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Some Biophysical Investigations on Diabetes Mellitus with Relevant to Oxidative Stress and Antioxidants

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Abstract: This study consists of 72 diabetic patients of type I (IDDM) and 52 diabetic patients of type II (NIDDM) who were taken from Al HusseinHospital and Sphinx Hospital besides 35 persons of matched age and sex. The following parameters were measured, blood sugar, immunoreactive insulin, antioxidant enzymes, thermodynamic parameters of hemoglobin (Hb), intrinsic viscosity and autoxidation reaction rate of Hb. Diabetes was shown to be inversely related to beta carotene concentration and lesser extend to lycopene which in its turn correlated with the baseline of fasting glucose. So, fasting insulin is inversely related to beta carotene as a result of the association of carotene deficiency with patients.The activity of SOD is less in NIDDM and IDDM in comparison with normal control. SOD is inhibited by HbAIc and is lowered in poorly controlled diabetes. The inactivity of SOD by glycosylation may be a dominant factor in the loss of SOD activity. An increase in the activity of GPX in diabetes mellitus may be an adequate or compensatory mechanism.High free radical in both cases (type I and type II) patients enhances the drive out of equilibrium and signifies the low affinity of Hb to oxygen and it occurs in both types of diabetic patients.A decrease of heat content of ΔH and activation energy as well as elevation of molecular disorder (entropy) of hemoglobin in both IDDM and NIDDM as compared to control means that Hb loses heat to the surrounding beside the defect in folding and unfolding which represents the dynamic motion of hemoglobin to carry oxygen. i.e. tissue hypoxia as a result of deficiency of antioxidants.Non appreciable changes in intrinsic viscosity of Hb in diabetic patients indicate a lack of changes in the dimensions of hemoglobin molecules were observed.The elevation of oxidative stress has an indirect effect on the degree of Hb folding and unfolding of diabetic patients as a result of high glucose content and HbAIc.The present data clearly indicate a defect in methemoglobin reductase system as a result of lack of insulin where it is important to maintain the autoxidation reaction rate in the red blood cells.Oxidative stress results in the lowering of antioxidant concentrations in people with glucose intolerance. Thus, it is conceivable that both endogenous and exogenous antioxidants could play a role in the pathogenesis of glucose intolerance.

Key words: Biophysics · Diabetes · Antioxidants

INTRODUCTION

Many evidences has indicated that some biochemical pathways strictly associated with hyperglycermia (nonenzymatic glycosylation, glucose antoxidation, polyol pathways) can increase the production of free radicals (Fig. 1) [1]. The glucose and fatty acids interact in the production of oxidative stress in vascular smooth muscle cells.

Non-enzymatic glycosylation of protein ensues exposure to hyperglycemia. Initially, glucose undergoes a nucleophilic addition reaction with proteins to form the Schiff base. Formed early glycosylation product, ketomine is chemically reversible and thus is dissociated when blood glucose level return to normal. However, it subsequently undergoes an Amadori compound (Fig. 2) [2]. Further reactions, rearrangements dehydration and cleavage irreversibly results in the formation of brown,

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insoluble, crosslinking complexes called advanced glycosylation end-products (AGEs). The Amadori products have been implicated in the formation of H_20_2 in vitro [3]. Amadori products could form H202 via two pathways [3]. One pathway is the 1,2-enolization pathway, which lead to 3 - deoxyglucosone formation under anaerobic conditions. In the presence of a suitable electron acceptor, however, enolization would occur to form H_2O_2 and glucosone [3]. The other pathway is 2.3 enolization pathway, which leads to 1-deoxyglucosone and the putative 1,4-deoxyglucosone [3]. Under oxidative conditions, however, the 2,3-enediol is thought to generate H_20_2 and carboxymethyllysine [3] 3-deoxyglucosones has been known to be a major and highly reactive intermediate in the non- enzymatic glycosylation and a potent cross-linker responsible for the polymerization of proteins to AGEs. AGEs tend to accumulate on long-lived macromolecules in tissues. Cross-linking AGE- protein with other macromolecules in tissues results in abnormalities of cell and tissue functions [4]. In addition, AGEs contribute to increased vascular permeability in both micro- and macro-vascular structure by binding to a specific macrophage receptor [4]. This process induced the synthesis and secretion of cytokines such as TNF and IL-1, which causes endothelial dysfunction and induces free radicals.

