A NEW ROUT FOR THE EVALUATION OF OXIDATIVE STRESS IN DIABETIC PATIENTS BY USING THE ACTIVITY OF SOME ENZYMATIC ANTIOXIDANTS

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Summary
Diabetes mellitus is a common health problem characterized by hyperglycemia resulted from absolute or relative decrease in insulin secretion from β-cells of the islets of Langerhans in pancreas. Diabetes mellitus is considered as a member of oxidative stress syndrome. It is associated with an imbalance between types of free radicals and scavengers system. This study is aimed to assess the oxidative stress status in patients with type 1 or 2 diabetes mellitus disease, and to explore the good free radical marker in this state.

ان داء السكري يعتبر من الامراض المعروفه بارتفاع مستوى السكر حيث يعتبر من الامراض التي تشارك في تكوين الجهد التاكسدي في جسم المريض بداء السكري وعليه فان هذه الدراسه تهدف الى توصيف مثل هذه التاثيرات وعلاقتها بمضادات الاكسده حيث دلت النتائج الى ان مثل هذه الحالات والتي تحتاج الى دراسه مفصله اخرى لامكانيه ان تكون كدالات لهذا الجهد

Introduction

Diabetes mellitus is a very complex chronic disease with syndrome hyperglycemia (1). It is result from absolute or relative decrease in insulin secretion from β -cell of the islets of Langerhans. Insulin is a polypeptide hormone that consists of a total of 51 amino acids two chains connected by two disulfide bridges; Insulin synthesized as a large single chain preproinsulin that is cleaved to a more immediate precursor proinsulin in the rough endoplasmic reticulum. 'Proinsulin is then packed into secretory granules, where it is broken down into equivocal amounts of insulin and an inactive C-peptide .

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, high glucose in urine, increased appetite, ketoacidosis (ketone bodies in blood), acid/base imbalance, and diabetic coma.Long-term complications of diabetes include blindness, neuropathy, kidney failure, lower extremity amputations, cardiovascular complication and pregnancy complication (2).

Free Radicals

A compound becomes a free radical through either oxidation i.e., the loss of an electron or through reduction i.e., the gain of an electron, however, a free radical is unstable and highly reactive in nature and may react with other nearby molecule also converting that molecule to a free radical, which can then initiate another reaction (3).

Theoretically, a single free radical can ultimately causes an endless number of reactions. This chain reaction is terminated either by the free radicals reaction with another free radical or by the free radicals reaction with an antioxidant (4).

Free Radicals and Diabetic Complications

Free radicals perform beneficial tasks such as aiding in the destruction of microorganisms and cancer cells. Excessive production of free radicals or inadequate antioxidant defense mechanisms, however, can lead to damage of cellular structure and enzymes (3).Damage to entire tissues can result from free radical-mediated oxidative alteration of fatty acids, also known as lipid peroxidation.There are well characterized reaction that leads to the formation of the $O_2^{\cdot -}$, H_2O_2, and the highly toxic $OH^{\cdot}$. The cytotoxic potential of the $O_2^{\cdot -}$ is mainly from its ability to be converted to the $OH^{\cdot}$ directly or via interaction with H_2O_2**.** The $O_2^{\cdot -}$ can also interact with $NO^{\cdot}$ to form peroxinitrite ($ONOO^-$) which can degrade to form the $OH^{\cdot}$(5).

