4 Quantitation of 8-hydroxy-2’ deoxyguanosine (8-OHdG)

4.4.3.4.1 Isolation of DNA from whole blood (Sambrook et al., 1989)

Principle

DNA was isolated from whole blood by Chloroform: iso-amyl alcohol extraction method.

Materials

I. 2 M Sodium acetate: 27.2 gm sodium acetate was dissolved in 100 ml distilled water.
II. 0.3 M Sodium citrate: 4.410 gm tri-sodium citrate was dissolved in 50 ml distilled water.
III. 3 M Sodium chloride (NaCl): 8.7 gm NaCl was dissolved in 50 ml distilled water.
IV. 20 X SSC solution: 0.3 M Sodium citrate and 3 M sodium chloride was mixed in 1:1 ratio and the pH was adjusted to 7.
V. 10 % SDS: 10 gm SDS was dissolved in 100 ml distilled water.
VI. 20 mg/ml Proteinase K: 20 mg proteinase K was dissolved in 1 ml distilled water.
VII. 0.5 M EDTA: 18.61 gm EDTA was dissolved in 50 ml distilled water and pH was adjusted to 8 with NaOH and the final volume was made up to 100 ml.
VIII. 20 X Tris-acetate-EDTA buffer (TAE): 4.8 gm Tris was dissolved in 1.14 ml glacial acetic acid and 2 ml of 0.5 M EDTA. Final volume was made up to 100 ml with distilled water.
IX. 1 X TE buffer: 0.010 ml of 0.5 M EDTA was added to 0.1 ml of 1 M Tris–Cl and the final volume was made up to 50 ml.

Method

1. 0.7 ml of frozen blood sample was taken and mixed with 0.8 ml of SSC solution. This was centrifuged at 7000 rpm for 5 mins at room temperature.
2. Supernatant was discarded and 1 ml of SSC was added to the pellet and vortexed thoroughly. This was centrifuged at 7000 rpm for 5 mins at room temperature.
3. Supernatant was discarded and 0.375 ml of 0.3 M sodium citrate, 25 µl 10 % SDS and 5 µl Proteinase K was added to the pellet and vortexed for 20 seconds. This was incubated at 56°C in water-bath for 12 hrs.
4. Equal volume of phenol/Chloroform and iso-amyl alcohol (0.120 ml) was added and vortexed for 30 seconds. Samples were spun at 10,000 rpm for 10 mins at room temperature.

5. Aqueous layer was gently transferred to another vial and 1 ml chilled ethanol was added to it. This was mixed gently by inverting the vials and kept at -20°C for 15 mins.

6. Samples were spun at 10,000 rpm for 2 mins at 4°C and supernatant was discarded. 180 µl TE buffer was added to the pellet, vortexed and incubated at 56°C for 10 mins.

7. 20 µl of 2 M sodium acetate buffer was added and mixed gently followed by a quick spin. 50 µl chilled ethanol was added and mixed gently to achieve a homogenous solution. Samples were spun at 10,000 rpm for 2 mins at 4°C.

8. Supernatant was discarded and washed twice with 1 ml of 70 % ethanol. The samples were spun at 10,000 rpm for 2 mins and supernatant was discarded.

9. The pellet was kept for 15 mins to air dry and 80 µl TE buffer was added to the pellet and incubated at 56°C for 12 hrs to dissolve and stored at -20°C.

4.4.3.4.2 Detection of 8-OHdG by an Enzyme Linked Immuno Absorbant Assay (ELISA) (Modak et al., 2009)

**Principle**

8-OHdG is measured using competitive ELISA where 8-OHdG monoclonal antibody reacts competitively with the 8-OHdG bound on the plate and the 8-OHdG in samples. Therefore, higher concentrations of 8OHdG in the sample solution reacts with 8-OHdG antibody in the solution allowing less amount of 8-OHdG antibody to bind with the 8-OHdG on the plate. The quantity of 8-OHdG in unknown sample is determined by comparing it with that of a known 8-OHdG standard curve.
Materials

I. **10 X PBS**: 80 gm NaCl, 2 gm KCl, 11.5 gm Na$_2$HPO$_4$ and 2 gm KH$_2$PO$_4$ were mixed in 900 ml distilled water. pH of the solution was adjusted to 7.4 and volume was made up to 1000 ml.

From this 1X PBS was prepared by diluting 10X PBS with distilled water.

II. **0.05% PBST**: 0.05% PBST was made by mixing 50 μl of Tween-20 in 100 ml 1X PBS.

III. **8-hydroxy-2-deoxyguanosine (8-OHdG, Sigma (USA))**: 1μg/μl 8-OHdG was prepared by dissolving it in distilled water.

IV. **Goat anti-mouse IgG conjugated biotin F (ab’) fragment (Bangalore Genei, India)**: 1:2000 dilution of this antibody was used. For this 5 μl of antibody was mixed with 10 ml of PBST.