In addition to direct glycosylation reactions, monosaccharides and fructose-lysine can spontaneously reduce molecular oxygen under physiological conditions [5]. The reduced oxygen products formed in the autoxidative reaction are superoxide, hydroxyl radical and hydrogen peroxide. All can damage lipids, as well as proteins, through crosslinking and fragmentation. Free radicals also accelerate the formation of advanced glycosylation end-products, which in turn generate more free radicals. This process is called as glucose autoxidation [6]. Although the potential importance of this process in vivo is only indirect and has been inferred from in vitro experiments, there is some evidence in vivo that transition-metal chelating agents can prevent autoxidation in animal diabetes.

Diabetic person needs vitamin and supplement different from people without diabetes. The best source of vitamins and minerals is a well balanced healthy eating plan. However, a recently published nutrition manual states that ‘there is probably no harm in taking a multiple vitamin- mineral supplement with doses no higher than 100% of the recommended daily allowances (RDA)’.

According to the American Diabetes Association more research is still needed to make a recommendation about any need for antioxidant vitamin and minerals that are different from the recommended amounts for all Americans. The ADA reinforces that the best way to get your supply of antioxidants is through an adequate supply of foods with the variety of antioxidants. However, experts suggest that there may be benefits to taking Vitamin E to decrease LDL-cholesterol [8].

They are potent antioxidants with the ability to quench singlet oxygen and other toxic species, also enhance T4 helper cell number, lymphocyte proliferation and activation of tumor necrosis factor and other cytokine production [9,10].

Beta carotene has the highest vitamin activity and provides about two thirds of vitamin A necessary of human nutrition, also beta carotene and lycopene are both effective scavengers of alkoxyl and peroxyl radicals [10 -12].

It is believed that IDDM diabetes is caused by the autoimmune beta cell destruction in pancreatic islets [13]. Beta cells normally secrete insulin in response to the increase of serum glucose. The destruction of beta cells results in deficiency and finally total loss of insulin secretion. What factors may trigger this autoimmune islet damage is concerned. Increasing evidence suggests that free radicals play as one of factors in the beta cells damage. This evidence includes hydrogen peroxide, nitric oxide and superoxide are toxic to the human, pig and rat islets in vitro [13].

Beta cells are prone to be destroyed by free radicals because of the low antioxidant enzyme nature [141. Immune-effect cells such as macrophages, T cells, nature killer cells and B cells are believed to produce free radicals that causes damage to beta cells [15,16].

The mechanism by which the antioxidant reserve still needs further studies. Protein damage due to the protein glycosylation may be a mechanism that lowers the activities of primary antioxidant enzymes [11]. Therefore, this study is devoted to oxidative stress and antioxidants with relevant to diabetes mellitus [17,18].

MATERIALS AND METHODS

Seventy two diabetic patients of type I (IDDM) and fifty two diabetic patients of type II (NIDDM) who were taken from outpatients of Al Hussein Hospital and from Sphinx Hospital with 35 normal controls of matched aged and sex. Blood glucose was controlled and all the family history, age, height, weight, any complications and the number of years since the onset of the disease were obtained. The blood samples were taken at fasting.

Determination of Blood Sugar: Plasma glucose levels were estimated by the glucose oxidase method (using-glucose kit) (bio Merieux) [19].

Determination of Insulin: Immunoenzymetric assay for the quantitative measurement of insulin in plasma according to (MEDGENIX-INS-EASIA) (Biosource Europe S.A., B-1400 Nivelles. Belgium, Code 40 125 00-February 7, 1990).

Determination of Fructosamine: It was performed by a colourmetric method with liquid reagents. In an alkaline ambient glicates reduce nitroblue tetrazoliurn formazan. To give a colour that can be measured at 530-550nm. The formed colour is directly proportional to fructosamine concentration. (Solo per uso diagnostic in vitro, Data Rilascio, 2001).

