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Biomarkers of oxidative stress in newly diagnosed Tunisian type 2 diabetes mellitus

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Abstract

Background: *Type 2 diabetes mellitus (DM), a metabolic disease that the percentage has increased in the world including Tunisia, is characterized by glycemic disorders. This disease is one of the main risk factors for cardiovascular diseases which are associated with activation of oxidative stress. Aim: The aims of this study were to evaluate the oxidative status in diabetic patients with and without cardiovascular diseases (CVD) and to determine the correlation between the different oxidative stress parameters.*
Methods: *Eighty newly diagnosed type 2 diabetes patients were recruited in this work. Forty diabetic patients without cardiovascular diseases (CVD), others forty diabetic patients with the history of cardiovascular diseases and twenty healthy controls patients were selected for this study.*
Results: *Our findings indicated that the plasma MDA levels, which is the most objective marker of intracellular oxidative stress levels, was significantly higher ($p<0.05$) in patients with both CVD and DM or with only DM in comparison to control patients. Plasma alpha tocopherol levels were decreased ($p<0.001$) in diabetic patients with CVD as compared to controls. The increase of homocysteine levels which may play an etiologic role in the pathogenesis of type 2 diabetes by promoting oxidative stress, systemic inflammation, and endothelial dysfunction, and a significant decrease of SOD activity in diabetic patients with cardiovascular diseases compared with controls were also noted.*
Conclusions: *The obtained results revealed the crucial association between oxidative stress in Tunisian newly diagnosed diabetic patients and the development of risk factors like cardiovascular diseases.*

Keywords*: Oxidative stress, MDA, Alpha tocopherol, type 2 diabetes; cardiovascular diseases.*

Introduction

Diabetes mellitus is a source of many disorders and diseases such as liver and kidneys dysfunctions, immunological toxicity, hypertension and particularly heart diseases. Patients with type 2 diabetes mellitus (T2DM) are recognized to be at an amplified risk of atherosclerotic diseases, including cardiovascular diseases (CVD) in addition to well-known atherosclerotic risk factors, such as dyslipidaemia and obesity [1] .Diabetic patients have been generally described as having high levels of oxidative stress biomarkers [2]. It has been shown that oxidative stress due to the disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defense plays a vital role in the pathogenesis of coronary atherosclerosis and its complications.

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Enhanced formation of ROS may affect four fundamental mechanisms that contribute to atherogenesis, namely: oxidation of low density lipoprotein (LDL), endothelial dysfunction, vascular smooth muscle cells growth, and monocytes migration [3].

Oxidative stress generally causes damage to the membrane polyunsaturated fatty acids leading to the generation of MDA [4].

Malondialdehyde (MDA) from the oxidative polyunsaturated fatty acids (PUFA) degradation is determined by the reaction of thiobarbituric acid (TBA) with MDA to generate the stable end product of MDA-TBA adduct. This MDA free radical has been demonstrated as a causative of the atherosclerosis pathogenesis [5]. Elevated MDA levels in T2DM patients are associated with cardiovascular diseases risk [6].

Homocysteine (hcy) is a thiol containing amino acid produced by demethylation of methionine. Half of the hcy formed goes through the trans-sulphuration pathway and the other half takes a methyl group from betaine or 5-methyltetrahydrofolic acid.

In addition, antioxidants like SOD, vitamins C and E provide a defense system against free radical- induced damage. In fact, antioxidant enzymes like SOD catalyses the reaction in which superoxide anion is converted to hydrogen peroxide and oxygen.

Earlier studies suggested that diabetics had lower plasma Total Antioxidant Status, vitamin C and E concentrations than those without DM [7]. Vitamin E had been studied extensively in the prevention of atherosclerosis. Vitamin E was classified as an antioxidant due to its ability to scavenge lipid radicals and terminate oxidative chain reactions [8]. It could terminate radical chain reactions by interacting with the lipid peroxyl radical, preventing it from generating a new radical and perpetuating the chain reaction by oxidizing other lipids. Following its oxidation, vitamin E can be recycled back to its native unoxidized form by various soluble antioxidants such as vitamin C and ubiquinol. This process prevented the accumulation of vitamin E radicals and their subsequent peroxidation of lipids, and was considered by some to be critical for the antioxidant activity of vitamin E [9]. Thus, α-Tocopherol is a potent lipid-soluble antioxidant and the most abundant isomer of vitamin E found in humans [10].

