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RESEARCH ARTICLE

OXIDATIVE STRESS STATUS IN PATIENTS WITH TYPE2 DIABETES MELLITUS.

Selma. M Osman. M. Yousif[1], Mohammed S. M. Abdalla[1], E. M. A. Elmahdi[2] and Manal. Mohammed. Ramadan[3].

1. Sudan Atomic Energy Commission, Khartoum.
2. University of Khartoum, Faculty of Medicine.
3. National Research Center in Cairo, Egypt.

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Abstract

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Background: Oxidative stress due to chronic hyperglycemia has been implicated in the pathogenesis of type 2 diabetes and its complications.
Objectives: To estimate the levels of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation marker i.e. malondialdehyde (MDA) in type 2 DM patients with and without complications i.e., neuropathy, nephropathy and cardiovascular diseases compared to normal subjects. To access the association between oxidative stress and diabetes mellitus and development of its complication.
Materials and methods: Plasma levels of SOD, CAT and MDA were estimated in 50 Control and 140 diabetic patients divided into: DWC group consisted of diabetic patients without complications, CAD group consisted of patients with coronary artery disease, DN group consisted of patients with diabetic nephropathy, DNe group consisted of patients with diabetic neuropathy. Spectrophotometry and enzyme-linked immunosorbent assay (ELISA) techniques have been applied for determination of MDA, CAT and SOD respectively.
Results: comparing the levels of SOD and CAT in all groups, it was observed that the lowest concentration was in CAD, DN, DNe groups followed by DWC and control groups. Oxidative stress was found increased in CAD, DN, DNe groups as compared to DWC and control groups since, the highest concentration of MDA levels were observed in these groups.
A significant inverse correlation was observed of HbA1c, insulin and MDA with SOD, CAT in all diabetic groups. And a significant direct correlation between HbA1C and MDA was also observed in all diabetic groups.
Conclusion: The present study confirms susceptibility of diabetic patients to oxidative stress and that poor glycaemic control is associated with free radical-mediated lipid peroxidation. The present study arrived to the conclusion that hyperglycemia and oxidative stress present a high risk for development of diabetic complications and need early intervention.

Corresponding Author:- Selma. M Osman. M. Yousif.
Address: - Sudan Atomic Energy Commission , Email: selmayousif@hotmail.com

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Introduction:-

Diabetes mellitus is a chronic disease with serious metabolic disturbances in carbohydrate, protein and fat metabolism characterized by hyperglycemia resulting from defects in insulin secretion, resistance to insulin action or both. The primary causative factor of oxidative stress in diabetes mellitus is hyperglycemia. Hyperglycemia is known to cause elevation in plasma free radical concentrations [1, 2]. Increased production of free radicals and diminishing antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. These consequences of oxidative stress can promote the development of complications of diabetes mellitus such as coronary artery diseases, nephropathy, neuropathy, and foot ulceration [3].

Enzymes like Superoxide Dismutase (SOD) and Catalase(CAT) comprise natural cellular defense mechanism against these free radicals. Superoxide dismutase (SOD) is the antioxidant enzyme, plays important protective roles against cellular and histological damages that are produced by ROS. It facilitates the conversion of superoxide radicals into hydrogen peroxide and in the presence of other enzymes hydrogen peroxide is converted into oxygen and water [4]. CAT is the antioxidant enzyme, plays an important role against oxidative stress-generated complications such as diabetes and cardiovascular diseases [5]. CAT acts as main regulator of a highly reactive small molecule, hydrogen peroxide metabolism. It enzymatically converts hydrogen peroxide into oxygen and water and thus neutralizes it [6]. CAT protects pancreatic-cells from damage by hydrogen peroxide [7]. Increased risk of diabetes has been documented in patients with catalase deficiency.

Malondialdehyde (MDA) has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress [8]. Increased lipid peroxidation in diabetes induced many secondary chronic complications through peroxidative injury, like atherosclerosis and neural disorders [9, 10]. Therefore the aim of this study was to evaluate plasma lipid peroxidation marker, malondialdehyde (an oxidant) and antioxidant enzyme (superoxide dismutase and catalase) in type 2 diabetes mellitus patients with and without complications i.e., neuropathy, nephropathy and cardiovascular diseases compared to normal subjects. And also, to access the association between oxidative stress and diabetes mellitus and development of its complication.

Materials and methods:-

Study Design and Population:-

A total of 140 type II diabetic patients were recruited from Jabir Abu Eliz (the public diabetes Center) and People Teaching Hospital of the heart and chest in Khartoum State. Medical records were screened by specialist physicians.