Determination of Glycosvlated Hemoglobin: The glycosylated hemoglobin assay has been validated as a reliable indicator of mean blood glucose level for an 8-12 week period prior to determination. Quantitative colorimetric determination of glycohemoglobin in whole blood was carried out by using Stanbio laboratory, procedure No. 035 [20].

Determination of C-Peptide: C-peptide concentration in a sample is determined by an enzyme immunoassay performed by an enzyme coated with anti c-peptide antibody (Medgenix Diagnosties S.A. kit, Fleurus, Balgium, 1996).

Autoxidation Rate Measurements: Measurement of the autoxidatiori rate was carried out spectrophotometrically as described by Wallace *et al.* [2l].

Determination of Electrical Conductivity: At constant temp the electric conductivity of various biochemicals will increase with increasing the amounts of several different absorbantes. The magnitude of the increase is dependent both on the absorbante and on the biochemical substrate. The electrical conductivity is measured using conductivity meter.

Viscosity Measurements: Viscosity measurements were carried out with a capillary ostwad iscorneter with distilled water flow time of 208 seconds. Ad viscosity measurements were carried out at a constant temperature of 20±0.05°C Temperature was controlled with circulating water through a jacked around the viscometer. The temperature of the circulating water was controlled through the use of a thermostate model-u/µH manufactured by VEB MLW company in Berlin.

Determination of Carotenoids: The HPLC system consisted of an AIC/GPC chromatograph system, used solvent tetrahydrofuran, hydroxybutyltoluene, a 5µm, with a guard column of aquapore ODS type RP-18,l5mm, 3- 9mm, 7mm [22].

Superoxide dismutase (SOD) is determined using xanthine and xanthine oxidase to generate superoxide radicals which react with 2-4 - iodophenyl 3-4-nitrophenol 5 -phenyltetrazalium chloride to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction using spectrophotometer [19].

Also Glutathione peroxidase (GPX) as an antioxidant enzyme was measured using paglia and valentine. Glutathione peroxidase (GPX) catalyses the oxidation of giutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted to NADPH to NADP with positive charge and the decrease in absorbance at 340nm is measured [12].

RESULTS

All values in the tables are expressed as mean plus or minus the standard error. Mean values of fasting insulin, glucose, HbAIc, fructosamine and c-peptide in IDDM, NIDDM and normal control are shown in Table 1 and Figs. 1-3. The obtained data revealed a very highly significant difference as compared to control ($p < 0.001$) in both cases of insulin dependent diabetes mellitus and non insulin dependent diabetes mellitus.

Mean values of antioxidants (beta carotene and lycopene) as well as antiperoxidative enzymes (superoxide dismutase and glutathione peroxidase) activaties in IDDM, NIDDM as compared to control are shown in Table 2. The obtained data revealed a highly significant decrease ($p<0.005$) concerning SOD, beta carotene and lycopene, concomitant with an increase in glutathione peroxidase activity.

Mean values of biophysical parameters (conductivity, intrinsic viscosity and the autoxidation expressed as reaction rate constant) are shown in (Table 3 & Figs. 4-8). The obtained data revealed that there is a very highly significant decrease, in Hb conductivity concomitant with an increase in reaction rate constant as compared to control while no variation observed in case of intrinsic viscosity.

