OXIDATIVE STRESS IN *DIABETES MELLITUS*

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Abstract. Our aim was to evaluate oxidative stress in *diabetes mellitus* by the measurement of biophysical parameters (changes) of hemoglobin macromolecule and some biochemical parameters on two groups of diabetic patients, non-insulin dependent *diabetes mellitus* (NIDDM), and insulin-dependent *diabetes mellitus* (IDDM). This study has been conducted on 45 NIDDM, 30 IDDM, compared to 20 healthy subjects. Blood glucose, glycosylated hemoglobin (HbA1c), fructosamine, plasma insulin, plasma malondialdehyde (MDA), erythrocyte reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-Red) were measured. Also the biophysical parameters, electrical conductivity of hemoglobin, auto-oxidation rate of hemoglobin, and hemoglobin derivatives were measured. Diabetic patients (NIDDM, IDDM) have significant increases in blood glucose, glycosylated (HbA1c), fructosamine with a concomitant significant decrease in Plasma insulin compared to control. Diabetic patients (NIDDM, IDDM) have a significant high level of MDA compared to control. Diabetic patients (NIDDM, IDDM) have a significant increase in SOD activity, and a significant decrease of GSH compared to control. Significant increases in electrical conductivity of Hb, hemoglobin auto-oxidation rate and hemoglobin derivatives in diabetic patient compared to control. IDDM have a higher level of hemoglobin auto-oxidation rate when compared to NIDDM. Diabetic patients undergo an important oxidative stress that is low in NIDDM compared to IDDM, suggesting metabolic differences between the two types of diabetes. Met-Hb is an important indicator for oxidative stress in diabetes.

Key words: IDDM, NIDDM, electrical conductivity, hemoglobin, SOD, MDA, GSH.

INTRODUCTION

Diabetes mellitus, a common metabolic disorder resulting from defects in insulin secretion or action or both, is characterized by hyperglycemia often accompanied by glycosuria, polydipsia, and polyuria [2, 8, 46]. During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation [3, 7, 44]. Free radicals are generated as by-products of normal cellular metabolism; however, several conditions are known to disturb the balance between ROS production and cellular defense mechanisms. This

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imbalance can result in cell dysfunction and destruction resulting in tissue injury. The increase in the level of ROS in diabetes could be due to their increased production and/ or decreased destruction by nonenzymic and enzymic catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)] antioxidants. The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes. Also this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses [18, 31, 44, 54].

Diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation [33], which is responsible for increased incidence of atherosclerosis [15], a major complication of *diabetes mellitus* [50]. An enhanced oxidative stress has been observed in these patients as indicated by increased free radical production [24], lipid peroxidation and diminished antioxidant status [6].

Free radicals may play an important role in the causation and complications of *diabetes mellitus* [37]. In *diabetes mellitus*, alterations in the endogenous free radical scavenging defense mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury.

Oxidative stress is currently suggested as a mechanism underlying diabetes and diabetic complications [22]. Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental *diabetes mellitus*, are thought to be the etiology of chronic diabetic complications [6]. In recent years, much attention has been focused on the role of oxidative stress, and it has been reported that oxidative stress may constitute the key and common event in the pathogenesis of secondary diabetic complications [10]. Free radicals are continually produced in the body as a result of normal metabolic processes and interaction with environmental stimuli.

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or both. Implication of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation, auto-oxidation of glucose [38], impaired glutathione metabolism [36], alteration in antioxidant enzymes [51], lipid peroxides formation [6] and decreased ascorbic acid levels [56]. In addition to GSH, there are other defense mechanisms against free radicals like the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) whose activities contribute to eliminate superoxide, hydrogen peroxide and hydroxyl radicals [49].

The measurement of electrical conductivity of aqueous solution has led to an understanding to what extent the substances are ionized in water, the combination of ions with surrounding molecules, and the way in which ions move in water. All of these topics have a great significance in biology [29].