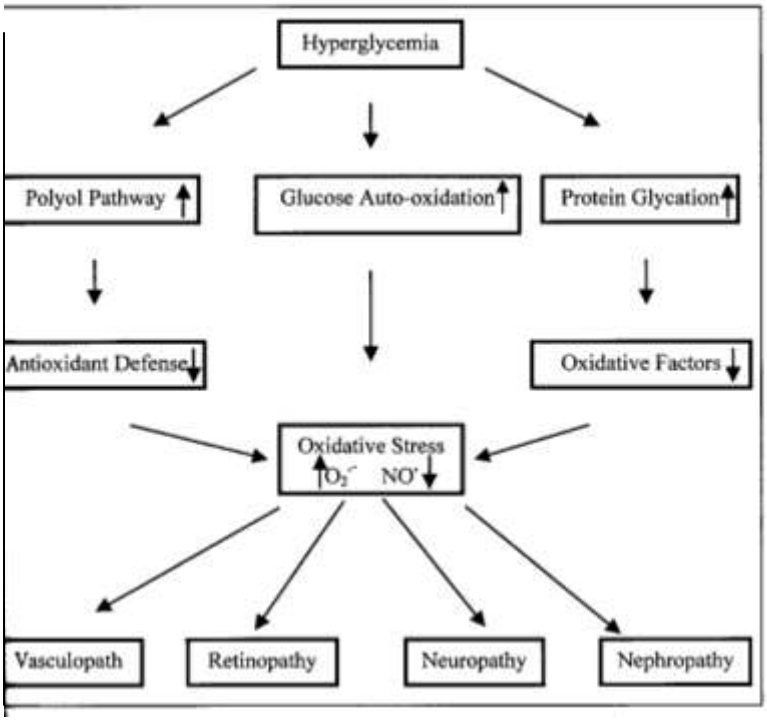


Figure (1): Possible links between hyperglycemia-induced oxidative stress and diabetic complications (6).

Diabetes Mellitus and Oxidative Stress Syndrome

The oxidative stress is significantly increased in diabetes because of prolonged exposure to hyperglycemia. Many evidence have indicated that some biochemical pathways strictly associated with hyperglycemia (non-enzymatic glycosylation, glucose auto-oxidation, polyol pathways) can increase the production of free radicals (6),

Specific Biological Alterations in Oxidative Stress Syndrome

Lipid Peroxidation

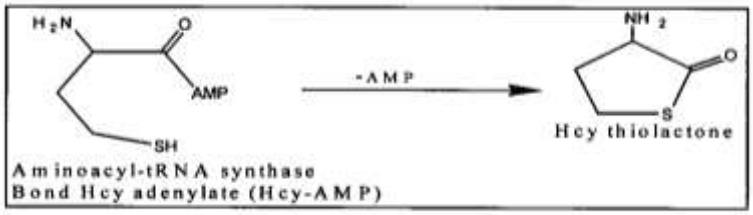
Lipid peroxidation is formed by oxidation of membrane polyunsaturated fatty acids (PUFA) that

contain double or triple bonds (interaction of peroxidation aldehyde with phospholipids) leads to release of short-chain aldehydes such as Malondialdehyde (MDA) (7).A low density lipoprotein (LDL) particles has 2200 molecules of free fatty acid, half of which is PUF A which is a highly susceptible substrate for free radical reaction. Patient with DM have an increased risk of premature atherosclerosis, which may be due in part to increased oxidizability of LDL (8).

Hyperhomocysteinemia

Homocysteine (Hcy) has become widely accepted as a novel risk marker associated with atherosclerotic cardiovascular disease (CVD) in the coronary, cerebral and peripheral vascular beds (9).

The important role of oxidative-redox stress and hyperhomocysteinemia is biologically plausible because Hcy promotes (oxidant in the cells, particularly the endothelium and endothelial nitric oxide synthase (eNOS) reaction through the auto-oxidation of Hcy, formation compounds of disulfide interaction of Hcy thiolactones, and protein homocysteinylation) (10). Additionally, Hcy may undergo complicated rearrangement to form Hcy thiolactone (acyclic thioester), which is chemically reactive and acylates free amino groups such as the side chain lysine groups in proteins. In the process of forming homocysteinylated protein further oxidative stress develops and may lose their biological activity(11).



Enzymatic Antioxidants

Several antioxidant enzymes are made by various cells in the body. The most three important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) which are made in the body in response to the presence of certain free radicals. Thus, if a body is under higher oxidative stress, and is producing more tree radicals, more of the antioxidant enzymes will be made to counterbalance the stress like glutathione S-transferase (GST), and glutathione reductase (GR), figure (2).

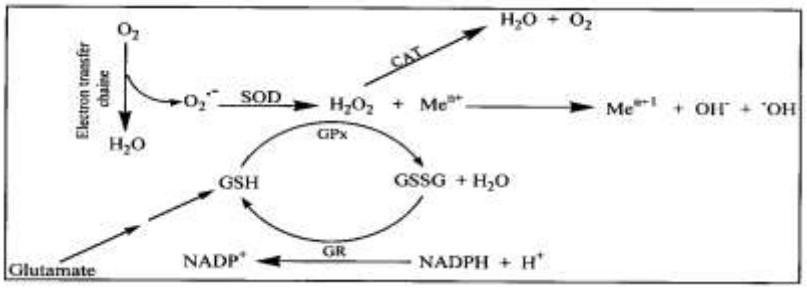


Fig.(2):Co-operation of antioxidant enzymes (12)