The aims of this work were to evaluate oxidative status of newly diagnosed type 2 diabetes mellitus through the measure of MDA, Hcy levels, thiol groups, the SOD activity and alpha tocopherol levels for two diabetic populations with and without cardiovascular diseases and to examine the correlation between these parameters.

Methods

1-Patients

Forty Tunisian patients with newly diagnosed (some months<age of diabetes<5years) type 2 diabetes mellitus (20 men and 20 women) were recruited from the Institute of Nutrition and food Technology (Tunis, Tunisia). Forty diabetic patients with cardiovascular diseases (especially myocardial infracts and ischemic diseases) recruited from the cardiology service of Charles Nicolle hospital (Tunis, Tunisia), were also implicated in this study. Another's twenty healthy subjects (12 men and 8 women) served as controls. The diagnosis of diabetes was based on a previous history of diabetes on American Diabetes Association criteria (Expert Committee on the Diagnosis and classification of Diabetes Mellitus, 1997).The age of diabetic patients is included between 40 and 65 years and were treated by diet or oral antidiabetic drugs (i.e., biguanides and/or sulfonylurea). The following inclusion criteria were used for the recruitment of patients: body mass index (BMI) less than 30 Kg/m^2 and absence of kidney failure and liver or thyroid disease

Ethics: This study was approved by the Ethics Committee of Tunisia in accordance with the principles described in the Declaration of Helsinki. Informed consent was obtained from all the patients prior to the application of the research protocols.

2-Laboratory procedures

Determination of lipid profile

Blood samples were obtained after at least 8 h overnight fast and congealed at-80 ° C until use. Total cholesterol, TG and HDL-C concentration were measured using enzymatic methods respectively Cholesterol SL, Cholesterol HDL Direct SL. from Eli Tech companies. France. LDL cholesterol was calculated using the Friedewald formula [11].

Determination of lipid peroxidation

The lipid peroxidation was evaluated as malondialdehyde (MDA) levels which is the end product of lipid peroxidation reacting with thiobarbituric acid (TBA) as a TBA Reactive Substance (TBARS) to produce a red colored complex with a peak absorbance at 532 nm. The MDA activity was expressed as mmoles per milligram of protein. Proteins concentrations were determined according to the method of by using bovine serum albumin as a standard. [12].

Determination of thiol groups

The measurement of thiol groups (SH) was carried out in plasma, by the method described by Ellman (1959) [13].

Antioxidant enzymes activity

The activity of superoxide dismutase (SOD) was measured according to the epinephrine method, based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome ([14]).

Determination of Alpha tocopherol levels

The Alpha tocopherol levels were determined by High Performance Liquid Chromatography (HPLC). The patient's plasma was treated with n-hexane. Vitamin E was extracted twice in hexane phase and the collected extract was dried in liquid nitrogen. The dried extract was solubilized in 0.5 ml methanol for HPLC. Injections were made in duplicate for each sample. The quantification was utilizing absorption spectra of 296 nm. HPLC separations were accomplished at room temperature with a Perkin-Elmer liquid chromatography system (Series 1100) consisting of a sample injection valve (Cotati 7125) with a 20 µl sample loop, an ultra-violet (UV) spectrophotometric detector (Cecil 68174), an integrator (HP 3395) and a Techsphere ODS-2 packed (5 *am* particle and 80 °A pore size) column (250 x 4.6 ID) with a methanol: acetonitrile: chloroform (47: 42: 11, v/v) mobile phase at 1 ml min^{-1} flow rate (Catignani, G.L. (1983), Miller, K.W., (1984)).