The aim of this study was to evaluate oxidative status in two types of *diabetes mellitus*: insulin dependent *diabetes mellitus* (IDDM) and non-insulin dependent *diabetes mellitus* (NIDDM) compared to a normoglycemic group through the measurements of some biophysical and biochemical parameters.

MATERIAL AND METHOD

SUBJECTS

Blood samples were obtained under fasting conditions from 95 patients with *diabetes mellitus*: 30 IDDM (15 females and 15 males) with a mean age of 50±16 years; 45 NIDDM (20 females and 25 males) with a mean age of 55±13 years and 20 healthy subjects (12 females and 8 males) with a mean age of 37.2±18 years. Duration of diabetes was 15±11 years for NIDDM, and 18±12 years for IDDM.

BIOCHEMICAL MEASUREMENTS

For erythrocyte enzymes, heparinized whole blood was kept in ice. Erythrocytes were separated and centrifuged at 4 °C for 20 min at 3500 rpm. Erythrocytes were rewashed for measurement of enzymes (≤ 24 hours). All colorimeteric measurements were carried out, using UV/Visible spectrophotometer type (Helios Alpha) 9423NC.

Hemoglobin concentration of the samples was measured by Drabkin's method [12] to express erythrocyte GSH, GSH-Px and SOD activity values in units per gram hemoglobin.

BLOOD GLUCOSE AND PLASMA INSULIN

Fasting blood glucose was estimated by O-toluidine method [47]. Plasma insulin was estimated by using enzyme linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany).

GLYCOSYLATED HEMOGLOBIN (HBA1C) AND FRUCTOSAMINE

Glycosylated hemoglobin was measured using the spectrophotometric method (procedure kit, Helena Laboratory). Serum concentration of fructosamine was determined by a colorimetric method (FRUC kit, Roche Diagnostics, Mannheim, Germany).

OXIDATIVE PARAMETERS

Malondialdehyde (MDA)

Plasma MDA concentration was determined by using the method described by Draper and Hadley [13, 23] based on TBA reactivity. Briefly, 2.5 mL of 10% trichloracetic acid and 0.5 mL of plasma were added into tubes and mixed. After incubating for 15 min at 90°C and cooling with cold water the mixture was centrifuged at 3000 rpm for 10 min. Two milliliters of supernatant were taken and 1ml of 0.675% TBA was added. The tubes were sealed and incubated at 90°C for15 min and then cooled to room temperature. The optical density was measured at 532 nm by a spectrophotometer.

ANTIOXIDANT PARAMETERS

Erythrocyte reduced glutathione (GSH)

Intra-erythrocyte GSH was determined with a colorimetric assay using Bioxytech GSH-400 kit (Oxis International, Portland, OR, USA) based on a two-step reaction: thioethers formation followed by a β-elimination under alkaline conditions. Thioethers obtained are transformed into chromophoric thiones which have a maximal absorbance wavelength at 400 nm.

Superoxide dismutase (SOD)

The activity of SOD was measured at 500 nm with a commercially available kit (Randox Laboratories, kit Ransod superoxide dismutase) by testing the inhibition degree of a tetrazolium salt oxidation reaction. The coefficient of variability between assays was 4.2%[52].

Glutathione peroxidase (GSH-PX)

The erythrocyte activity of GSH-Px was measured with a commercially available kit (Ransel glutathione peroxidase, Randox Laboratories) in erythrocytes at 340 nm by measuring the decrease of NADPH absorbance. This method is based on that of Paglia and Valentine [43]. The coefficient of variability between assays was 4%.

Glutathione reductase (GSH-Red)

GSH-Red activity was measured in erythrocytes with a commercially available kit (kit GR, Randox Laboratories) by measuring the decrease of absorbance of NADPH at 340 nm. The coefficient of variability between assays was 4%.

BIOPHYSICAL PARAMETERS

Electrical conductivity of hemoglobin

Electrical conductivity was measured by a conductivity meter (digimeter L21) range from 0–200 μS/cm and automatic temperature compensator.