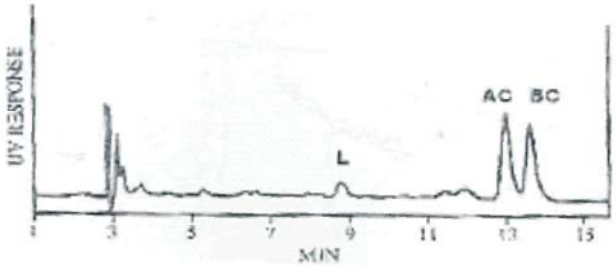

Fig. 1: The HPLC chromograms of beta carotene and jycopene in IDDM group

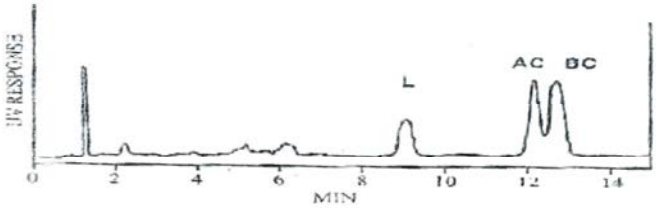

Fig. 2: The HPLC chromograms of beta carotene and jycopene in NIDDM group

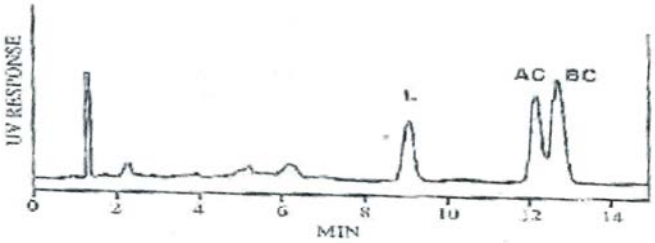

Fig. 3: The HPLC chromograms of beta carotene and jycopene in control group

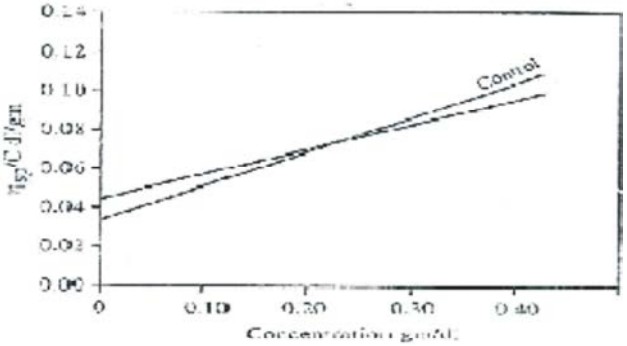

Fig. 4: Intrinsic viscosity of hemoglobin of IDDM, NIDDM and control groups

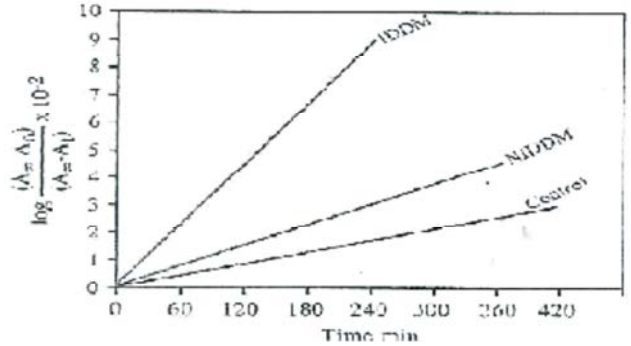

Fig. 5: Autoxidation rate of hemoglobin of IDDM, NIDDM and control groups

Table 1: Mean values of the insulin, glucose, HbAIc, fructosamine and c-peptide in insulin dependent group, non insulin dependant group as compared to control group

Studied parameters	IDDM group N=72	NIDDM group N=52	Control group N=35
Insulin (µmole/L)	15.463±0.137 a	18.165±0.2 b	22.579±0.232 a b
Glucose (ngm/dl)	95.865±0.195 a	184.640±0.3 b	74.913±0.456 a b
HbAlc(µmole/L)	26.257±O.14a	28.llO±O.129b	18.343±0.181 ab
Fructosamine (p.mole/L)	292.036±0.197 a	297.775±0.336 b	216.074±0.299 a b
C-peptide ngm/dl)	0.503±0.011 a	1.554±0.012 b	2.425±0.019 2

Means with same superscripts are significantly different from each other at level p<0.001).

Table 2: Mean value of antioxidant parameters in insulin dependant group, non insulin dependant group as compared to control group.

Studied parameters	IDDM group N=72	NIDDM group N=52	Control group N=35
SOD (1.U/mgHb)	2.528±0.011 a	2.684±0.009 b	3.385±0.017 a b
GPX (1.U/mgHb)	7.470±0.045 a	6.504±0.069 b	5.529±0.047 a b
Beta carotene (µmole/L)	0.227±0.006 a	0.343±0.003 b	0.450±0.005 a b
Lycopene (µmole/L)	0.127±0.001 a	0.162±0.02 b	0.342±0.002 ab

Means with same superscripts are significantly different from each other at level P< 0.001).

Table 3: Mean value of the biophysical parameters in insulin dependant group. non insulin dependant group as compared to control group.

