Oxidative stress markers as early predictors of neuropathy in type 2 diabetic patients

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Abstract: Backgrounds and aim of the work: Diabetic neuropathy is the most common complication of diabetes mellitus (DM). Diabetic peripheral neuropathy involves the presence of symptoms or signs of peripheral nerve dysfunction in people with diabetes. The aim of this study was to assess the level of oxidative stress markers in patients with type 2 diabetes with peripheral neuropathy in compared to healthy subjects. **Subjects & methods:** The study **was** performed on 100 outpatients with type 2 diabetes matched with Age, BMI and Gender with 25 healthy subjects was selected from the outpatient's clinics of National Institute for Diabetes and Endocrinology. All groups were subjected to estimation of Fasting blood glucose, Glycosylated haemoglobin (HbA1c) and lipid profile in addition to ox-LDL, LPO and TAOS (TAC). **Results:** Fasting blood glucose and HbA1c showed significant increase in the diabetic patients groups compared to control group ($P<0.05$). Serum total cholesterol, Triacylglycerol and LDL-c were significantly higher in diabetic patients groups as compared to control group ($P<0.05$) while HDL-c level showed significant increase in the control group when compared to diabetic patients groups. The level of ox-LDL and LPO represented significant increase ($P<0.0001$) in the diabetic groups comparison to control group, while the level of TAOS (TAC) represented significant elevation ($P<0.0001$) in the control group in comparison to the diabetic groups.

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1. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia together with biochemical alterations of glucose and lipid peroxidation. The oxidative stress in diabetes was greatly increased due to prolonged exposure to hyperglycemia and impaired of oxidant\ antioxidant equilibrium ***(Ramakrishna and Jailkhani, 2008).*** Neuropathy is the most common complication of diabetes, affecting approximately 50% of patients over the course of their disease ***(Edwards, J. L., et al, 2008).*** Typical symptoms of diabetic neuropathy include pain, numbness, tingling, weakness, and difficulties with balance. The disease is associated with substantial morbidity, including depression, susceptibility to foot or ankle fractures, ulceration and lower-limb amputations ***(Gandhi, R. A., et al, 2010).*** Oxidative stress is an important factor in the pathogenesis of many chronic diseases, including diabetes. Chronic exposure to elevated levels of glucose, which is the main characteristic of the diabetic milieu, increases the production of reactive oxygen species (ROS) and generates oxidative stress in islet cells ***(O SAVU, et al, 2012).*** An important form of cholesterol is called oxidized low density lipoprotein (oxLDL), which is known to have a critical function in the development of atherosclerosis. Along with its receptor, the lectin-like oxLDL receptor (LOX)-1***) Kita T, et al, 2001),*** both oxLDL and LOX-1 contribute to intracellular oxidative stress and inflammation injury within vascular tissues as well as in the immune system ***(Esterbauer H, et al,1993).*** This may exist in an additive state with DM, as both oxLDLs and hyperglycemia increase LOX-1 expression ***(Li L, et al, 2004).*** In murine models, high fat ingestion leads to significantly increased plasma oxLDL levels in combination with morphological and functional evidence of peripheral neuropathy before hyperglycemia develops. Also, oxLDLs contribute to oxidative stress and injury in dorsal root ganglia sensory neurons in vitro via a LOX-1 mechanism related to activation of nicotinamide adenine dinucleotide phosphate oxidase ***(Vincent AM, et al, 2009).*** These data not only suggest that oxLDL and LOX-1 may contribute to DPN, but that they may also lead to development of peripheral neuropathy even in the absence of DM. Lipids (cholesterol, polyunsaturated fatty acids (PUFA) are a main target of oxidative attack and this leads to the formation and the accumulation of lipid oxidation (LPO) products ***(Sies H., 1997).*** The progressive accumulation of LPO products is a starting point mechanism of tissues and cellular dysfunction involved in ageing and in well-defined diseases of liver, kidney, neurological and cardiovascular systems, cancers, endocrine and metabolic disorders, diabetes and its complications and other oxidative stress related pathologies ***(ANNE***

NEGRE-SALVAYRE, et al, 2010). Plasma antioxidant status is the result of interaction and cooperation of various antioxidants. The concept of total antioxidant capacity (TAC) was developed considering the synergistic role of those antioxidants rather than the simple sum of individual antioxidant action ***(Wang Y, et al, 2013).***

The purpose of this study was to assess the level of oxidative stress markers in patients with type 2 diabetes with peripheral neuropathy in compared to healthy subjects.