The patients of type2 diabetes mellitus were divided into four groups: DWC group consisted of diabetic patients without complications; CAD group consisted of patients with coronary artery disease, diagnosed by clinical symptoms of angina pectoris, electrocardiogram examination Or documented myocardial infarction; DN group consisted of patients with diabetic nephropathy, evaluated by significant renal impairment such as abnormal creatinine or macroalbuminuria; DNe group consisted of patients with diabetic neuropathy(nervous system damage), diagnosed by the clinical examination of the patients. The diabetic patients received oral hypoglycemic agents like sulphonylureas or metformin. No patient was taking insulin in all the groups of diabetic patients.

Healthy control group, C group was selected. Healthy Control subjects and diabetic patients were matched with respect to age, sex, body mass index (BMI) as determined by the weight and height of patients. All individuals were non smokers. None had taken antioxidant vitamin supplements.

This study was conducted after taking ethical clearance from The Ministry of Health Khartoum state-planning management and training. Written informed consent was taken from the subjects prior to the study.

Samples Collection & Preparation:-

Fasting venous blood samples (5ml) were collected. Whole blood was used to for analysis of glycosylated hemoglobin (Hb A1C). For plasma preparation, Blood samples were collected in heparinized tubes, centrifuged 20-min at the speed of 2000-3000 r.p.m, for immediate analysis of glucose. Aliquots of plasma were frozen at −20 C^{o} for further determinations of insulin, SOD, CAT and MDA.

Biochemical Analysis:-

Determinations of fasting blood glucose, HbA1c and insulin:-

Glucose was measured spectrophotometrically by oxidase/peroxidase method using reagents and standards obtained from BioSystems S.A [11]. HbA1c was measured quantitatively by Ichroma ^{TM} HbA1c fluorescence immunoassay (FIA), using reagents and standards obtained from Boditech Med Incorprated [12]. Insulin was measured quantitatively by insulin [^{521}I] IRMA assay system using reagents and standards obtained from IZOTOP.

Parameters of oxidative stress:-

Malonaldehyde (MDA) was estimated spectrophotometrically by thiobarbituric acid assay, using reagents and standards obtained from Biodiagnostic Co., Cairo, Egypt. Thiobarbituric acid (TBA) reacts with Malonaldehyde(MDA) to form thiobarbituric acid reactive product [13].

Superoxide dismutase (SOD) was measured by SOD ELIZA assay, using reagents and standards obtained from SunLong Biotech Co., LTD. Catalase (CAT) was estimated by calorimetrically enzymatic assay kit, using reagents and standards obtained from Biodiagnostic Co., Cairo, Egypt [14].

Statistical analysis:-

All analyses were performed using SPSS software (version20) for Windows. Statistical significance between the groups was analyzed by Independent Samples T –Test. and correlation between variables was studied by using Pearson's correlation coefficient test. Multivariate model was performed with diabetes mellitus as a control variable; to find out the effect of variables independently for the development of diabetic complications. All parameters will be given as mean± standard error (SE). The criterion for significance will be $p < 0.001$.

Results:-

General Characteristics of the Study Participants:-

The general characteristics and biochemical parameters of all the study participants were summarized in Table 1. All Observations were recorded as (Mean± SE or %).

Plasma glucose, HbA1C, insulin and duration of the disease:-

Significant difference was observed between diabetic groups (DWC DN, DNe, CAD) and the control group for plasma glucose mean level which was high in all diabetic patients regardless of complications (107.8±2.7, 171.4±13, 183.0±13, 191.5±12.2) versus (88.8±0.9) for the control group, (P<0.001, P <0.001, P <0.001, P <0.001) respectively. The difference was also found to be statistically significant in DN, DNe, CAD groups when compared with DWC group, (P <0.001, P <0.001, P <0.001) respectively as shown on Table1.

Similar to glucose, Significant difference was found between diabetic groups (DWC, DN, DNe, CAD) and control group for plasma HbA1C mean level which was high in all diabetic patients regardless of complications (5.8±0.08, 8.9±0.37, 10.0±0.42,10.3±0.32,) versus (4.9±0.05), (P<0.001, P <0.001, P <0.001, P <0.001) respectively. The difference was also found to be statistically significant in DN, DNe, CAD groups when compared with DWC group (P <0.001, P <0.001, P <0.001) respectively, as shown on Table1.