Superoxide Dismutase (SOD)

SOD (EC.1.I5.I.I) is a metalloenzyme. There are three major isoforms of SOD; intracellular Cu/Zn-SOD, Extracellular Cu/Zn-SOD (Ec-SOD), and Mn-SOD, with copper and zinc or manganese ions at the active sites, respectively . Despite different structures, the three isoforms catalyze the same reaction, that is dismutase O_3 to H_20_2 (12).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

In diabetic, the wide variability among studies, which are shown decreased, increased and unchanged in SOD isoforms activity (13,14) which does not allow conclusions to be drawn as to whether SOD isoform activities are abnormal in diabetic patients, again differences in methodology or study design do not completely plain the conflicting findings among studies.

Catalase (CAT)

Catalase (EC 1.11.1.6) is an oligomer with four 60,000 Dalton subunits. It is a hemo protein containing four heme groups. Subcellularly, CAT is mainly localized to the peroxisomes, as much as 40% of the total protein content of the peroxisomes has been estimated to be CAT (15). The peroxisomes are cytoplasmic organelles in which several oxidative processes occur, e.g. the oxidation and detoxification of several toxic compounds resulting in large amounts of H_20_2 . CAT is found in blood, bone marrow, mucous membranes, kidney, and liver. CAT catalyses the direct composition of H_20_2 to ground state O_2:

$$2H_20_2 \xrightarrow{CAT} 2H_2O + O_2$$

Many studies are found decreased levels of CAT in patients with DM (16,12).

Glutathione S- Transferase (GST)

The (GST) are recognized as important catalysts in the bio-transformation of xenobiotics, including drugs as well as environmental pollutants. Multiple forms exist, and numerous transferase from mammalian tissues, insects, and plants have been isolated and characterized. Enzymatic properties, reactions with antibodies, and structural characteristics have been used for classification of the glutathione transferase (17).

Creatine Kinase (CK)

Creatine Kinase (CK, EC 2, 7.3.2) is a dimmer consisting of two sub units. There are three isoenzyme have been designated as CK-BB (brain type), CK-MB (hybrid type), and CK-MM (muscle type) . CK is a major phosphotransfer system in cells with high-energy demand, and it acts in concert with other enzymatic system to facilitate intracellular energetic communication (18). During conditions of oxidative stress, the enzyme CK appears to be inactivated via oxidation of the active-site thiol (19). Ck is relatively unstable, and its activity is apparently lost as a result of disulfide formation which investigated by workers to show also the effect of endogenous extracellular glutathione concentration on serum CK activity, and they found in patients with multiple organ failure, low serum CK activities , where accompanied by extremely low serum glutathione concentration. (20).

Material and Methods

Place of work

This research was conducted in Al-Qadisiya Governorate, Al-Diwaniya General Hospital; Department of Biochemistry, College of Medicine, Al-Nahrain University, and Department of Chemistry, College

of Science, Babylon University.

Subjects

The study samples included 50 Patients suffering from type 2 of diabetes (28 males, and 22 females) aged between 35 and 65 years; and 50 patients with type 1 diabetes (32 males and 18 females) aged between 13 and 65 years, controlled with 50 healthy individuals (38 males and 12 females) aged between 15 and 65 years. The practical work was expended 15 months period, beginning in October 2003 and ending in December 2004.

Samples

The study samples of patients were collected from Al-Diwaniya General Hospital between October 2003 to December 2004, and the blood was drawn from venous of fasting patients recently diagnosed with diabetes mellitus (type 1 and type 2) and healthy subjects were used as control.

All tests were performed on serum, some of the blood was allowed to clot on crushed ice, and then centrifuged (450 X g for 10 minutes) to be used serum samples immediately for detection of variable in this study, and others was stored at deep freezing (-20°C) until using.

Determination of Serum Malondialdehyde

Principle:-The principle of the following method was based on the spectrophotometric measurement of the color, occurred during the reaction with thiobarbutric acid (T B A) with MDA.. (21)

Determination of Serum Total Homocysteine (tHcy)

Procedure:-

All test were performed on serum, which was stored at -20°C until HPLC analysis. The separation was carried out on shimadzu LC-6A system with two pumps and SCI-6A system controller, 100 µl of sample was injected on to shimpack CIC-ODS column (250 X 4.6 mm i.d) protected by ODS guard column (50 X 4.6 mm i.d). (22,23)

Assay the Activity of Serum Superoxide Dismutase (SOD)

Principle; -

Ec-SOD activity in serum was determined using a modified Photochemical nitroblue tetrazolium (NBT) method utilizing sodium cyanide as peroxidase inhibitor. (24).