2. Subjects and Methods

The study was performed on 100 outpatients with type 2 diabetes and 25 healthy subjects. All patients were selected from outpatients Clinic of National Institute of Diabetes and Endocrinology (NIDE), Cairo, Egypt. Type 2 DM was diagnosed according to ***(The Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2006).*** Peripheral neuropathy was made from a standard consultation and clinical examination. Diabetic neuropathy was defined as a positive sign of pain, numbness, tingling, weakness, and difficulties with balance. Type 2 diabetic patients were taking the same insulin therapy. Demographic data was recorded for each subject using self-made questionnaire. Approval had been taken from the research ethics committee of General Organization of Teaching Hospitals and Institutes. An informed consent was obtained from all patients and healthy subjects that described the aim of the study and the procedures that would be required from them. The study included 5 groups of subjects, Group I: type 2 diabetic patients with peripheral neuropathy (n = 25), the mean age 52.84±8.57years; the mean duration of the disease was12.12±5.6years, Group 2: type 2 diabetic patients with peripheral neuropathy and neuropathic ulcer (n = 25), the mean age 52.28 ±7.31 years, the mean duration of the disease was 12.56±5.6 years, Group 3: type 2 diabetic patients with peripheral neuropathy and Charcot neuroarthropathy (n = 25), the mean age 52.28±7.74 years, the mean duration of the disease was 12.88±5.23 years, Group 4: type 2 diabetic patients with peripheral neuropathy, neuropathic ulcer and Charcot neuroarthropathy (n=25) the mean age 52.48±8.27 years, the mean duration of the disease was 12.72±5.28years Group 5: control group (n =25), the mean age 52.44±7.85 years. The following variables were also recorded: age, BMI, blood pressure. BMI was calculated as weight (kg) divided by height squared meter (Kg/m2) according to ***(Shiwaku et al., 2004).*** Blood pressure measurements were performed by trained technicians or nurses with a mercury sphygmomanometer and the first and fifth Korotkoff sounds were recorded to represent the systolic and diastolic pressure, two measurements were obtained and averaged. Hypertension was considered if the systolic blood pressure was G140mm Hg or diastolic blood pressure G90 mm Hg, or use of medication for hypertension. Patients with any history of smoking, alcohol habits, respiratory disorder, clinical or laboratory signs of liver disease, thyroid function impairments, chronic inflammation and were significant infectious diseases were excluded. The healthy control subjects were matched with age and sex as patients groups with no recognizable diseases and not receiving any medications. Blood samples were collected after 12 h overnight fasting from healthy subjects and diabetic patients into three types of vacutainer tubes first vacutainer tube without additive. Blood was centrifuged at 3000 rpm for 10 minutes. Serum was rapidly separated and subdivided into aliquots one of them to measure lipid profile, and the others were stored at -80° C until the measurements of ox-LDL, TAS/TAC and LPO. Second part of collected blood was taken on EDTA for determination of HbA1c level. Hemolysed samples were excluded. Third part of collected blood was taken on fluoride for determination of Plasma glucose level at once by glucose oxidase method according to ***(Barham and Trinder; 1972).*** Serum total cholesterol was determined by the enzymatic method according to ***(Allain et al., 1974).*** Triacylglycerol was assayed by peroxidase-coupled method according to ***(Mc Gowan et al., 1983).*** HDL-c was measured by enzymatic method according to ***(Finley et al., 1978)***, LDL-c was measuring according to ***(Friedewald et al., 1972).*** Sampling, reagent delivery, mixing, processing, calculating and printing were full automatically performed by the Dimension® RxL Max Integrated chemistry system (SEIMENS instruments Inc, USA), and HbA1c was assayed using ion-exchange high performance liquid chromatography (HPLC) with Bio-Rad Variant Hemoglobin Testing System (Bio-Rad Laboratories, USA) according to method of ***(Lahousen et al., 2002).*** Serum ox-LDL concentration was measured using commercially available enzyme-linked immunosorbent assay (ELISA) Kit Immunodiagnostic (Catalog No K 7810) (Bensheim, Germany). Serum TAS/ TAC concentration was measured using commercially available enzyme-linked immunosorbent assay (ELISA) Kit Immunodiagnostic (Catalog No KC 5200) (Bensheim, Germany). Serum LPO concentration was measured using commercially available enzyme-linked immunosorbent assay (ELISA) Kit Glory Science (Catalog No 11004) (Glory Science Co., USA).