Determination of homocysteine levels

The blood for measuring total homocysteine (t Hcy) was collected in tubes containing EDTA and kept on ice until centrifuged (3500 rpm/minute for 15 minutes). The plasma was stored at −80°C until assayed.

The concentrations of t Hcy in plasma were evaluated by using an automatic analyzer (Immulite 1000 DPC; Siemens Medical Solutions, Los Angeles, CA) based on a competitive immunoassay. The Hcy assay involves a preliminary manual pretreatment step; Hcy is released from its binding proteins and converted to S-adenosyl-homocysteine (SAH) by an off-line 30 minute incubation at 37°C in the presence of S-adenosyl-Lhomocysteine hydrolase and dithiothreitol (DTT) [15].

3 – Statistical methods

Statistical analyses were realized using SPSS version 19. All data are expressed by the mean ± S.D. To compare between means of two continuous variables the student's test was used; to compare categorical variables, Chi-square test (χ^2) was used. At *P<.0.5*, the test is considered significant. Pearson correlation test was carried between all the parameters and linear regression was studied for MDA.

Results

1- Lipid disorders in diabetic patients with and without cardiovascular diseases

Table 1 demonstrated lipid disorders in diabetic patients with and without cardiovascular diseases. These disorders were associated with lipid abnormalities which were the hypertriglyceridemia and the decrease of HDL level accompanied with the increase of LDL level. The lipid disorders were revealed in the two groups of diabetes with and without cardiovascular diseases. The HDL/LDL ratio decreased in the two diabetic with and without cardiovascular when compared with healthy patients. The LDL/HDL ratio increased in diabetic patients with and without CVD compared with controls.

Table 1: Lipid profile of Healthy, Diabetic patients with and without Cardiovascular diseases

	H	D	D with C VD
Age	49.1 ± 1.59	51.15± 1.66	56.05 ± 3.08 #
Sex ratio (M/F)	12/8	20/20	20/20
BMI (Kg/m²)	< 30	<30	< 30
TG	1.27± 0.23	1.83±0.15*	2.17± 0.37 # #
T C	3.96± 0.20	4.79± 0.32*	4.64±0.27 #
HDL	1.08± 0.11	0.81± 0.29*	0..72± 0.18#
LDL	2.30± 0.20	3.16± 0.25*	3.04±0.29#
TC/ HDL	4.35± 0.49	7.09±0.41*	7.32±0.35##
LDL/ HDL	2.74±0.16	4.86±0.14*	5.66±0.22#

H: Healthy patients; D: Diabetic patients; D with CVD: Diabetic patients with cardiovascular disease
*: significant difference (p<0.05) H vs D;
#: significant difference (p<0.05) H vs D with CVD

2- Determination of oxidative stress parameters

Figure 1, 2 and 3 showed different oxidative stress parameters in diabetic patients with and without CVD. This difference is more pronounced in the two sexes in diabetic patients with CVD. A significant increase in hcy levels, MDA levels and a decrease in SOD activity were registered in diabetic patients with and without CVD compared with controls.

Figure 4 revealed the variation Alpha tocopherol levels in control group, the diabetic patients with and without cardiovascular diseases. A significant decrease in Alpha tocopherol levels in diabetic patients with and without CVD compared with controls was noted.

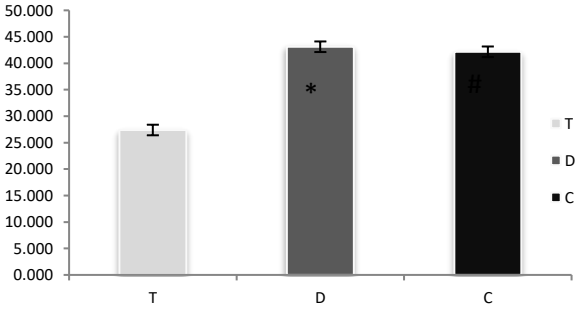

Figure 1: The MDA levels in healthy, diabetic patients and diabetic patients with CVD
H: Healthy patients; D: Diabetic patients; C: Diabetic patients with cardiovascular diseases
*: significant difference (p<0.05) H vs D;
#: significant difference (p<0.05) H vs D with CVD