V. **Avidin Horse radish peroxidase (HRP) (Bangalore Genei, India)**: 1:10000 dilution of this antibody was used. For this 1 μl of antibody was mixed with 10 ml of PBST.

VI. **ABTS buffer (pH 8)**:

Solution A: **0.2 M Sodium phosphate dibasic anhydrous**: 2.83 gm of sodium phosphate was dissolved in 100 ml distilled water.
**Solution B: 0.2 M Citric acid:** 2.1 gm citric acid was dissolved in 100 ml of distilled water.

25 ml of solution A and 25 ml of solution B was mixed together and the volume was made up to 100 ml with distilled water.

**VII. 0.01% ABTS:** 1 mg of ABTS was dissolved in 10 ml of ABTS buffer containing 6 μl of H$_2$O$_2$.

**Method**

1. Polyvinylchloride flat bottom microtiter plate was pre-coated with 100 μl of 0.03% protamine sulphate and incubated for 8 hours at 37°C.
2. Protamine sulphate was discarded and the plate was coated with 100 ng/well 8-OHdG and incubated at 4°C overnight.
3. The antigen was discarded and the plate was incubated with monoclonal antibody (1 μg/ml) having either standard 8-OHdG or sample DNA (single stranded, 100 ng/well) for 3 hrs at 37°C. After incubation the plate was washed five times with PBST for 5 mins each.
4. 100 μl (1:2000) of goat anti mouse IgG conjugated with biotin F (ab') fragment in PBS was added and incubated at 37°C for 30 mins. After incubation the plate was washed five times with PBST for 5 mins each.
5. The plate was then incubated with 100 μl (1:10000) avidin conjugated with peroxidase at 37°C for 30 mins.
6. The plate was then washed twice with PBST for 5 mins each and thrice with phosphate citrate buffer for 5 mins each.
7. The plate was then incubated with 0.1 ml of ABTS solution and incubated for 10 mins. Following 10 mins incubation, the absorbance was read at 410 nm using ERBA Microscan Elx800 (USA) ELISA reader and quantitated using standard.

**Calculations**

Concentration of 8-OHdG was calculated by extrapolating the absorbance on a standard curve by plotting absorbance versus log of concentration and expressed as nmoles/100 ng DNA.

**5. Statistical analyses**

Data is presented as mean ± SD. The baseline difference of OSP between diabetic patients and non-diabetic subjects was tested using t test. Age and body size
(weight, BMI, waist, hip) in the two groups were significantly different and therefore the baseline difference of OSP in the two groups was further tested using multiple linear regression analysis, adjusted for gender, age and body size (BMI). Association of OSP with glycemia and lipids was tested using multiple linear regression analysis (adjusted for gender, age and BMI). Longitudinal analysis was performed using repeated measure ANOVA to test differences in serial measurements and generalized estimating equation (GEE) to test association of OSP with FPG (Jos and Twisk et al., 2003).

Twelve different OSP were measured which have different units and behave differently during the study period. To facilitate the interpretation of total oxidative stress in diabetic patients, oxidative stress score (OSS) was calculated by computing gender specific Z scores of each parameter relative to the measurements in control group. The formula used for calculating Z score was as follows:

\[
Z \text{ score} = \frac{\text{Value of individual parameter} - \text{mean value of that parameter}}{\text{Standard deviation in the parameter}}
\]

In the calculation of OSS, lower activities of CAT, GPx, GR and SOD, and lower concentrations of vitamin C, GSH and protein sulphhydryl were considered as higher OSS. On the other hand, higher concentrations of bilirubin, uric acid, protein carbonyl, TBARs and 8-OHdG were considered as higher OSS. This allows us to interpret and compare widely different measures using comparable and similar units. Association of OSS with fasting glucose, insulin, HOMA-IR and HOMA-β were tested using correlation analysis. Statistical analyses were performed using statistical package R 2.9.2 and SPSS 16.0 (SPSS Inc., Chicago, US).
RESULTS

1. Demographic, anthropometric and biochemical parameters

Fifty four type 2 diabetic patients (28 males, 26 females) and 50 non-diabetic subjects (25 males, 25 females) were recruited for this study. A proforma of detailed information gathered from a representative non-diabetic subject and diabetic patient is attached in Annexure II.

Table 1 shows the demographic and anthropometric characteristics of non-diabetic subjects and diabetic patients at baseline. Diabetic patients were older, more obese (BMI) and more centrally obese (waist circumference) compared to non-diabetic subjects ($p<0.01$, all).