Significant difference was found between diabetic groups (DWC, DN, DNe, CAD) and control group for plasma insulin mean level which was high in all diabetic patients regardless of complications (15.2±1.5, 18.1±2.1, 19.0±1.8, 19.8±2.7). versus (9.0±0.35), (P<0.001, P <0.001, P <0.001, P <0.001) respectively, as shown on Table1.
The patients of diabetes with complications have a longer duration of disease than those without complication ($p < 0.001$), as shown on Table1.

Plasma Malonaldehyde (MDA) level:-

MDA level was significantly increased in all groups of type II diabetes mellitus (DWC, DN, CAD, DNe) when compared with control (9.2±0.2, 10.5±0.4, 11.4±0.4, 12.4±0.4) versus (6.7±0.1), (P<0.001, P <0.001, P <0.001, P <0.001) respectively. The difference was also found to be statistically significant in DN, CAD, DNe groups when compared with DWC group (P =0.014, P <0.001, P <0.001) respectively as shown on Table1.

Plasma Superoxide (SOD) and Catalase (CAT) levels:-

SOD level was significantly lower in all groups of type II diabetes mellitus (DWC, CAD, DNe, DN) when compared with control (254.9±4.9, 174.7±1.3, 150.0±1.4, 126.8±1.4) versus (363.9±4.9), (P<0.001, P <0.001, P <0.001, P

<0.001) respectively. The difference was also found to be statistically significant in CAD, DNe, DN groups when compared with DWC group (P<0.001, P <0.001, P <0.001) respectively as shown on Table1.

CAT level was significantly lower in all groups of type II diabetes mellitus (DWC, CAD, DN, DNe,) when compared with control (299.4±3.2, 245.9±5.2, 194.7±5.1, 151.8±2.7) versus (413.9±3.4), (P<0.001, P <0.001, P <0.001, P <0.001) respectively. The difference was also found to be statistically significant in CAD, DN, DNe groups when compared with DWC group (P<0.001, P <0.001, P <0.001) respectively, as shown on Table1.

Correlation of HbA1c with SOD, CAT, MDA and duration of diabetes:-
The study showed significant inverse correlation of HbA1c with SOD, CAT and significant direct correlation with MDA and duration of diabetes in all diabetic groups as shown on Table2.

Correlation of MDA with SOD, CAT and duration of diabetes:-
The study showed significant inverse correlation of MDA with SOD and CAT and significant direct correlation with duration in all diabetic groups as shown on Table3.

Correlation of insulin with SOD, CAT:-
The study showed significant inverse correlation of insulin with SOD and CAT in all diabetic groups as shown on Table3.

Association of oxidative stress parameters with diabetic complications:-
To find out the effect of oxidative stress parameters independently for the development of diabetic complications (coronary artery disease, nephropathy, neuropathy) we performed the multivariate model with diabetes mellitus as a Control variable. Multivariate analysis revealed that SOD, CAT, MDA, HbA1c, insulin and duration of diabetes were associated with CAD, DN and DNe as shown on Table4.

Table1:- Demographic and biochemical parameters of diabetic and control Subjects

	Control Subjects	Diabetic Patients Type11			
	C Group	DWC Group	CAD Group	DN Group	DNe Group
Number	50	35	35	35	35
M/F ratio	17/18	17/18	19/16	19/16	18/17
Age (years)	54.5 ± 0.9	54.3 ± 1.1	57.4±1.1	57.0±1.3	56.1 ±1.3
BMI (kg/m2)	25.0 ± 0.5	26.9 ± 0.9	26.6±0.9	26.4±0.9	26.0±0.9
FBS (mg/dl)	88.8±0.9	107.8±2.7*	191.5±12*a	171.4±13*a	183.0±13*a
HbA1C (%)	4.9±0.05	5.8±0.08*	10.3±0.32*a	8.9±0.37*a	10.0±0.42*a
Insulin(mIU/L)	9.0±0.35	15.2±1.5*	19.8±2.7*	18.1±2.1*	19.0±1.8*
MDA (nmol/ml)	6.7±0.1	9.2±0.2*	11.4±0.4*a	10.5±0.4*a	12.4±0.4*a
SOD (U/L)	363.9±4.9	254.9±4.9*	174.7±1.3*a	126.8±1.4*a	150.0±1.4*a
CAT (U/L)	413.9±3.4	299.4±3.2	245.9±5.2*a	194.7±5.1*a	151.8±2.7*a

C, control; DWC, diabetic without complications; CAD, coronary artery disease; DN, diabetic nephropathy; DNe , diabetic neuropathy; BMI , Body mass index; FSB, fasting blood sugar; HbA1C; glycatedhemoglobin; MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase. Values are given as mean± standard error. *p<0.001 when C group compared with DWC, CAD, DN and DNe groups. p<0.001=a when DWC group compared with CAD, DN and DNe groups.