Hemoglobin auto-oxidation rate

Measurement of the auto-oxidation rate was carried out spectrophotometrically as described by Guillochon *et al*. [20].

Hemoglobin derivatives

HbO2, HbCO, SHb and Met-Hb were measured by using the multicomponent spectrophotometric method for the simultaneous determination of hemoglobin derivatives described by Atef *et al*. [5].

STATISTICAL ANALYSIS

Data were analyzed using SPSS statistical software (SPSS/10 for Windows). All the measurements were done in triplicates. A student's t-test was used to estimate differences between the groups. All parameters were given as mean± standard error (SE). The criterion for significance was $p < 0.05$.

RESULTS

Clinical and biochemical data of healthy and diabetic subjects are summarized in Table 1 while antioxidant and oxidative stress parameters results in Table 2.

Table 1 shows the levels of glycosylated hemoglobin, fructosamine, and plasma insulin, in the diabetic patients (IDDM, NIDDM) and normoglycemic subjects. There were significant increases in the levels of glycosylated hemoglobin and fructosamine with a concomitant decrease in the level of plasma insulin in the diabetic patients (IDDM, NIDDM) when compared with normoglycemic subjects.

Table 2 shows the level of lipid peroxidation marker, malondialdehyde (MDA), and antioxidant defense system components in the diabetic patients (IDDM, NIDDM) and normoglycemic subjects. MDA is significantly higher in the diabetic patients (IDDM, NIDDM) as compared to control. Erythrocyte GSH is significantly lower in the diabetic patients (IDDM, NIDDM) compared to control. SOD activity is significantly enhanced in diabetic patients (IDDM, NIDDM). Enzymatic activities of GSH-Px and GSH-Red are higher in diabetic than in healthy subjects but the difference is not significant.

Table 1

Clinical and biochemical parameters in diabetic patients (IDDM, NIDDM) as compared to control

Parameters	**Control**	**IDDM**	**NIDDM**
n	30	30	45
Female/male	12/8	15/15	20/25
Age (years)	37.2 ± 18	50 ± 16[a]	55 ± 13*
Duration of diabetes (years)		20 ± 14	14 ± 10
HbA1c (%)	4.5 ± 0.8	9.7 ± 2*	12.5 ± 2.5*
Fructosamine (μmol/L)	230 ± 13	430 ± 16*	510 ± 18*
Plasma insulin (μU/mL)	14 ± 0.7	5.5 ± 0.3*	6.9 ± 0.42*

*$p < 0.05$ diabetic patients vs. controls
[a]$p < 0.05$ IDDM vs. NIDDM

Table 2

Oxidative and antioxidant parameters in diabetic patients (IDDM, NIDDM) as compared to control

Parameters	**Control**	**IDDM**	**NIDDM**
MDA (μmol/L)	1.3±0.3	2.6±0.5*	3.0±0.7*
GSH (mmol/L)	3.5±0.42	2.95±0.65[a,]*	2.30±1.3*
SOD (U/g Hb)	1321±250	3250±790*	3820±770*
GSH-Px (U/g Hb)	30±10.1	45.1±17	43.2±16.6
GSH-Red (U/g Hb)	6.5±0.9	10.32±2.5	12.2±3

*$p < 0.05$ diabetic patients vs. controls
[a]$p < 0.05$ IDDM vs. NIDDM

Table 3 provides the level of met-Hb % in the diabetic patients (IDDM, NIDDM) and normoglycemic subjects. There was a significant increase in met-Hb % level in the diabetic patients (IDDM, NIDDM) when compared with normoglycemic subjects.

Table 3

Hemoglobin derivatives of diabetic patients (IDDM, NIDDM) as compared to control

Parameters	Hemoglobin derivatives			
	HbO_2 (%)	HbCO (%)	S-Hb (%)	Met-Hb(%)
Control	96	0.3	0.7	3
IDDM	83.3	1.7	2	13
NIDDM	87.4	0.7	1.9	10

HbO_2, oxyhemoglobn, HbCO Carboxy-hemoglobin, S-Hb Sulpho-hemoglobin, Met-Hb methemoglobin.