Assay the activity of serum Catalase (CAT)

Principle; - Catalase(CAT)activity was determined by the decrease in absorbance due to H_2O_2 consumption (ε= 0.04 mM^{-1} cm^{-1}) (25).

Assay the activity of serum (GST)

Principle; -

The activity was determined using l-chloro-2,4-dintrobenzene (CDNB) as substrate(26) .

Assay the activity of serum Creatine Kinase (CK) by Using Commercially available Kit (Randox-U.K.)

Statistical Analysis

The data were analyzed by using student's T-test taking $P \leq 0.05$ as the lowest limit of significant of difference and simple linear correlation between two quantitative parameters, and correlation considered significant at $P \leq 0.05$.

Results And Discussion :

Sampling characteristics

One hundred patients with DM (50 with type 1 DM, and 50 with type 2 DM), and 50 healthy

individuals served as control were enrolled in the present study (Table 1).

Table (1) :The demographic sampling characteristics of the study.

The characteristics	Type 1 No.=50	Control No.=25	Type 2 No.=50	Control No.=25
Age (mean ± SE)	32.08± 2.327	27.04± 1.196	51.24±1.228	47.04± 1.750
Duration of DM (mean ± SE)	10.364± 1.394	-	5.616± 0.846	-
Family history (No.)	17	-	38	-
Smokers (No.)	11	11	17	13
Non-smokers (No.)	39	14	33	12
Males (No.)	32	21	28	17
Females (No.)	18	04	22	08

The mean age ± SE of patients with type 1 DM was (32.08 ± 2.327 years *vs* 27.04 ± 1.196 years of control), while in type 2 DM was (51.24 ±1.228 years *vs* 47.04 ± 1.750 years of control). There was no significant difference in age of control and diabetic patients with type 1 DM and type 2 DM.

Duration of DM disease

The mean ± SE of duration of DM was (10.364 ± 1.394 years) in patients with type 1 DM, while in type 2 DM was (5.616 ± 0.846 years).

Assessment of reactive oxygen species

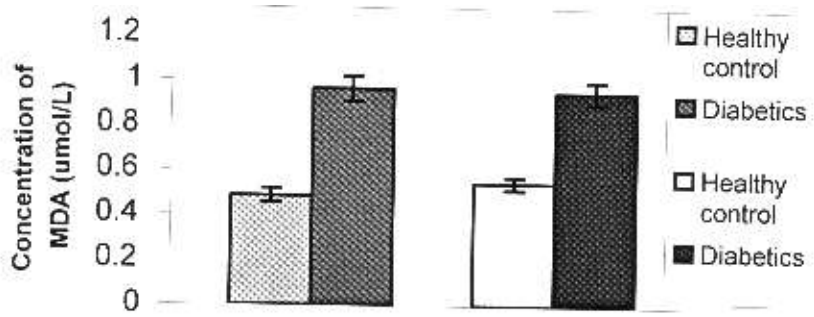
The assessment of generation of reactive oxygen species (ROD) in DM disease was achieved via determining the level of total homocysteine (tHcy), and MDA; the end product of lipid peroxidation as shown in table (2).

Table (2): Results of specific biochemical tests in serum of patients with type 1 or type 2 DM and control.

			MDA (µmol/L)	tHcy (µmol/L)
Type 1 DM No.=50	Mean±SE		0.397± 0.056	2.768± 0.391
	SD		0.397	2.768
	Range		1.605 – 0.386	26.25 – 14.66
	95% C.I.	Lower	0.869	19.624
		Upper	1.057	20.936
Control no.=25	mean±SE		0.486 ± 0.031	10.741± 0.197
P-Value			S	S
Type 2 DM No.= 50	mean±SE		0.948±0.363	21.97± 0.395
	SD		0.051	2.799
	Range		1.81 – 0.383	30.11 – 18.1
	95%C.I.	Lower	0.863	21.307
		Upper	1.033	22.633
Control No.=25	mean±SE		0.545±0.029	10.633±0.181
P-Value			S	S

Lipid Peroxidation

Dien Conjugation (DC) and thiobarturic acid reactive species (TBARS) are widely used as indicators of lipid peroxidation. DC is a measure of early events of lipid peroxidation reactions whereas TBARS measure end products of lipid peroxidation; MDA, (27). In this work, there was significant difference in Malondialdehyde (MDA) levels between controls and diabetic patients (with type 1, and type 2 DM) $P \leq 0.05$, but there was no significant difference between type 1 DM and type 2 DM. Therefore serum MDA levels in patients with type 1, and type 2 DM are higher than control by 1.981 and 1.739 times of control levels respectively, figure (3), table (3).