Table 1. Demographic and anthropometric characteristics of non-diabetic subjects and diabetic patients at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic subjects (n=50)</th>
<th>Diabetic patients (n=54)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Male/female)</td>
<td>25/25</td>
<td>28/26</td>
<td></td>
</tr>
<tr>
<td>Age (Years)</td>
<td>33 ± 11</td>
<td>48 ± 10</td>
<td>0.00</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.8 ± 3.7</td>
<td>158.8 ± 7.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>58.8 ± 9.3</td>
<td>65.5 ± 12.5</td>
<td>0.02</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>23.9 ± 2.3</td>
<td>26.1 ± 3.9</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Anthropometric</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>84.2 ± 12.9</td>
<td>97.5 ± 9.8</td>
<td>0.00</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>89.8 ± 12.9</td>
<td>103.0 ± 9.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Waist: hip ratio</td>
<td>0.93 ± 0.03</td>
<td>0.94 ± 0.03</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Values are mean ± sd. $p$ values are calculated using t test.
Biochemical characteristics of non-diabetic subjects and diabetic patients at baseline and successive visits are shown in Table 2. In addition to higher FPG and HbA1C, diabetic patients also had higher cholesterol and triglyceride concentrations at baseline ($p<0.01$, all). Diabetic patients had higher insulin and HOMA-IR indicating low insulin sensitivity (IR), but lower HOMA-β indicating decreased insulin secretion due to β-cell dysfunction at baseline ($p<0.01$, all). Concentration of hemoglobin and HDL were similar in the two groups ($p>0.05$, both). Renal and liver functionality was assessed by quantitating creatinine; and alanine transaminase (ALT) and aspartate transaminase (AST), respectively, which were similar in the two groups ($p>0.05$, all). Resting ECG was normal for all the diabetic patients and non-diabetic subjects suggesting absence of any cardiovascular event.

Over the 8 weeks of study period, FPG concentrations showed minor and clinically insignificant changes in non-diabetic subjects. After diagnosis, all diabetic patients were advised on diet, physical activity and received oral anti-diabetic treatment: 23 (42%) patients were treated with the combination of metformin and sulfonylurea (glimepiride or gliclazide) whereas 30 (55%) patients were treated with Saxagliptin (DPP-4 inhibitor). For glycemic control, 1 (3%) patient who did not receive oral anti-diabetic treatment was kept under diet control regimen. Fifteen (28%) patients received statins.

In diabetic patients, anti-diabetic treatment led to a significant decrease in FPG concentration by 30% during the two months of treatment ($p<0.01$), however it did not achieve the level similar to that of non-diabetic subjects. HbA1C concentrations fell significantly by 28% during the study period ($p<0.01$, all). HOMA-β values increased serially and significantly in diabetic patients by the end of 8 weeks ($p<0.01$), however, insulin and HOMA-IR values did not change significantly ($p>0.05$). Creatinine and cholesterol concentrations changed significantly in diabetic patients over the duration of study ($p<0.001$), however this change was not associated with change in glucose concentrations in different visits.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-diabetic subjects (n=50)</th>
<th>Diabetic patients (n=54)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>4.8 ± 0.4</td>
<td>5.0 ± 0.6</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5.5 ± 0.4</td>
<td>------</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>53.4 ± 24.0</td>
<td>61.2 ± 27.6</td>
<td>57.6 ± 28.8</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.6</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>118 ± 35</td>
<td>123 ± 39</td>
<td>116 ± 39</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.2 ± 0.9</td>
<td>4.4 ± 0.8</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.0 ± 0.5</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>24 ± 13</td>
<td>25 ± 14</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>24 ± 6</td>
<td>24 ± 8</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>78.6 ± 16.8</td>
<td>80.4 ± 14.1</td>
<td>83.1 ± 17.6</td>
</tr>
</tbody>
</table>

Values are mean ± SD. \( p \) values refer to significance of difference in serial measurements.
2. Oxidative stress parameters (OSP) and their association with glycemia

OSP were associated with gender, age and BMI. There was a significant difference in OSP between non-diabetic subjects and diabetic patients. Therefore in multiple linear regression analysis we adjusted for these parameters. Results for non-diabetic subjects and diabetic patients for three visits are summarized in Table 3A. Majority of OSP were stable in non-diabetic subjects over the study period. Among non-diabetic subjects, FPG was inversely associated with GR (std β = -0.60), SOD (std β = -0.58) and protein sulphhydryl (std β = -0.46) and directly with bilirubin (std β = 0.46) and TBARs (std β = 0.85) (p< 0.01, all). Antioxidant defense status was impaired and oxidative markers elevated in the diabetic patients at the time of diagnosis, compared to non-diabetic subjects. Among the antioxidant enzymes, activities of CAT, GPx and GR were significantly lower in diabetic patients (p< 0.01), however, the activity of SOD was similar in two groups. Among diabetic patients, FPG was inversely associated with all the antioxidant enzymes (CAT (std β = -0.47), GPx (std β = -0.42), GR (std β = -0.55), SOD (std β = -0.56)), vitamin C (std β = -0.36) (p< 0.01, all).