Statistical Analysis

Data was expressed as the M ± SD. Statistics were calculated and appropriate graphs and histograms were plotted when needed for the entire study cohort, using GraphPad Prism 5 (For Windows, © 1992- 2007 Graphpad software Inc., V 5.01, USA) which was used to test the significance of differences between groups in the present study. To analyze more than two sets of data, ordinary one way analysis of variance (ANOVA) for parametric data was first tried, followed by Tukey-Kramer multiple comparison test.

Furthermore, analysis was performed to examine the possibility for any correlation between different parameters. For clinical correlations, the correlation co-efficient was calculated using least square method. AUC was determined by receiver operating characteristics curve method "ROC" using GraphPad Prism 5 (For Windows, © 1992-2007 Graphpad software Inc., V 5.01, USA).

3. Results

Table (1): Demographic characteristics of the diabetic patients groups and the control group (M±SD).

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
Age (years)	**52.84±8.57**	**52.28 ±7.31**	**52.28±7.74**	**52.48±8.27**	**52.44±7.85**
Gender(F/M)	**12/13**	**13/12**	**12/13**	**13/12**	**12/13**
Duration of diabetes (years)	**12.12±5.6**	**12.56±5.6**	**12.88±5.23**	**12.72±5.28**	----------
BMI (Kg/m2)	**32.2±6.57**	**32.3±6.1**	**32.57±6.3**	**32.3±6.22**	**32.04±6.13**
SBP (mmHg)	**127.2±8.9**	**126.8±13.4**	**127.8±16.2**	**129.0±13.3**	**117.8±4.1**
DBP (mmHg)	**78.6±5.8**	**81.4±12.3**	**81.2±11.5**	**80.6±8.8**	**76.0±5.2**

Demographic characteristics of the patients groups and control group are represented in table (1).No significant differences were found between diabetic groups and control group with respect to age, gender and BMI (P> 0.05). The mean systolic blood pressure showed significant increase in diabetic groups when compared to control group but the diastolic pressure showed no significant between the diabetic groups and control. In the diabetic groups the mean duration of diabetes in peripheral neuropathy, peripheral neuropathy and neuropathic ulcer, peripheral neuropathy and Charcot neuroarthropathy and peripheral neuropathy, neuropathic ulcer and Charcot neuroarthropathy were 12.12±5.6, 12.56±5.6, 12.88±5.23 and 12.72±5.28 respectively.

Table 2: Biochemical characteristics of glycemic index profile in the diabetic patients groups and the control group (M±SD).

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
FBG (mg/dl)	**259.78±78.16**	**286.12±107.31**	**271.1±111.06**	**298.6±59.9**	**95.2±11.08**	**0.000*****
HbA1c (%)	**10.28±2.5**	**10.91±1.9**	**11.36±1.94**	**12.46±1.23**	**5.5±0.4**	**0.000*****

Table (2): showed the biochemical characteristics of glycemic index in the diabetic groups and control group, plasma fasting blood glucose and HbA1c showed significant increase in the diabetic patients groups compared to control group (P<0.05).

Table 3: Serum lipid profile in the diabetic patients groups and the control group (M±SD).