Type 1 DM Type 2 DM

Figure (3): Level of Malondialdehyde (MDA) in serum of healthy control And patients with type 1 and type 2 diabetes mellitus DM) disease. The values are the mean ± SE .

Elevated levels of lipid peroxidation products in serum of diabetic subjects and rats have been shown in several studies (28,29). The results of this study indicated that serum MDA levels are elevated in diabetic patients. Higher levels of MDA is associated with reduced of antioxidant activity and increased oxidative stress (30,31,32). Low density lipoprotein particles has 2200 molecules of free fatty acids, half of which is PUFA which is a highly susceptible substrate for free
radical reaction to form ashort-chain aldehyde such as Malondialdehyde, therefore elevated level of MDA might increase susceptibility of diabetic patients to premature atherosclerosis, which may be due in part to increased oxidizability of LDL.

Total Homocysteine (tHcy) Level

In plasma, about 80% of homocysteine is bound to proteins (especially albumin), the remainder being present in a free form, either as a disulphide with itself or cysteine, or as reduced homocysteine itself. Usually the sum of these fractions is measured in the laboratory and called serum tHcy concentration.. Hyperhomocysteinemia (elevated level of tHcy) plays an extremely important role for additional oxidative-redox stress regarding the accelerated cardiovascular complications in diabetic patients (33). There is no obvious direct biochemical link between methionine-homocysteine metabolism and glucose/carbohydrate metabolism other than insulin induced protein synthesis, which may decrease serum methionine level (34). Therefore, elevated level of tHcy may be associated with insulin deficiency that leads to enhanced homocysteine catabolism in the liver, a phenomenon that was reversible upon insulin administration (35). As shown in table (2), the mean ± SE of serum tHcy levels in patients with type 1 DM was (20.28 ± 0.391 µmol/L *vs* 10.741 ±0.197 µmol/L of control), while in patients with type 2 DM was (21.970 ± 0.395 µmol/L *vs* 10.633 ± 0.905µmol/L of control). There was significant elevation in serum tHcy levels of diabetic patients (with type 1 and type 2 DM) by about 50% from control, $P \leq 0.05$, figure (4).

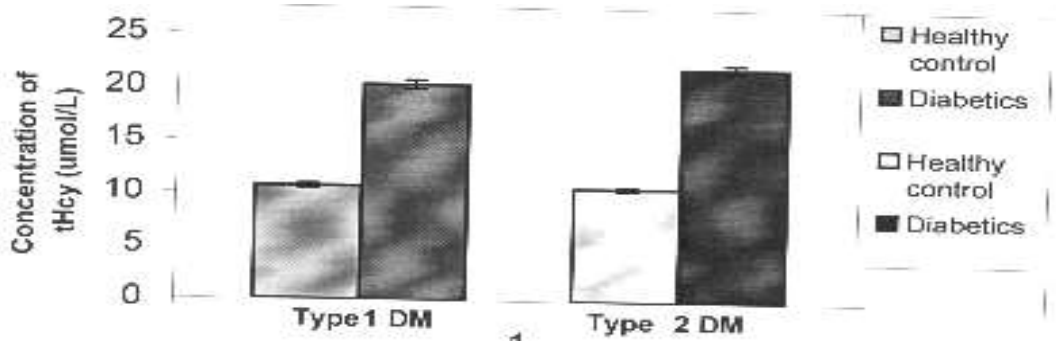


Figure (4): level of total homocystiene (tHcy) in serum of healthy control and patients with type 1 and 2 diabetes mellitus (DM) disease. The values are the mean ± SE.

Several studies pointed out elevated levels of serum tHcy in diabetic patients and suggested that homocysteine (Hcy) shows stronger relationship with cardiovascular diseases complications and death in patients with diabetes than in non-diabetes :

1. The mortality risk associated with tHcy is about 2 times stronger in diabetics than in non-diabetics (36).
2. The association between retinopathy and tHcy is much stronger in patients with diabetes compared to subjects without diabetes (36).
3. Despite similar plasma Hcy levels, a strong correlation between the extent of coronary abnormalities and plasma Hcy level is found in diabetics, while this relationship is not significant in non-diabetics. (37). The results of this study indicate the percentage (%) of antioxidant that assessed in this study table(3),(figure 4).