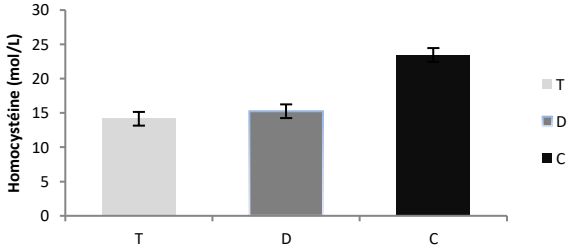

Figure 2: Variation of homocysteine levels in healthy, diabetic patients and diabetic patients with CVD
H: Healthy patients; D: Diabetic patients; C: Diabetic patients with cardiovascular diseases
*: significant difference (p<0.05) H vs D;
#: significant difference (p<0.05) H vs D with CVD

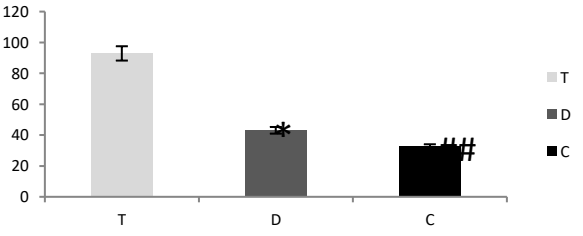

Figure 3: The SOD activity in healthy, diabetic patients and diabetic patients with CVD
H: Healthy patients; D: Diabetic patients; C: Diabetic patients with cardiovascular diseases
*: significant difference (p<0.05) H vs D;
##: very significant difference

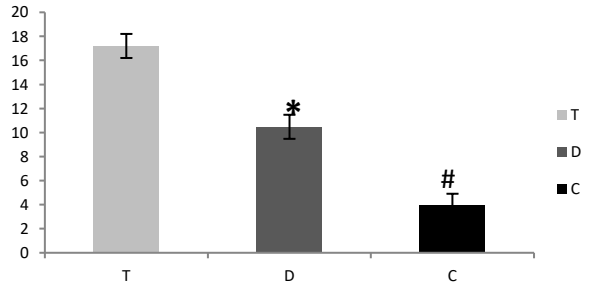

Figure 4: The Alpha tocopherol levels in healthy, diabetic patients and diabetic patients with CVD

H: Healthy patients; D: Diabetic patients; C: Diabetic patients with cardiovascular diseases

*: significant difference (p<0.05) H vs D;

##: very significant difference (p<0.01) H vs D with CVD (p<0.01) H vs D with CVD

Figure 5 showed the variation of thiol groups in controls, the diabetic patients with and without CVD. A significant increase was noted in diabetic patients with and without CVD when compared with controls.

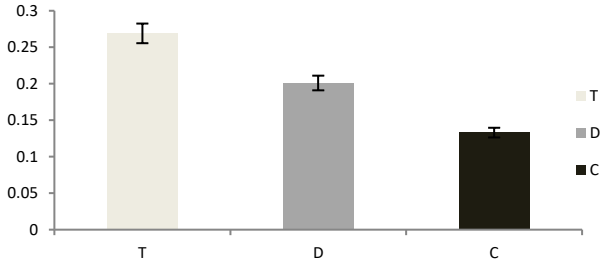

Figure 5: The thiol groups levels in healthy, diabetic patients and diabetic patients with CVD

H: Healthy patients; D: Diabetic patients; C: Diabetic patients with cardiovascular diseases

*: significant difference (p<0.05) H vs D;

##: very significant difference (p<0.01) H vs D with CVD (p<0.01) H vs D with CVD.

Table 2 indicated MDA linear regression for healthy, diabetic patients with and without cardiovascular diseases.