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Cholesterol(mg/dl)	**200.4±36.0**	**213.6±42.36**	**223.16±34.13**	**239.76±29.5**	**191.28±34.2**	**0.000*****
Triacylglycerol (mg/dl)	**180.28±62.1**	**193.76±49.4**	**197.8±50.6**	**157.2±61.3**	**126.3±49.6**	**0.000*****
HDL-c (mg/dl)	**38.96±7.3**	**35.48±5.21**	**33.96±3.9**	**32.36±3.88**	**46.4±10.9**	**0.000*****
LDL-c (mg/dl)	**123.12±23.5**	**129.4±23.19**	**149.96±29.44**	**158.2±24.39**	**123.0±35.1**	**0.000*****

Table (3): Showed lipid profile in the diabetic patients groups and control group, serum total cholesterol, Triacylglycerol and LDL-c were significantly higher in diabetic patients groups as compared to control group (P<0.05) while HDL-c level showed significant increase in the control group when compared to diabetic patients groups.

Table (4): Serum level of ox-LDL, LPO and TAOS (TAC) in the diabetic patients groups and the control group (M±SD).

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
ox-LDL(ng/ml)	11.38±3.1	12.2±3.15	12.07±4.5	13.7±3.0	7.34±2.95	0.000***
LPO((nmol/l)	278.52±104.0	281.3±104.3	197.7±85.3	287.44±66.0	27.72±16.12	0.000***
TAOS(TAC) (µmol/l)	129.7±51.1	119.56±59.9	107.24±49.9	99.4±51.7	196.5±27.5	0.000***

Table (4): The level of ox-LDL and LPO represented significant increase (P<0.0001) in the diabetic groups comparison to control group, while the level of TAOS (TAC) represented significant elevation (P<0.0001) in the control group in comparison to the Diabetic groups.

Table (5): Pearson's correlation analyses revealed that, in diabetic patient groups FBG correlated positively with HbA1c (r^2= 0.398, P=0.0001). HbA1c was correlated positively with LDL-c with (r^2= 0.237, P=0.018).Cholesterol was correlated positively with T.G and LDL-c with (r^2= 0.198, P=0.048 and r^2= 0.475, P=0.0001) respectively. Also HDL-c was correlated negatively with LDL-C and ox –LDL with (r^2= - 0.309, P=0.002 and r^2= - 0.230, P=0.021) respectively.

Table 5: The correlations among parameters in type 2 diabetic patients (no=100).

Parameters	r2	P- value
FBG and HbA1c	0.398	0.0001***
HbA1c and LDL-c	0.237	0.018*
Chol and T.G	0.198	0.048*
Chol and LDL-c	0.475	0.0001***
HDL-c and LDL-c	-0.309	0.002**
ox-LDL and HDL-c	-0.230	0.021*