Enzymatic Antioxidant

Table (3): levels of several enzymatic antioxidant in serum of patients with type 1 or type 2 DM, and control.

			SOD (U/ml)	CAT (K/ml)	CK (U/L)	GST (U/L)
Type 1 DM No.=50	Mean±SE		1.553 ± 0.039	0.383± 0.010	168.56±3.897	4.731±0 199
	SD		0 276	0.074	27.562	1.413
	Range		2.875– 0.579	0.595 – 0.084	259 – 75	7.59 – 2.2
	% 95C.I.	Lower	1.487	0.366	162.029	3.056
		Upper	1.618	0.400	175.090	5.064
Control No.=2	Mean±SE		1.012±0.054	1.626±0.062	192±5.955	2.988±0.051
P-Value			S	S	S	S
Type 2 DM No.= 50	Mean±SE		1.327±0.027	0.506±0.018	158.52±3.026	4.014±0.197
	SD		0.197	0.133	21.402	1.398
	Range		2.526 – 0.137	0.776 – 0.332	184 – 86	7.691 – 2.32
	%95C.I.	Lower	1.281	0.475	153.448	3.683
		Upper	1.373	0.537	163.592	4.345
Control No.=25	Mean±SE		1.032±0.030	1.625±0.059	226.8±5.662	3.053±0.033
P-Value			S	S	S	S

Extracellular Souperoxide Dismutase (Ec-SOD) Activity.

The primary natural defense against free radicals is to prevent their formation by various enzymes, especially SOD, which is regulating superoxide anion ($O_2^{\cdot-}$) levels in catalyzing the dismutation of $O_2^{\cdot-}$ to H_2O_2 (38). The activity of Cu/Zn-SOD increases in many disease causing oxidative stress (39). Therefore, several studies pointed out high levels of Ec-Cu/Zn-SOD activity in diabetic patients (with type 1) ($O_2^{\cdot-}$) that is generating during illumination of riboflavin in the presence of electron donor substance . However, as seen in the present study, there is a significant increase in serum Cu/Zn-SOD activity levels in patients with type 1 and type 2 DM, $P \leq 0.05$, figure (5). Table (3) shows the mean ± SE of serum Cu/Zn-SOD activity in patients with type 1 DM was (1.553 ± 0.039 U/ml vs 1.012 ± 0.054 U/ml of control), while type 2 DM was (1.327 ± 0.027 U/ml vs 1.032 ± 0.030 U/ml of control).

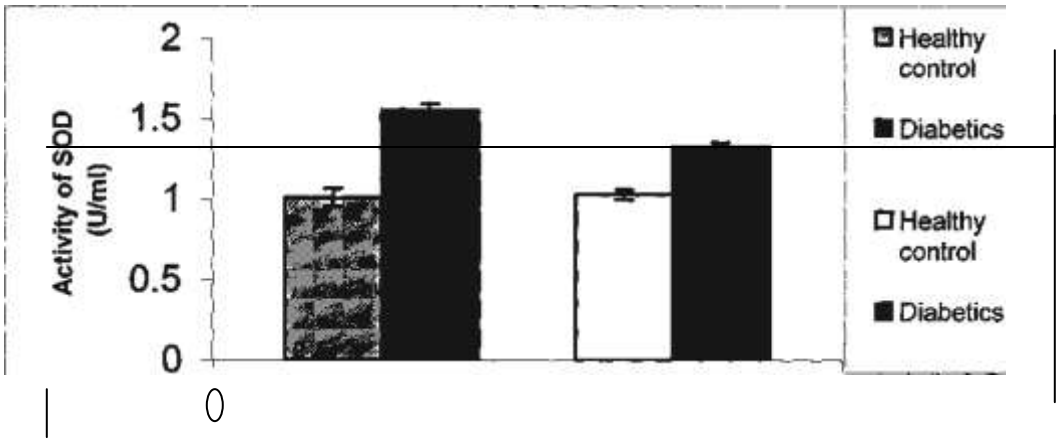
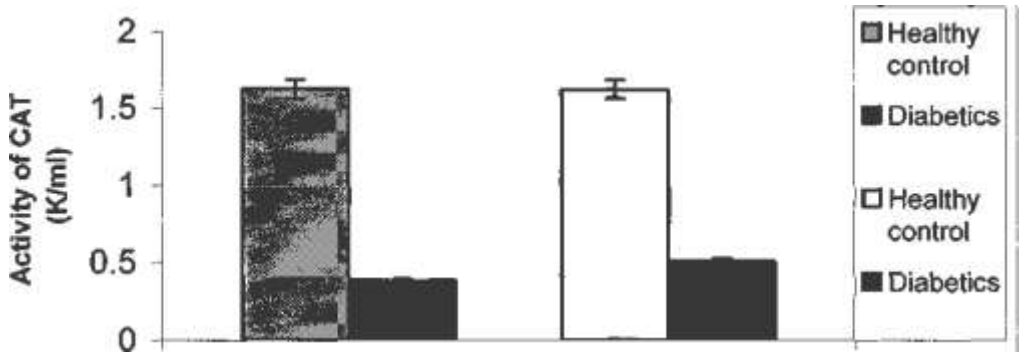