Table2:-correlation of HbA1c with SOD, CAT , MDA and duration in diabetic patients

	DWC Group		CAD Group		DN Group		DNe Group	
	R	P	R	P	R	P	R	P
HbA1c/ SOD	-.57	<0.001**	-.86	<0.001**	-.78	<0.001**	-.80	<0.001**
HbA1c/ CAT	-.64	<0.001**	-.84	<0.001**	-.78	<0.001**	-.82	<0.001**
HbA1c/ MDA	.33	0.002*	.73	<0.001**	.67	<0.001**	.61	<0.001**
HbA1c/ duration	.13	.253ns	.56	<0.001**	.62	<0.001**	.52	<0.001**

Correlation is significant at the 0.01 level (2- tailed). **; Correlation is significant at the 0.05 level (2-tailed).* ; correlation is nonsignificant,ns

Table3:-Correlation Of Mda And Insulin With Sod, Cat And Duration In Diabetic Patients

	DW C Group		CAD Group		DN Group		DNe Group	
	R	P	R	P	R	P	R	P
MDA/SOD	-.62	<0.001**	-.78	<0.001**	-.70	<0.001**	-.80	<0.001**
MDA/CAT	-.70	<0.001**	-.76	<0.001**	-.69	<0.001**	-.80	<0.001**
MDA/ Duration	-.10	.518ns	.39	<0.001**	.43	<0.001**	.39	<0.001**
insulin/ SOD	-.43	<0.001**	-.46	<0.001**	-.47	<0.001**	-.53	<0.001**
Insulin/ CAT	-.46	<0.001**	-.44	<0.001**	-.48	<0.001**	-.55	<0.001**
Correlation is significant at the 0.01 level (2-tailed). **; Correlation is								
Significant at the 0.05 level (2-tailed).* ; correlation is nonsignificant, ns.								

Table 4:-Multivariate model determining biochemical variables associated with diabetic complications

	Parameter	B	Std. error	t	F	Adjusted R^2	P	95% CI
CAD								
	SOD	.491	.1	2.7	7.4	.085	0.008*	.131 - .850
	CAT	.743	.2	3.4	11.8	.135	0.001*	.312 – 1.17
	MDA	.737	.1	4.4	19.8	.214	<0.001**	.407 – 1.06
	HbA1c	1.36	.5	2.1	4.8	.052	.032*	.125 – 2.61
	Insulin	.683	.1	4.3	18.7	.205	<0.001**	.368 – 997
	Duration Of Diabetes	.529	.2	2.4	6.2	.070	.015*	.106 - .953
DN								
	SOD	.563	.2	2.0	4.2	.045	0.040*	.017 – 1.10
	CAT	.724	.3	2.0	4.1	.043	0.042*	.011 – 1.43
	MDA	.390	.1	2.1	4.8	.053	.031*	.036 - .744
	HbA1c	1.0	.4	2.0	4.2	.045	.043*	.035 – 2.12
	Insulin	.342	.1	2.4	5.8	.066	.018*	.061 - .623
	Duration of Diabetes	.533	.2	2.6	7.1	.081	.010*	.134 - .932
DNe								
	SOD	.541	.2	2.3	5.6	.063	0.020*	.087 - .994
	CAT	1.02	.4	2.2	4.8	.053	0.031*	.099 – 1.95
	MDA	.502	.2	2.2	5.1	.056	.027*	.060 - .944
	HbA1c	1.7	.5	2.7	7.3	.084	.008*	.461 – 3.02
	Insulin	.518	.1	4.1	17.4	.193	<0.001**	.271 - .765
	Duration of Diabetes	.794	.1	19.6	4.4	.213	<0.001**	.437 - 1.15

CAD, coronary artery disease; DN, diabetic nephropathy; DNe , diabetic neuropathy; HbA1C; glycatedhemoglobin; MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase; CI, confidence interval. *P < 0.05.

Discussion:-

Type II diabetes mellitus is characterized by chronic hyperglycemia which induces an increase of oxidative stress leading to an overproduction of reactive oxygen species (ROS) and free radical species associated with an impairment of antioxidant defence systems [15].