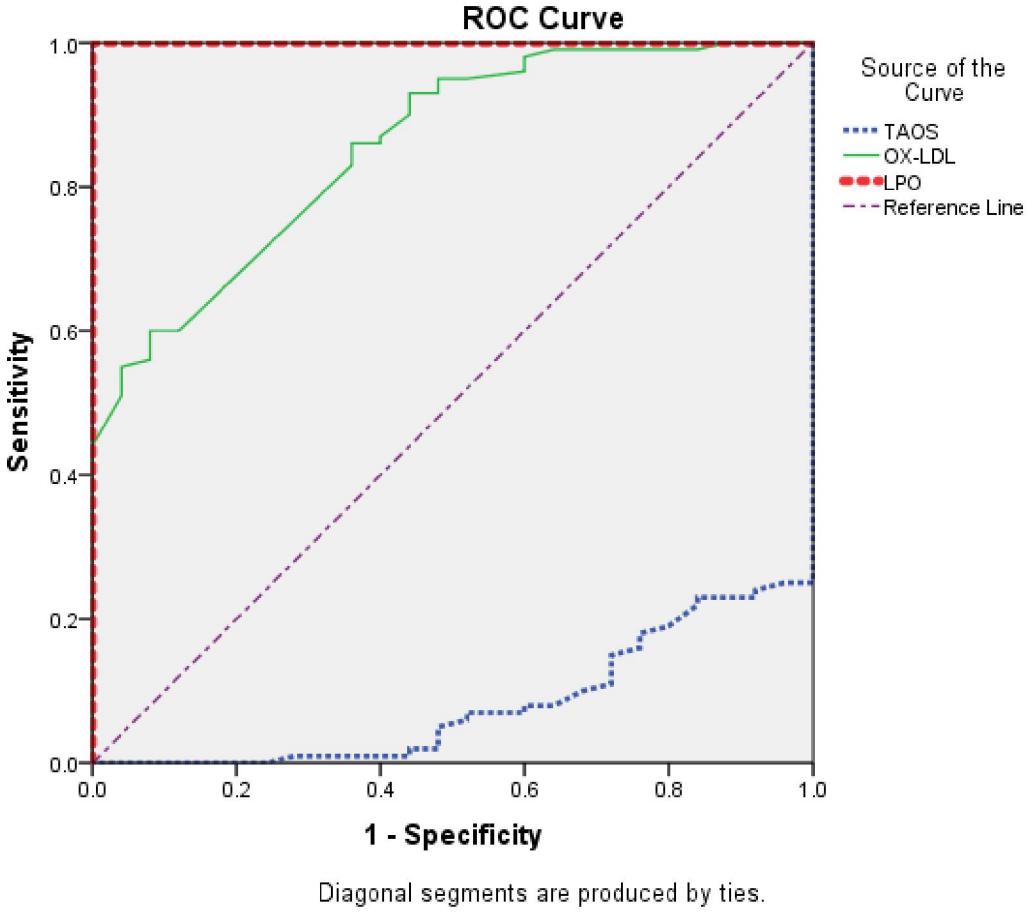

Figure 1: Illustrates the ROC analysis of ox-LDL, LPO and TAOS (TAC).

ROC analysis

Receiver operating characteristic "ROC" curves for ox-LDL, LPO and TAOS (TAC) in the diagnostic of diabetic patient with neuropathy and its complications indicated that area under the curve AUC = 0.855±0.038 for ox-LDL, AUC =1.000±0.0001 for LPO and AUC for TAOS (TAC) was 0.081±0.025.

4. Discussion

Diabetic polyneuropathy (DPN) is a common and disabling complication of human diabetes mellitus (DM) seen in up to 50% of patients, leading to sensory, motor and/or autonomic dysfunction ***(Zochodne, 2008).*** There are several potential mechanisms postulated to contribute to development of DPN, including: excessive sorbitol-aldose reductase pathway flux; protein kinase C (PKC) isoform(s) overactivity; increased oxidative and nitrative stress; microangiopathy; accumulation of advanced glycation end products (AGEs) and interaction with their receptor (RAGE); and failure of neurotrophic support***(Alma, et al;2014).***