Figure (5): Level of superoxide dismutase (SOD) in serum of healthy Controls and patients with type 1 and type 2 diabetes mellitus (DM)disease. The values are the mean ± S

The results of this study reports that high levels of serum Cu/Zn-SOD activity in diabetic patients are indicating to the presence of high level of superoxide anion ($O_2^{\cdot-}$), which represents an indicator to high degree oxidative stress in those patients, therefore increased amounts of substrate of $O_2^{\cdot-}$ this results in a stimulant to increase synthesis of serum Cu/Zn-SOD in diabetic patients and may protect against free radicals damage (40).

Catalase (CAT) Activity

In this study, the results reported that measurement of serum CAT activity in diabetic patients represents the powerful indicator to evaluation of the oxidative stress. However, there was a significant reduction in serum CAT activity levels of diabetic patients (with type 1 and type 2 DM) than controls, $P \leq 0.05$, figure (6), therefore the mean ± SE of serum CAT activity levels in patients with type 1 DM was (0.383 ± 0.010 K/ml vs 1.626 ± 0.062 K/ml of control), while in patients with type 2 DM was (0.506 ± 0.018 K/ml vs 1.625 ± 0.059 K/ml of control), table (4), these found results were in agreement with a great number of previous publications (41).



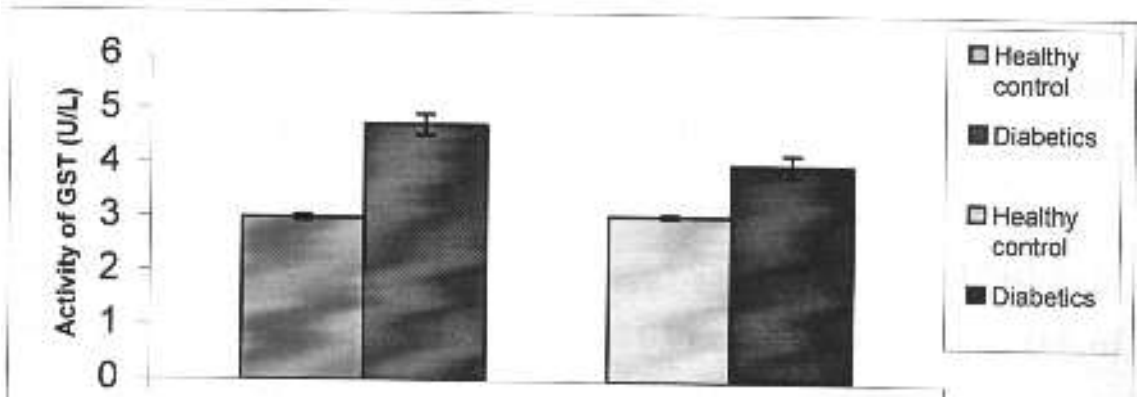
Type 1 DM Type 2 DM

Figure (6): Level of catalase (CAT) activity in serum of healthy Controls and patients with type 1 and 2 diabetes mellitus (DM) Disease. The values are the mean ± SE.