In response to anti-diabetic treatment over a period of two months activities of CAT and GPx increased significantly by 14 and 142%, respectively (p< 0.01), whereas those of GR and SOD although changed by 13 and 4 %, respectively, this change was not significant. Figure 1 represents the box plot showing the median, inter-quartile range and outlying values ( ●) of antioxidant enzymes in three different visits in non-diabetic subjects represented as □ and diabetic patients represented as ▪.
**Figure 1.** Box plots representing activity of different antioxidant enzymes in non-diabetic subjects and diabetic patients for three different visits.

A. **CAT**

![Box plot for CAT activity](image)

B. **GPx**

![Box plot for GPx activity](image)
C. GR

Among the antioxidant molecules (Figure 2), uric acid was significantly higher whereas GSH was significantly lower in diabetic patients \( (p < 0.01) \), concentration of bilirubin and vitamin C were similar in the two groups. Anti-diabetic treatment during the duration of study led to a decrease in the concentration of

D. SOD

Among the antioxidant molecules (Figure 2), uric acid was significantly higher whereas GSH was significantly lower in diabetic patients \( (p < 0.01) \), concentration of bilirubin and vitamin C were similar in the two groups. Anti-diabetic treatment during the duration of study led to a decrease in the concentration of
bilirubin and uric acid by 16 and 21\%, respectively, whereas GSH increased significantly by 135\% (p < 0.0, all). Vitamin C concentration showed a significant increase by 22\% at the end of 4 weeks of treatment but fell to pre-treatment levels by 8 weeks. Among diabetic patients, FPG was inversely associated with GSH (std β = -0.36) whereas it was directly associated with bilirubin (std β = 0.82), uric acid (std β = 0.44), (p< 0.01, all).

**Figure 2.** Box plot representing concentration of antioxidant molecules in non-diabetic subjects and diabetic patients at baseline and successive visits.

**A. Uric acid**

![Uric acid box plot](image)

**B. GSH**

![GSH box plot](image)
C. Bilirubin

D. Vitamin C
Table 3A. Antioxidant defense status in study participants at baseline and at follow-up visits.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic subjects (n=50)</th>
<th>Diabetic patients (n=54)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
<td>P value</td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
<td>P value</td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td><strong>A. Antioxidant enzymes</strong></td>
<td></td>
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<tr>
<td>Catalase (U/mg Protein)</td>
<td>1761 ± 293</td>
<td>1722 ± 284</td>
<td>1735 ± 245</td>
<td>0.04</td>
<td>1379 ± 205</td>
<td>1628 ± 310</td>
<td>1567 ± 259</td>
<td>&lt;0.001</td>
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<tr>
<td>GPx (U/mg Protein)</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.04</td>
<td>0.19 ± 0.04</td>
<td>0.23</td>
<td>0.07 ± 0.02</td>
<td>0.16 ± 0.07</td>
<td>0.18 ± 0.04</td>
<td>&lt;0.001</td>
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<tr>
<td>GR (U/mg Protein)</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.23</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.08</td>
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<tr>
<td>SOD (U/mg Protein)</td>
<td>66 ± 11</td>
<td>66 ± 11</td>
<td>67 ± 10</td>
<td>0.48</td>
<td>58 ± 6</td>
<td>61 ± 9</td>
<td>61 ± 10</td>
<td>0.19</td>
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<tr>
<td><strong>B. Antioxidant molecules</strong></td>
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<tr>
<td>Bilirubin (mg%)</td>
<td>0.14 ± 0.07</td>
<td>0.15 ± 0.07</td>
<td>0.15 ± 0.05</td>
<td>0.79</td>
<td>0.19 ± 0.08</td>
<td>0.14 ± 0.07</td>
<td>0.15 ± 0.06</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>Uric Acid (mg%)</td>
<td>4.10 ± 0.49</td>
<td>4.26 ± 1.07</td>
<td>4.24 ± 0.97</td>
<td>0.56</td>
<td>5.07 ± 1.29</td>
<td>4.07 ± 0.83</td>
<td>4.01 ± 0.67</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vitamin C (mg%)</td>
<td>1.4 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>1.6 ± 0.4</td>
<td>0.01</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.6</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (nmoles/ml)</td>
<td>865 ± 338</td>
<td>851 ± 302</td>
<td>826 ± 278</td>
<td>0.4</td>
<td>143 ± 122</td>
<td>198 ± 80</td>
<td>334 ± 132</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
</tbody>
</table>

Values are mean ± sd. p values refer to significance of difference in serial measurements.
Among the oxidative damage markers (Figure 3), concentrations of protein carbonyls and 8-OHdG were significantly higher whereas protein sulphydryls were significantly lower in the diabetic patients ($p<0.01$) and concentration of TBARs was similar in the two groups at baseline. Anti-diabetic treatment led to a significant decrease in the oxidative damage markers in diabetic patients (Table 3B). Concentration of protein carbonyls and TBARs decreased by 28 and 22 %, respectively; whereas protein sulphydryls increased by 29 % ($p<0.01$, all). Among diabetic patients, FPG was inversely associated with protein sulphydryl (std $\beta = -0.30$) whereas it was directly associated with protein carbonyls (std $\beta = 0.30$), TBARs (std $\beta = 0.32$) and 8-OHdG (std $\beta = 0.40$) ($p<0.01$, all).