Table 2: MDA linear regression for healthy, Diabetic patients with and without Cardiovascular diseases

	H (N= 20)	D (N= 40)	C (N= 40)
glycemia	F= 2.127 , R^2 = 0.106, P = 0.162	F= 1.313 , R^2 = 0.033, P = 0.25	F= 0.005 , R^2 = 0.06, P = 0.94
chol	F= 0.677 , R^2 = 0.036, P = 0.42	F= 0.66 , R^2 = 0.002, P = 0.79	F= 0.000 , R^2 = 0.000, P = 1
TG	F= 0.57 , R^2 = 0.031, P = 0.45	F= 1.49 , R^2 = 0.038,P = 0.229	F= 0.29 , R^2 = 0.008, P = 0.10
HDL	F= 0.522 , R^2 = 0.028, P = 0.47	F= 5.038 , R^2 = 0.01, P = 0.031*	F= 0.45 , R^2 = 0.012, P = 0.50
LDL	F= 1.108 , R^2 = 0.006, P = 0.74	F= 0.27 , R^2 = 0.01, P = 0.87	F= 0.45 , R^2 = 0.001, P = 0.82
CHOL/ HDL	F= 1.14 , R^2 = 0.06, P = 0.29	F= 10.657 , R^2 = 0.21, P = 0.002*	F= 0.047 , R^2 = 0.001, P = 0.82
LDL/HDL	F= 0.97 , R^2 = 0.051, P = 0.33	F= 13.28 , R^2 = 0.25, P = 0.001**	F= 0.03 , R^2 = 0.000, P = 0.95

H: Healthy patients; D: Diabetic patients; D with CVD: Diabetic patients with cardiovascular disease

*: significant difference (p<0.05) H vs D; **: very significant difference (p<0.001) H vs D.

Discussion

The number of subjects with diabetes is continuously increasing all over the world. People with diabetes are at two to three-fold increased risk of developing cardiovascular diseases (CVD), and CVD remains the major cause of death in patients with diabetes [16].

The oxidative stress plays a significant role in the development of T2DM-related CVD [17]. There is considerable evidence that induction of oxidative stress is a key process in the onset of diabetic complications. The precise mechanisms by which oxidative stress may accelerate the development of complications in diabetes are only partly known.

In addition, Lipid peroxidation of the cellular structures, a consequence of increased oxygen free radicals, is thought to play an essential role in atherosclerosis and microvascular complications of T2 DM [18]. In both type 1 and type 2 diabetes, the late diabetic complications in nerve, vascular endothelium, and kidney arise from chronic elevations of glucose and possibly other metabolites including free fatty acids (FFA)[19].

In fact, Malondialdehyde (MDA) was a major player in low density lipoprotein (LDL) modification and was a product of the peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acids. MDA, the three-carbon dialdehyde, can exist in many forms in the aqueous circulation. This chromophore was termed thiobarbituric acid reacting substances.

In the current study, a significant increase of MDA levels was noted in diabetic patients with CVD. Oxidised-LDL (ox-LDL) results from the interactions between aldehydes such as MDA and lysine residues in apoB-100 of LDL [20].

The obtained results showed a significant increase in hcy levels in diabetic patients with CVD.

In fact, Hcy level is well thought-out as a risk factor for the development of atherosclerosis. It has been suggested that Hcy enhances inflammatory response that is recognized for their role in atherosclerotic disease [15].

Also, Hcy is known to take part in the development of atherosclerosis and vascular injury and it has been suggested to contribute to the atherosclerotic process of diabetes mellitus. Several studies have demonstrated that plasma Hcy levels are elevated in diabetic patients [21].

Our findings indicated that vitamin level decreased significantly in diabetic patients with CVD.