Figure 1 shows the electrical conductivity of hemoglobin in the diabetic patients (IDDM, NIDDM) and normoglycemic subjects. There was a significant increase in the electrical conductivity of hemoglobin in the diabetic patients (IDDM, NIDDM) when compared with normoglycemic subjects.

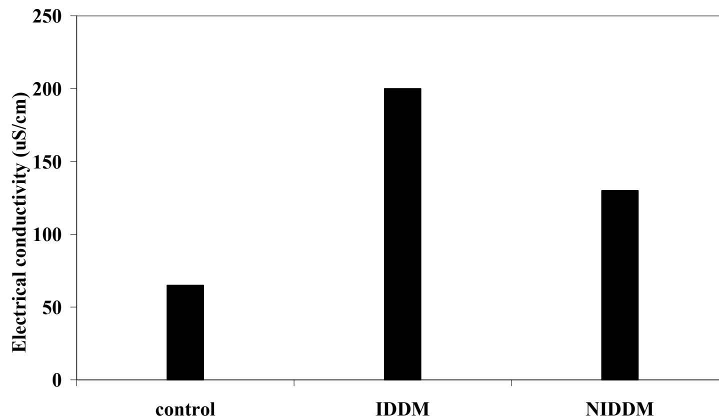

Fig. 1. The electrical conductivity of hemoglobin of diabetic patients (IDDM, NIDDM) as compared to control.

Figure 2 provides the auto-oxidation rate of hemoglobin in the diabetic patients (IDDM, NIDDM) and normoglycemic subjects. There was a significant increase in the auto-oxidation rate of hemoglobin in the diabetic patients (IDDM) compared to control, while the auto-oxidation rate is a significant decrease in (NIDDM) when compared to control. IDDM patients have a significant higher auto-oxidation rate of hemoglobin than NIDDM patients.

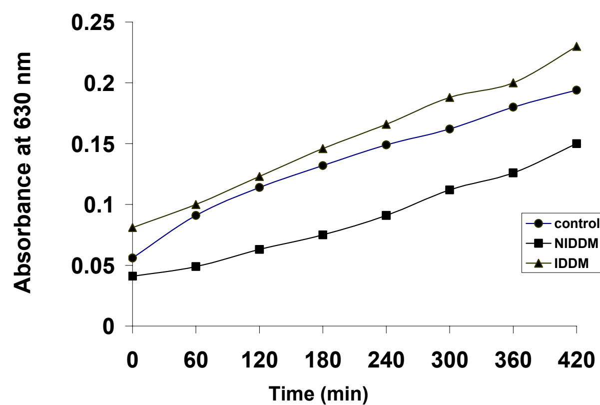

Fig. 2. The auto-oxidation rate of hemoglobin of diabetic patients (IDDM, NIDDM) as compared to control.

DISCUSSION AND CONCLUSION

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems [14]. There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [28, 45]. Free radicals are formed disproportionately in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins [41]. Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of *diabetes mellitus* [34].

In the present study, we examined oxidative stress pathway markers in the diabetic patients (NIDDM, IDDM) as compared to normoglycemic subjects.

From the results obtained, it is evident that the diabetic patients had much higher glucose levels and decreased insulin level when compared with normoglycemic subjects. The increase in blood glucose level and decreased insulin level depends upon the degree of β-cell destruction [19].

The increased level of glycosylated hemoglobin was observed in the diabetic patients and this increase is directly proportional to the blood glucose level [55]. This suggests the increase in oxidative stress due to hyperglycemia.