**Figure 3.** Box plot showing concentration of oxidative damage markers in non-diabetic subjects and diabetic patients at baseline and successive visits.

### A. Protein sulphydryl

![Box plot showing concentration of oxidative damage markers in non-diabetic subjects and diabetic patients at baseline and successive visits.](image-url)
Although, the antioxidant defense in diabetic patients showed an improvement along with a decrease in the concentration of oxidative damage markers, however, not all the OSP achieved the level similar to that of non-diabetic subjects. Amongst the OSP, the activity of GPx and concentration of bilirubin, uric acid and TBARs were comparable to those in non-diabetic subjects after eight weeks of treatment. Serial changes in all the OSP were significantly associated with the changes in the FPG concentration ($p < 0.01$), but there was no difference in the pattern of OSP with respect to the anti-diabetic treatment.
Table 3B. Oxidative damage markers in study participants at baseline and at follow-up visits.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic subjects (n=50)</th>
<th>Diabetic patients (n=54)</th>
<th>P value</th>
<th>Non-diabetic subjects (n=50)</th>
<th>Diabetic patients (n=54)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
<td>Baseline</td>
<td>4 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Protein Carbonyls</td>
<td>4.7 ± 1.6</td>
<td>5.1 ± 1.4</td>
<td>5.1 ± 1.5</td>
<td>0.08</td>
<td>8.9 ± 3.7</td>
<td>7.3 ± 2.5</td>
</tr>
<tr>
<td>(nmoles/mg Protein)</td>
<td></td>
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</tr>
<tr>
<td>Protein Sulphhydryls</td>
<td>1.55 ± 0.40</td>
<td>1.58 ± 0.39</td>
<td>1.55 ± 0.39</td>
<td>0.51</td>
<td>0.82 ± 0.44</td>
<td>1.12 ±0.34</td>
</tr>
<tr>
<td>(nmoles/mg Protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TBARs</td>
<td>2.28 ± 0.68</td>
<td>2.17± 0.67</td>
<td>2.24± 0.50</td>
<td>0.22</td>
<td>2.93 ± 1.30</td>
<td>2.38± 0.85</td>
</tr>
<tr>
<td>(nmoles MDA/L)</td>
<td></td>
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<td></td>
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<tr>
<td>8-OHdG</td>
<td>0.8 ± 1.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>5.1 ± 7.0</td>
<td>----</td>
</tr>
<tr>
<td>(nmoles/100 ng DNA)</td>
<td></td>
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</tbody>
</table>

Values are mean ± sd. *P* values refer to significance of difference in serial measurements.
3. Oxidative stress score (OSS), glucose, HOMA-IR and HOMA-β

Twelve different OSP were combined into a single comprehensive score, referred to as OSS. It represented total oxidative stress state in diabetic patients and was calculated by computing relative Z scores of twelve different OSP relative to the measurements in non-diabetic subjects. Expectedly, total OSS was higher in diabetic patients compared to non-diabetic subjects at the baseline \( (p < 0.001) \) and showed progressive and significant fall with control of hyperglycemia at the end of eight weeks \( (p < 0.01) \) (Figure 4).

Figure 4. Oxidative stress score (OSS) in non-diabetic subjects and diabetic patients. OSS was calculated taking into account all the parameters measured to assess oxidative stress and compared between diabetic and non-diabetic subjects at baseline, 4 weeks and 8 weeks.

![Figure 4: Oxidative stress score (OSS) in non-diabetic subjects and diabetic patients.](image)