Vitamin E has been studied extensively in the prevention of atherosclerosis. A recent meta-analysis of these trials has emphasized the ineffectiveness of vitamin E in atherosclerosis prevention with a possibility of harm at higher dosages. However, vitamin E has several isomers, with the alpha form being available via dietary supplements and the gamma form being available via dietary foodstuffs. The gamma form of vitamin E demonstrates several superior properties (such as trapping reactive nitrogen species and detoxifying nitrogen dioxide) compared with alpha vitamin E. All

clinical trials have utilized the alpha isomer with little concern that this isomer of vitamin E may actually suppress the gamma isomer of vitamin E. Basic research has provided credible mechanisms by which vitamin E might exert cardiovascular (CV) benefit, including inhibition of oxidation of low-density lipoprotein (LDL) cholesterol in plasma. Observational epidemiologic studies suggested that individuals who consumed high amounts of vitamin E through diet or supplements had decreased rates of CV diseases.

The antioxidant vitamin E had been a major subject of controversy in the prevention of atherosclerosis. The initial basic science studies suggested that vitamin E had beneficial effects on several different stages of the atherosclerotic process [22]. Vitamin E not only had antioxidant properties, but also non-antioxidant effects, including modulation of signal transduction pathways [23]. This was followed by observational, cross-sectional studies in patients with no initial coronary artery disease, which also suggested that vitamin E supplementation lowered the risk of major coronary heart disease [24]. However, subsequent large randomized controlled trials have shown that vitamin E supplementation had no clear benefit in the primary or secondary prevention of cardiovascular disease, with some trials suggesting harmful effects. Of the clinical trials showing benefit, most of them studied patients with evidence of increased oxidative stress, and in addition, gave vitamin C supplements with the vitamin E. Vitamin C, a water soluble antioxidant, has been shown to regenerate vitamin E (a lipid soluble antioxidant). Three recent trials using high dosages of both vitamins E and C together, in populations with high oxidative stress, have shown a delay of coronary arteriosclerosis when compared with placebo. Supplementation of both vitamins, in individuals with increased oxidative stress, may therefore be more effective than either vitamin taken alone. All of the above cited clinical trials utilized the alpha tocopherol isomer form of vitamin E (alpha Vitamin E). This was the composition of vitamin E sold in over-the-counter supplements.

Several studies had suggested that alpha tocopherol supplementation may decrease serum levels of gamma tocopherol in man. However, no variable dose response studies of the effects of alpha tocopherol on gamma tocopherol had been published so that the overall effects of alpha tocopherol are unknown. Since a wide range of alpha tocopherol dosages had been utilized in clinical trials, this information was critical to evaluate the effects of vitamin E on atherosclerotic risk. In addition, surrogate markers of atherosclerosis provide a useful approach to understanding the effects of an intervention on different components of the atherosclerotic process, including, oxidative stress, inflammation, and hyper coagulation. Therefore, It was the intent of this study to examine the potential benefits of alpha tocopherol supplementation (plus Vitamin C), by administering different dosages of these vitamins to type 2 diabetic individuals (a population with high oxidative surrogate markers of atherosclerosis). A protective effect of vitamin E may exist within the range of intake available from food. This effect may go undetected within studies of high-dose supplement use, which appears to hold no additional protective benefit. It had been suggested that all of the other biological functions of vitamin E were actually a result of its antioxidant activity [25].

Furthermore, a decrease in serum SOD levels was noted in the current study, which has a central role in the antioxidant defense system. The reduction in SOD activity was unregistered in diabetic patients with and without cardiovascular diseases. Previous studies have reported a decrease in SOD level in blood in diabetic patients [26-27]. Therefore, it is believable that the capacity of SOD to remove superoxide could be a major determinant of the degree of super- oxide-induced oxidative and nitrosative stress under chronic hyperglycemia. Taking these findings together, the reduction of SOD antioxidant defense capacity appears to be responsible for the increased level of oxidative and nitrosative stress in diabetic with and without CVD.

The presence of conventional risk factors cannot sufficiently account for the excess risk of atherosclerosis in patients with non–insulin-dependent diabetes mellitus (NIDDM). Oxidative modification of LDL has been implicated in the pathogenesis of coronary atherosclerosis.

Conclusion

Our results revealed the implication of the oxidative stress in the complication of type 2 diabetes especially CVD which need the control of oxidative stress parameters beside the glucose levels and the lipid profile to prevent CVD complication in type 2 diabetic patients.

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