Hypoinsulinaemia in diabetes increases the activity of the enzyme fatty acyl coenzyme A oxidase, which intiates β-oxidation of fatty acids, resulting in lipid peroxidation [25]. Increased lipid peroxidation impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. The products of lipid peroxidation are harmful to most cells in the body and are associated with a variety of diseases, such as atherosclerosis and brain damage [1]. In our study, a significant increase of MDA was observed in the plasma of diabetic patients.

Glutathione, a tripeptide present in millimolar concentrations in all the cells, is an important antioxidant [32]. Reduced glutathione normally plays the role of an intracellular radical scavenger and is the substrate of many xenobiotic elimination reactions [17]. A marked decreased level of reduced glutathione is reported in the plasma of diabetic patients. Results of our study is in agreement with other studies [10, 16, 26, 27, 39, 40, 42, 48, 53,]. GSH systems may have the ability to manage oxidative stress with adaptational changes in enzymes regulating GSH metabolism. There is a negative correlation between GSH and HbA1c in diabetic patients [16, 48] which confirms the link between hyperglycemia and GSH depletion. Indeed, in hyperglycemia conditions, glucose is preferentially used in polyol pathway [30], that consumes NADPH necessary for GSH regeneration by the GSH-Red enzyme. Hyperglycemia is therefore indirectly the cause of GSH depletion. As GSH is an important antioxidant molecule, its depletion leads to the increase of oxidative stress.

Superoxide dismutase is considered a primary enzyme since it is involved in the direct elimination of reactive oxygen species [21]. SOD is an important defense enzyme which catalyzes the dismutation of superoxide radicals [35]. GPx was considered biologically essential in the reduction of hydrogen peroxide. In the present study, the elevation in the antioxidant enzyme activities of SOD and GPx in diabetic patients was analyzed. There is a positive correlation between SOD, GPx and MDA. Our result is consistent with the result of Dominguez *et al.* [11] who reported an increase in antioxidant enzymes such as SOD, and GPx in *diabetes mellitus* which gives an evidence of increased reactive oxygen species production .

Increased oxidative stress as measured by the index of lipid peroxidation has been shown to be increased in both insulin-dependent (IDDM), and non-insulin-dependent (NIDDM) *diabetes mellitus* [4] and it could cause initial β cell damage in type I diabetes or impaired insulin production, release, or function in type II diabetes [7, 54].

Hyperglycemia will promote the conversion of oxyHb to metHb, and consequently the fractions of unstable Hb molecule that undergo abnormal dissociation(auto-oxidation) to metHb, SHb, and HbCO increased with increasing hyperglycemia as indicated in Table 3.

In this work, hyperglycemia will promote the conversion of oxyHb (diamagnetic) to metHb (paramagnetic) as indicated in Table 3. Consequently, the fraction of unstable Hb molecules that undergo abnormal dissociation (auto-oxidation) to metHb increased with the disease. The oxidation of hemoglobin molecules leads to unfolding of the globular protein with the formation of a new group exposed to the surface besides the polar hydrophilic groups and consequently increasing electrical conductivity (Fig. 1). As indicated in figure 1 the electrical conductivity of hemoglobin molecule in diabetic patients was more than in normoglycemic subjects. This indicates to the increase in the overall charges of hemoglobin molecule due to the increase in the free radical production. Hyperglycemia influences the electrical charge distribution on the surface of the cell membrane.

Also as indicated in figure 2, the auto-oxidation rate of hemoglobin molecule in the diabetic patients was more than that of normoglycemic subjects. This indicates to the effect of reactive oxygen species and free radical on the oxidation rate of oxyhemoglobin to met-hemoglobin.

Conclusively, Diabetic patients undergo an important oxidative stress when compared to control. Oxidative stress is comparatively low in NIDDM when compared to IDDM suggesting metabolic differences between the two types of diabetes. Methemoglobin is an important measure of oxidative stress in diabetic patients. The biophysical parameters such as electrical conductivity, hemoglobin derivatives and auto-oxidation rate of hemoglobin molecule explain the oxidative stress on the molecular level.

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