The present results indicate that decrease levels of serum CAT activity in diabetic patients may be due to hyperglycemia that causes degradation of peroxisomes (40) or may be due to inactivation of the enzyme CAT that may occur through glycation governed by prevailing glucose concentration. Thus increased glycation in diabetics and subsequent reactions of proteins may effect amino acids close to the active site of the enzyme or disturb the stereo chemical configuration and causes structural and functional changes in the molecules (42). Furthermore decline levels of serum CAT activity is a good indicator to high concentration of hydrogen peroxide (H_2O_2) in diabetics that increases oxidative stress and diabetic complications.

Glutathione S-Transferase (GST)Activity

GST is an antioxidant enzyme that catalyzes the reaction between reduced glutathione (GSH) and drugs, xenobiotics and other toxic compounds, rendering them more water soluble and finally exerted from the body (43). Previous studies have shown that some chemical compounds, which augments oxygen products, have generated toxic effect such as many drugs and xenobiotics, which contain quinone groups cause free radical. The metabolism of quinone compounds includes formation of semiquinone radical. This radical reduces the molecule oxygen immediately, the superoxide anion($O_2^{\cdot-}$) comes into existence and, following that other ROS are generated (44). However, most studies have reported increased levels of serum GST activity contributed to the increased oxidative stress found in type 1 and type 2 DM subjects (45,46). The results of this study indicate a significant difference was observed in serum GST activity levels between control and diabetic patients (with type 1 and type 2 DM), $P \leq 0.05$,
figure (7).



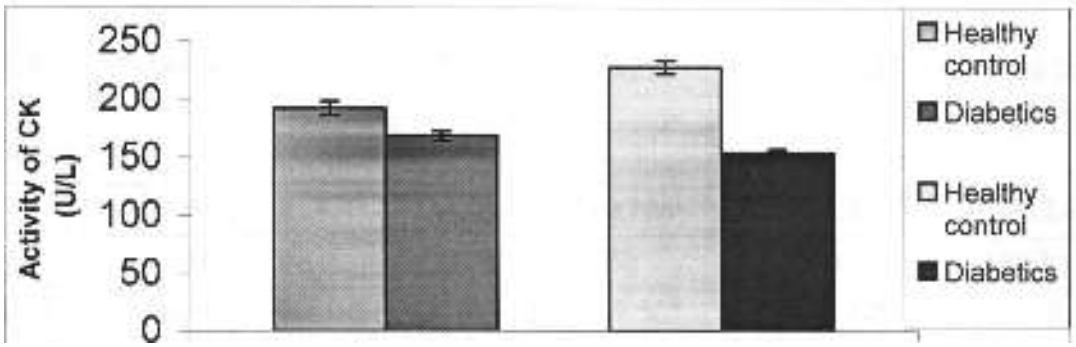
Type 1 DM Type 2 DM

Figure(7): Level of glutathione S-transferase (GST) activity in serum of healthy control and patients with type l and 2 diabetes mellitus (DM) disease. The values are the mean ± SE.

Thus the mean ± SE of serum GST activity levels of patients with type 1 DM was (4.731 ± 0.199 U/L *vs* 2.988 ± 0.051 U/L of control), while in patients with type 2 DM was (4.014 ±0.197 U/L *vs* 3.053 ± 0.033 U/L of control), table (3), shows higher levels of serum GST activity in diabetic patients which may be due to increased synthesis of this enzyme under oxidative stress to protect the body from toxic compounds (47).

Creatine Kinase (CK) Activity

Serum CK activity is an indicator commonly used in the diagnosis of heart and skeletal muscle disorder. Several studies pointed out that levels of serum CK is decreased in diabetic patients and suggested that decreased levels of serum CK may contribute to diabetic cardiomyopathy complications (48,29). In this work, serum CK activity in diabetic patients (with type 1 and type 2 DM) was significantly lower than controls activity, $P \leq 0.05$, figure (8). The mean ± SE of serum CK activity levels of patients with type 1 DM was (168.56 ± 3.897 U/L vs 192 ± 5.955 U/L of control), while in patients with type 2 DM was (158.52 ± 3.026U/L vs 226.8 ± 5.662 U/L of control), table (3).



Type 1 DM Type 2 DM

Figure(8): Level of creatine kinase (CK) activity in serum of healthy control and patients with type 1 and 2 diabetes mellitus (DM) disease. The values are the mean ± SE.

Serum CK is a protein contain thiol groups that is particularly susceptible oxidation (49), therefore decreased the activity of serum CK results from the oxidation of the essential -SH group of CK by ROS that generated in diabetic patients because prolonged exposure to hyperglycemia and inducing an inactivation of the enzyme (50).

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