Antioxidant enzyme score was significantly low in diabetic patients at baseline and increased by 82% after four weeks (Table 5A). On the other hand, antioxidant molecules score and oxidative damage score was significantly high in diabetic patients and decreased by 78% and 85%, respectively after 4 weeks. There was 80 % reduction in total OSS in diabetic patients by 4 weeks which then remained stable after 8 weeks. The total score, however, remained higher in diabetic patients at eight weeks compared to non-diabetic subjects (Table 5B).
Table 5A. Oxidative stress scores (OSS) of participants. OSS for diabetic patients was calculated by computing gender specific Z scores of individual antioxidant enzymes and molecules relative to the measurements in non-diabetic subjects at baseline and at follow-up visits.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic subjects</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
</tr>
<tr>
<td><strong>A. Antioxidant enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>0.07 (-0.23, 0.38)</td>
<td>-0.06 (-0.36, 0.23)</td>
</tr>
<tr>
<td>GPx</td>
<td>0.23 (-0.09, 0.55)</td>
<td>0.05 (-0.34, 0.45)</td>
</tr>
<tr>
<td>GR</td>
<td>0.81 (0.43, 1.20)</td>
<td>0.60 (0.23, 0.98)</td>
</tr>
<tr>
<td>SOD</td>
<td>-0.04 (-0.33, 0.24)</td>
<td>-0.02 (-0.32, 0.27)</td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>1.08 (0.29, 1.87)</td>
<td>0.57 (-0.26, 1.41)</td>
</tr>
<tr>
<td><strong>B. Antioxidant molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>-0.01 (-0.49, 0.46)</td>
<td>0.07 (-0.34, 0.50)</td>
</tr>
<tr>
<td>Uric acid</td>
<td>-0.06 (-0.30, 0.17)</td>
<td>0.18 (-0.32, 0.70)</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>-0.10 (-0.47, 0.27)</td>
<td>0.24 (-0.18, 0.66)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.06 (-0.27, 0.40)</td>
<td>0.09 (-0.28, 0.30)</td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>-0.03 (-0.95, 0.87)</td>
<td>0.01 (-0.99, 1.03)</td>
</tr>
</tbody>
</table>

Values are mean (95% CI).
Table 5B. Oxidative stress scores (OSS) of participants. OSS for diabetic patients was calculated by computing gender specific Z scores of individual oxidative damage markers relative to the measurements in non-diabetic subjects at baseline and at follow-up visits.

<table>
<thead>
<tr>
<th>Oxidative damage markers</th>
<th>Non-diabetic subjects</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.17 (-0.52, 0.17)</td>
<td>0.13 (-0.18, 0.45)</td>
</tr>
<tr>
<td>Protein sulphydryl</td>
<td>0.18 (-0.19, 0.56)</td>
<td>0.29 (-0.08, 0.67)</td>
</tr>
<tr>
<td>TBARs</td>
<td>0.18 (-0.18, 0.54)</td>
<td>-0.04 (-0.38, 0.28)</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>0.06 (-0.24, 0.38)</td>
<td>-----</td>
</tr>
<tr>
<td>Total OSS</td>
<td>-1.2 (-3.4, 0.9)</td>
<td>-0.7 (-2.9, 1.4)</td>
</tr>
</tbody>
</table>

Values are mean (95% CI).
In both non-diabetic subjects and diabetic patients OSS was strongly associated with glucose (r = 0.79, r = 0.83, p< 0.001, both, respectively). In non-diabetic subjects, it was directly associated with HOMA-IR (r = 0.32, p< 0.01) and inversely with HOMA-β (r = -0.17, p< 0.05). In diabetic patients there was no association between OSS and HOMA-IR but there was a strong inverse association with HOMA-β (r = -0.56, p< 0.001). Fall in OSS with treatment was associated with improvement in HOMA-β (std β = -0.38, p< 0.01).
DISCUSSION

The present work was carried out to comprehensively study antioxidant enzymes, antioxidant molecules and oxidative stress markers in fifty-four newly diagnosed diabetic patients and to repeat the measurements serially after four and eight weeks during which diabetic patients were treated for control of hyperglycemia. Fifty non-diabetic subjects served as controls. There was a significant impairment in antioxidant defense and an increase in OS in newly diagnosed diabetic patients compared to non-diabetic subjects. This was clearly reflected in lower activities of antioxidant enzymes, lower concentrations of antioxidants vitamin C & GSH, and elevated concentrations of oxidative damage markers despite elevation in endogenous antioxidants (bilirubin and uric acid). Serial measurements in non-diabetic subjects showed a stable status of OSP over eight weeks. Upon diagnosis, diabetic patients were put on anti-diabetic treatment which led to a significant and gradual decrease in the concentration of glucose and glycosylated hemoglobin by the end of eight weeks. Additionally, in diabetic patients, antioxidant defense status improved in response to anti-hyperglycemic treatment. This was reflected in elevated activity of antioxidant enzymes and concentrations of antioxidant molecules and decreased concentration of oxidative stress markers. Improvement in OSP in diabetic patients was not related to the different anti-diabetic drugs received by them. Among OSP, concentrations of GPx, uric acid, bilirubin and TBARs became comparable to those in non-diabetic subjects. Level of glycaemia was a strong predictor of OS at baseline in diabetic patients, and fall in glucose concentration over the study duration was a strong predictor of improved antioxidant status. Additionally, OSS, calculated by collating twelve different OSP, showed strong correlation with glucose and HOMA-β, both in non-diabetic subjects and diabetic patients. In non-diabetic subjects, but not in diabetic patients, OSS, an indicator of total systemic OS, had a weak association with HOMA-IR. Improvement in hyperglycemia and reduction in OSS were not associated with HOMA-IR.