Studied parameters	IDDM group N=72	NIDDM group N=52	Control group N=35
Conductivity (µs/cm)	58.356±0.186 a	55.863±0.243 b	77.994±0.219a b
intrinsic viscosity (dl/gm)	0.044±0.000 a	0.044±0.0002 b	0.043±0.0002a b
Autoxidation (min^{-1})	0.000209±0.000006 a	0.000156±0.000007 b	0.000083±0000002 a b

Means with same superscripts are significantly different from each other at level (P<0.001)

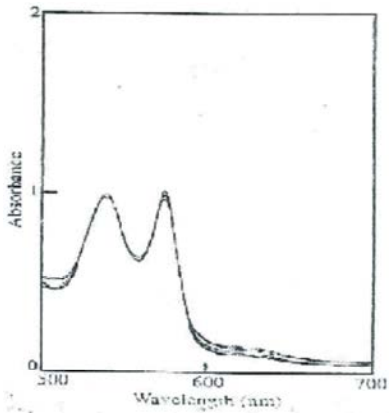

Fig. 6: Absorption spectra of autoxidation in control gropup

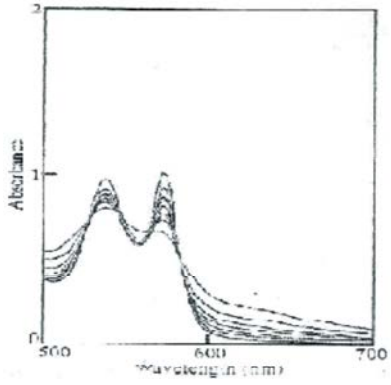

Fig. 7: Absorption spectra of autoxidation rate in IDDM

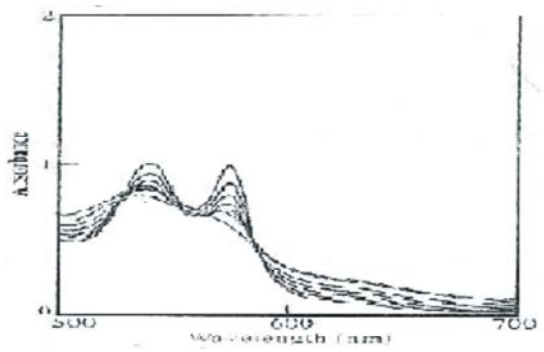

Fig. 8: Absorption spectra of autoxidation rate in NIDDM group

DISCUSSION

The steady state balance between pro-oxidants and antioxidants in the human may be disturbed in case of depletion of antioxidants which may occur endogenously or as a consequence of a diminished dietary intake or disease like diabetes. ROS generation in mononuclear cells was greater in IDDM and NIDDM patients than their respective controls [23].

Abnormalities in the regulation of ROS and transition metal metabolism are postulated to result n diabetes as well as its longer term complications, diabetes is associated with an increase risk of premature vascular disease. Vascular research is currently focusing on a potential role of adhesion molecules in diabetes mellitus

as classical risk factors, including hyperlipidemia and hypertension, not completely account for the increased incidence of atherosclerosis in diabetes. After the expression of adhesion molecules on the cell surface, they are shed into plasma. Soluble adhesion molecules are detectable at low levels in healthy people but are increased in various disorders [24].

In contrast to IDDM at the diagnosis, the insulin secreting cells have largely disappeared from the pancreas. So, the plasma immunoreactive insulin is either very low or undetectable, in NIDDM there is only moderate reduction in the total mass or islet tissue which related to the blood glucose level & reduced concentration of insulin in plasma [25].

The C-peptide formed during cleavage of insulin from proinsulin, has no known biological activity. It is released from the beta cells in equimolar amounts with insulin, so it is related to insulin and more accurate [26].

Glucose metabolic control (HbAIc and Fructosamine), glycosylated hemoglobin reflect the state of blood glucose control during the previous two months in diabetic patients, the glycosylated hemoglobin may affect the oxygen releasing capacity of the red cells by two reversible abnormalities. Firstly, due to increase amount of glycosylated hemoglobin which has an increased affinity for oxygen Secondary a compensatory rise in the red cells 2,3 diphosphoglycerate which is an important regulator of the intracellular hemoglobin function. It binds strongly to deoxyhemoglobin than oxyhemoglobin thus causing marded reduction in the affinity of hemoglobin to oxygen [27].