The aldehydic product of lipid peroxidation (MDA) is a biomarker of intensified lipid peroxidation and also indirect evidence of high free radical production in diabetes [16]. In the present study, the MDA level was found to be significantly increased in type II diabetic patients with different complications compared to patients without complications and to healthy subjects. The findings of this study are also in a good agreement with the findings of a previous study which showed that MDA levels in patients with diabetic neuropathy are ~40% higher than diabetics without neuropathy and almost three times higher than healthy controls [17].

In this study, Superoxide dismutase (SOD) and catalase (CAT) levels were found to be significantly reduced in type II diabetic patients with different complications compared to patients without complications and to healthy subjects. The present findings were in a good agreement with the observations of Kimura et al [18] who have reported decreased SOD level in type II diabetic patients. Uzel et al [19] and Kedziora-Kornatowska et al [20] have also reported low SOD and catalase activities in type II diabetics compared to controls. In contrast, Aydin and his coworkers [21] have noticed that SOD activity is elevated while catalase activities are normal in erythrocytes of type II diabetics.

In this study, the insulin hormone level was found to be significantly increased in type II diabetic patients compared to healthy subjects. This result agrees with the theory of insulin resistance associated with hyperinsulinemia that promotes higher production of free radicals by NADPH-dependent mechanisms [22].

This study specifically focused on the correlation of HbA1c and insulin with antioxidant enzymes, superoxide dismutase and catalase. The study findings showed a significant inverse correlation of HbA1C and insulin with superoxide dismutase and catalase in diabetic patients and even stronger inverse correlation in diabetic patients with different complications. This was consistent with the Hunt et al's proposition that chronic hyperglycemia and hyperinsulinemia may increase oxidative stress [23].

This study also focused on the correlation of MDA with antioxidant enzymes, superoxide dismutase and catalase. The study showed significant inverse correlations of MDA with antioxidant enzymes, superoxide dismutase (SOD) and catalase in diabetic patients, and even stronger inverse correlations were found in diabetic patients with different complications. This evidently showed that oxidative stress in terms of MDA was present in diabetic patients and was still significantly increased in diabetic patients with different complications; this is consistent with the findings of other studies [24, 25, and 26]. Increase in lipid peroxidation products (as indicated by the level of MDA) due to the decrease of the antioxidant enzymes is in line with previous reports [27, 28]. Hence complications of diabetes may be the result of this high level of free radicals (increase in the level of MDA and peroxidation index) and the reduction in antioxidant defences.

The findings of the present study clearly demonstrated that oxidative stress occurred early in diabetes and increased in the course of the disease, leading to diabetic complications. This finding was in line with those of other authors [29].

The present study also focused on the correlation of HbA1c with the MDA. The present study showed significant direct correlation of HbA1c with the MDA in diabetic patients and even stronger significant direct correlation in diabetic patients with different complications.

The direct correlation between glycemic control parameter (HbA1c) and MDA in diabetic patients has been reported by other authors [30, 31], and it can be partially explained by the existing correlation between hyperglycemia, and increased lipid peroxidation.

The present study showed significant direct correlation of HbA1C and MDA with the duration of diabetes in diabetic patients and even stronger significant direct correlation in diabetic patients with different complications. This is in accordance with the study of Kesavulu MM [32] and Sundaram RK [33].

The present study have assessed the effect of malonaldehyde (MDA), antioxidant enzymes; superoxide dismutase and catalase and other factors; glycated hemoglobin (HbA1c), insulin and duration of diabetes independently for the development of diabetic complications i.e., neuropathy, nephropathy, and cardiovascular diseases using the multivariate model .In this multivariate model diabetes mellitus was taken as a control variable for comparison. The

outcome of this multivariate was that, all the oxidative stress biomarkers were found to be independently significantly associated with diabetic complications. Therefore, the overall effect of the individual biomarkers may aggravate the situation and speed up the development of complications. The present study findings have shown that poor glycemic control and long duration of diabetes were associated with nephropathy, neuropathy and cardiovascular disease. This is similar to that reported in several other studies [34, 35].

Conclusions:-

1. The present study confirms susceptibility of diabetic patients to oxidative stress and that poor glycaemic control is associated with free radical-mediated lipid peroxidation.
2. Significantly decreased levels of SOD and catalase, increased levels of MDA in diabetic patients with different complications suggest that oxidative stress plays an important role in the pathogenesis of diabetic complications
3. The present study findings have shown that glycemic control, insulin, MDA, antioxidant enzymes; superoxide dismutase and catalase and duration of diabetes were associated with diabetic complications.

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