Several clinical studies have showed an impaired concentration of biomarkers of OS as suggested by lowered status of antioxidant defense and elevated oxidative damage in diabetic patients with and without complications (Matteucci and Giampietro 2000; Aydin et al., 2001; Maharjan et al., 2008). However, there are only a few reports on the antioxidant defense status of patients newly diagnosed with
diabetes (Domínguez et al., 1998; Martin-Gallan et al., 2005; Pasaoglu et al., 2004; Song et al., 2007) and even fewer studies wherein the effect of glycemic control on OSP is serially measured (Song et al., 2007) in response to anti-diabetic treatment. There is an agreement in the literature that level of antioxidant defense is severely compromised and oxidative stress markers are elevated in diabetic patients with and without diabetic complications. However, there is no consensus on the pattern of antioxidant enzymes in diabetic patients: some studies have shown an increase (Aydin et al., 2001; Kesavulu et al., 2001; Gallan et al., 2003; Martinez-Sanchez et al., 2005; Moussa, 2008; Bandeira et al., 2012) while others a decrease (Bhatia et al., 2003; Abou-Seif and Youssef, 2004; Martin-Gallan et al., 2005; Ramakrishna and Jailkhani, 2007; Pasupathi et al., 2009; Arif et al., 2010) in the activity of antioxidant enzymes. This discrepancy could be attributed to the difference in the study design of these studies with respect to type and duration of diabetes, glycemic status, anti-diabetic treatment and also the methods used to measure different parameters. Additionally, all the studies where association between OSP and glycaemia or diabetic complications is demonstrated are cross-sectional where it is difficult to assert causality. Song et al., (2007) demonstrated that OS is increased even in pre-diabetic state i.e., impaired glucose tolerance (IGT) as reflected in high concentration of TBARs and low activity of SOD. This indicated that biomarkers of OS are impaired in diabetic patients before the establishment of frank hyperglycaemia, a characteristic of type 2 diabetes.

Intrinsic antioxidant defense machinery of a cell comprises of several antioxidant enzymes and antioxidant molecules which scavenge ROS directly and/or indirectly, thereby protecting the cell from its deleterious effects. However, in diabetic patients this defense machinery is severely impaired, thereby exacerbating the harmful effects of ROS. The possible mechanisms underlying the compromised antioxidant defense status are poorly understood. It is attributed to glycosylation and oxidation of proteins, lipids and nucleic acids (Hinokio et al., 1999; Kalousová et al., 2002; Miyazawa et al., 2012). Disturbances in the redox buffer of a cell such as NADH/NAD and GSH/GSSG owing to the excess generation of free radicals also could contribute to the low defense status. Low concentration of GSH in turn fails to replenish antioxidants like vitamins C and E (Young and Woodside, 2001) and further impairs the antioxidant defense mechanism. Another antioxidant, uric acid, constitutes over half the antioxidant capacity of blood plasma (Simic and Jovanovich, 1989; Young and Woodside, 2001) by scavenging free radicals such as, O$_2^-$, $^1$O$_2$, and `OH.
and by chelating transition metals. On the contrary, it is known to act as a pro-oxidant at higher concentrations (Becker, 1993; Strazzullo and Puig, 2007). High levels of uric acid in diabetic patients could therefore represent a compensatory response or a primary cause of OS. Another important endogenous antioxidant in the serum namely bilirubin is known to scavenge peroxyl radicals more effectively than α-tocopherol and suppresses the oxidation of lipids and lipoproteins (Stocker et al., 1987). Despite these endogenous sources of antioxidants, excess ROS which escape the antioxidant defense mechanism, damage biomolecules such as proteins, lipids and DNA.