The activity of SOD is less in NIDDM and IDDM in comparison with normal control. Dietary glutathione supplementation normalized SOD levels, but had no effect on blood glucose. SOD is inhibited by HbAIc and is lowered in poorly controlled diabetes. Due to the absence of protein synthesizing machinery in the erythrocytes, the inactivation of SOD by glycosylation may be a dominant factor in the loss of SOD activity [28].

An increase in GPX activity in diabetic patients may be due to an increase selenium levels in tissues, like in diabetic children, also dietary factors other than selenium intake affect GPX activity [29].

So, the increase in the activity of GPX in diabetes mellitus may be an adaptive or compensatory mechanism developed to deal with the increased generation of free radicals and also with secondary complications.

The carotenoid concentration is associated with insulin resistance and glucose tolerance status. The evidence was strongest for beta carotene and lycopene. Significantly higher proportions of men and women with known NIDDM had carotene deficiency than did healthy control.

The carotenoids were inversely related to fasting insulin concentration, supporting an association between serum carotenoid concentrations and insulin resistance and thus raising the possibility that carotenoids may favourably affect glucose tolerance by influencing insulin resistance, authors used fasting insulin as a marker for insulin resistance [30]. A high level of beta carotene, a lipid soluble antioxidant, were found to be associated with decreased risk of NIDDM [31].

Bioenergetics, intrinsic viscosity and conductivity of Hb may refer to the complication that occur in Hb molecule which in its turn illustrates the degree of hypoxia.

The value of intrinsic viscosity of hemoglobin of diabetic patients, indicates a lack of changes in the dimensions and shape of hemoglobin molecules. The values of apparent activation energy of viscous flow for distilled water are consistent with values obtained by Rizk And Girgis [32]. The observed changes in activation energy of viscous flow (Ea) of Hb solution is in complete agreement with the changes in the intermolecular interaction, i.e. quaternary structure of hemoglobin.

The degree of Hb folding and unfolding depends mainly on the hydrophobic groups that appeared on the molecular surface. Electrical conductivity of hemoglobin of normal control revealed highly significant increase as compared to patients. The new groups exist in the surface of this globular proteins are responsible for this value of electrical conductivity. This mainly depends on hydrophobic/ hydrophilic ratio that possess certain value for physiological function of Hb, so the elevation of oxidative stress or free radicals suggests its indirect effect on the degree of Hb folding and consequently its physiological function.

A degree of Hb folding of diabetic patients was detected as a result of high glucose content which predominant at least three months ago that referred from HbAIc.

The conformational change reflects the stability of Hb molecule against autoxidation. Rates of autoxidation reactions of normal oxy-Hb, after the removal of all other redox active red cell components, were determined by Wallace *et al*. [21]. The present data clearly indicate a defect in methemoglobin reductase system as a result of lack of insulin where it is important to maintain the autoxidation reaction in the red blood cells [33].

The best source of vitamins and minerals is a well balanced healthy eating plan. There is probably no harm in taking a multiple vitamin- mineral supplement with doses no higher than 100% of the recommended daily allowances (RDA).

All carotenoids are produced in plants, therefore eating fruits and vegetables provides carotenoids. Alpha lipoic acid is a potent, natural antioxidant which works with vitamins C & E. together, they form an "antioxidant network" which works to neutralize free radicals and bridge nutritional "gaps" often found among people with diabetes [34].

According to the American Diabetes Association more research is still needed to make a recommendation about any need for antioxidant vitamin and minerals that are different from the recommended amounts for all Americans. The ADA reinforces that the best way to get a supply of antioxidants is through an adequate supply of foods with the variety of antioxidants. However, experts suggest that there may be benefits to taking Vitamin E to decrease LDL-cholesterol. A daily array of fruits and vegetables, is to eat at least five servings of fruits and vegetables and nine servings are optimal [35].

Beta carotene has the highest vitamin activity and provides about two thirds of vitamin A necessary of human nutrition, also beta carotene and lycopene are both effective scavengers of alkoxyl and peroxyl radicals.

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