Pathogenesis of diabetic neuropathy is complex. Chronic hyperglycemia is a major factor which induces nerve fibers injury. Chronic hyperglycemia causes oxidative stress in tissues prone to complications in patients with diabetes ***(Rosen, et al; 2001).*** High levels of glucose stimulate the polyol pathway causing osmotic stress, enhance reactive oxygen species generation, and play an important role in diabetic angiopathy development. So the progression of diabetic neuropathy is dependent on glycemic control in both type 1 and 2 diabetes patients ***(Sima, 2006).***This study elucidates that fasting blood glucose and HbA1c levels showed pronounced increase in the diabetic patients groups in compared to control group. So these findings are in agreement with the previous studies which suggest that hyperglycemia can induce oxidative stress that contribute in the development of diabetic vascular and neural dysfunction ***(Yorek, 2003).*** Also chronic and acute hyperglycemia cause oxidative stress in the peripheral nervous system that can promote the development of diabetic neuropathy ***(Vincent, et al; 2004)*** as in table (2). Clinical epidemiological studies have now demonstrated a similar strong association between dyslipidemia and microvascular complications, including neuropathy in both type 1 and type 2 diabetes. Dyslipidemia is, therefore, an important modifiable parameter in the prevention and treatment of neuropathy in diabetes ***(Fioretto, et al; 2010).*** There is clinical evidence that triglyceridemia contributes to progression of already existing DPN ***(Wiggin, et al;2009)*** while each of total cholesterol, low density lipoprotein and triglyceride levels have been positively associated with increasing cumulative incidences ***(Tesfaye, et al;2005)*** or greater severity ***(Sachedina and Toth, 2013)*** of diabetic peripheral neuropathy. Furthermore, a low high density lipoprotein level may also relate to presence of DPN ***(Maser, et al; 1989).*** This in accordance with our finding in which there is an increase in the total cholesterol, Triacylglycerol and LDL-c in diabetic patients groups in compared to control group as in table (3). In diabetes, plasma lipoproteins are subject to an oxidizing environment. Peripheral sensory neurons, like vascular endothelial cells, express scavenger receptors for oxidized LDLs (oxLDLs), including oxidized LDL receptor 1 (LOX1) and Toll-like receptor 4 ***(Ishiyama, et al; 2010).*** These neurons also express RAGE, which binds glycated LDL ***(Vincent, et al; 2007).*** The receptors internalize oxLDL and glycated LDL, releasing potentially injurious triglycerides and fatty acids, and initiate an inflammatory signaling pathway that results in activation of NADPH oxidase ***(Vincent, et al; 2009)***. This enzyme produces substantial cellular oxidative stress by generating superoxide radicals and by depleting NADPH levels. Oxidative stress in diabetes leads to increased expression of oxLDL and RAGE via p38 mitogen-activated protein kinase (MAPK) signaling; producing a positive-feedback mechanism of injury ***(Toth, et al, 2008).*** Our results found that there is an increase in the ox-LDL level in the diabetic patients groups with peripheral complications in compared to control group. This finding is agreement with other studies in which preclinical studies have identified potential mechanisms by which oxLDL may contribute to neurodegeneration, particularly in the presence of hyperglycemia related to DM ***(Alma, et al; 2014)***. Also, Along with oxLDL, LOX-1 contributes to intracellular oxidative stress and inflammation injury ***(Esterbauer, et al; 1993).*** Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems. Once this imbalance appears, cellular macromolecules may be damaged by the predominant free radicals. This leads to oxidative modifications of the genome, proteins, structural carbohydrates, and lipids; in the latter case, lipid peroxidation (LPO) occurs. LPO is a free radical-related process, that in biologic systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or nonenzymatically. This latter form is, as mentioned above, associated mostly with cellular damage as a result of oxidative stress, and a great variety of aldehydes is formed when lipid hydroperoxides break down in biological systems, among them, malondialdehyde (MDA) and 4-hydroxynonenal

(HNE) ***(Francisco, et al;1998).*** This in accordance with our result in the study in which there is an increase in the LPO level in the diabetic patients with peripheral neuropathy and its complications groups in compared to control group as in table(4). The increased presence of free radicals has been suggested to be one of the major causes of diabetic complications, having implications on the pathogenesis of type 2 diabetes mellitus ***(Sagara, et al; 1998).*** Several hypotheses have been tested to evaluate the possible causal mechanism of increased free radicals in diabetes ***(Bayness, et al; 1999).*** Some studies suggest enhanced free radicals due to elevated glucose concentrations. Other studies focus on reduced antioxidant defense in diabetes ***(Maxwell, et al; 1997).*** This is in accordance with our result in which there is a decrease in the total antioxidant capacity in the diabetic patients with peripheral neuropathy and its complications in compared control group as in table (4).

In conclusion: the results suggest that ox-LDL, LPO, TAC could be used as a markers for the detection of diabetic neuropathy with its complications and may be a valuable future tools in diagnosis, prognosis and assessment of diabetic neuropathy and its complications in patients with type 2 diabetes mellitus.

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