Prolonged exposure of proteins to ROS causes severe spontaneous post-synthetic modifications such as oxidation. Direct oxidation of lysine, arginine, proline and threonine residues in proteins yields carbonyl derivatives, which are early and stable markers of protein oxidation. This mechanism contributes to the inactivation of CAT, SOD and GPx, thus perpetuating oxidative damage. Additionally, thiol groups (-SH) bound to proteins (protein sulphydryls) existing in both intracellular and extracellular matrix are amongst one of the first crucial plasma sacrificial antioxidants that scavenge aqueous peroxyl radicals. Low concentration of thiol groups in diabetic patients (Baskol et al., 2008; Pandey et al., 2009) is attributed to their increased consumption to scavenge free radicals. ROS mediated degradation of polyunsaturated fatty acids, incorporated in all biological membranes, lead to the formation of malondialdehyde (MDA) through a series of chain reactions. This increase in lipid peroxidation in diabetic patients could be a result of inefficient antioxidant scavenger mechanism. Excess ROS oxidizes DNA to 8-OHdG and has been measured most frequently to indicate the extent of oxidative damage in diabetic patients with complications (Xu et al., 2004; Goodarzi et al., 2010). Increased concentration of oxidative damage markers in this study is in accordance with previous reports where elevated concentration of lipid peroxides, protein carbonyls and 8-OHdG in addition to depleted concentration of protein sulphhydryl groups in diabetic patients (Jain et al., 1989; Dandona et al., 1996; Odetti et al., 1999; Davi et al., 2003; Nishikawa et al., 2003) has been demonstrated. Out of these biomolecules, only DNA can be repaired whereas lipids and proteins are turned over. In the long run, this damage leads to disturbances in the integrity and physiological function of a cell, thereby contributing to various complications like vascular damage in diabetic patients. Since, OS plays an important role in the pathogenesis of both β-cell dysfunction and IR, inter-relation between oxidative stress status, β-cell function and IR was investigated. Glucose
clamp is the gold standard in assessment of insulin sensitivity (DeFronzo et al., 1979). Since it is reported that HOMA-IR is strongly related to glucose clamp-measured insulin resistance in both non-diabetic and diabetic subjects (Matthews et al., 1985; Emoto et al., 1999; Bonora et al., 2000), HOMA-IR was used as an index for insulin sensitivity and HOMA-β as an index for β–cell function. The increased degree of IR, as observed in the present study, represented a compensatory mechanism, perhaps by protecting adipocytes and muscle cells from OS, which is a key element in the pathogenesis of IR and in pancreatic β-cell dysfunction. Progressive decline of β-cell function in pre-diabetic condition is partially attributed to increasing ‘hyperglycemia’ (glucotoxicity) (Poitout and Robertson, 2002; Fonseca, 2009). This effect is further amplified in β-cells owing to their poor antioxidant defense status (Grankvist et al., 1981; Lenzen et al., 1996) which causes β-cells to succumb to ROS assault. Improvement in HOMA-β in diabetic patients with improvement of hyperglycemia is indicative of restoration of β-cell function as a consequence of reduced OS. However, improvement in hyperglycemia and reduction in OSS were not associated with HOMA-IR, insulin sensitivity index, suggesting its irreversibility in short duration.

Since, damage to proteins, lipids and DNA in diabetes is mediated by hyperglycemia induced excess free radical production, controlling glycemia in turn would prevent the cells from oxidative damage. Anti-diabetic treatment for duration of eight weeks led to a reduction in the OS biomarkers and also restored the activity of antioxidant enzymes. Although metformin (Formoso et al., 2008) and gliclazide (O’Brien et al., 2000) have been reported to exhibit antioxidant property and are known to restore antioxidant function in type 2 diabetic patients, there is no information available on specific antioxidant properties of Saxagliptin, which was administered to 55% diabetic patients in this study. Thus improvement in the antioxidant defense status and oxidative stress markers in response to anti-hyperglycemic treatment, observed in this study, could be a direct consequence of the reduction in hyperglycemia mediated ROS production.

In summary, these findings draw attention towards two major things. Firstly, an elevated level of OS, reflected by increased oxidative damage to the bio-molecules and compromised antioxidant defense status is evident at the onset of type 2 diabetes i.e., much before the development of secondary complications and was also associated with reduced β-cell function. Secondly, these findings also clearly demonstrate that controlling hyperglycemia in diabetic patients for duration of eight weeks improves
the antioxidant defense status of individuals and partially restores the oxidative damage irrespective of the type of anti-diabetic treatment. Improvement in OS in diabetic patients was associated with an improvement in β-cell function suggesting a reduction in the oxidative damage caused by ROS at the end of eight weeks. However, anti-diabetic treatment for a longer duration is likely to further restore the activity of antioxidant enzymes and consequently reduce the oxidative damage to cells. Therefore, it is interesting to study the effect of stricter and longer glycemic control regimen on OS and its potential benefits on β-cell function and other forms of tissue damage.

Additionally, the present study also potentiates the importance of controlling OS in conjunction with glucose control. Several intervention studies have been undertaken in this regard to determine if dietary antioxidants exert beneficial effects for type 2 diabetic patients. Treatment with antioxidants such as vitamin C and E, either individually or in combination, have been reported to improve early oxidative stress markers in the plasma, urine, and circulating cells, as well as endothelial dysfunction and microalbuminuria (Beckman et al., 2001; Gaede et al., 2001; Venugopal et al., 2002). Oral administration of Vitamin E has also been demonstrated to restore plasma antioxidant defenses by maintaining GSH/GSSG ratio and improves insulin action in diabetic patients (Paolisso et al., 1993). On the contrary, some studies have reported that intake of vitamins C and E in diabetic patients is not associated with risk of diabetic complications (Millen et al., 2004). These contrasting reports could be due to rampant usage of antioxidants which affect the regulatory role of ROS. Nevertheless, importance of antioxidant supplementation in diabetes cannot be denied and the adverse effect of antioxidants can be prevented by maintaining a delicate balance between the production of ROS and their neutralization by antioxidants.