Endothelial Dysfunction and Plasminogen Activator Inhibiror-1 (PAI-1) alteration as Risk Factors for Coronary Heart Diseases

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Abstract: Background: Insulin resistance is known to be a common feature of type 2 diabetes mellitus and regarded as an important mechanism in the pathogenesis of this disease. The key pathogenetic mechanisms of insulin resistance progression are free fatty acids metabolism impairment and enhanced activity of plasminogen activator inhibitor-1 (PAI-1). Both oxidized LDL (ox-LDL) and PAI-1 were recognized as risk factors for coronary heart disease. **Objective:** To analyze simultaneously the correlation between hyperglycemia and biochemical markers related to stress, endothelial dysfunction, blood coagulation disorders and cardiovascular diseases. **Methods**: This study was carried out on 82 male non smoker subjects classified into three groups: group 1 included 35 diabetic uncomplicated patients; group 2 enrolled 35 diabetic cardiovascular complicated patients and group3 consisted of 12 healthy subjects taken as control group. The following parameters were analyzed: fasting blood glucose; glucose metabolism factors [glycated hemoglobin (HbA_{1c}); insulin; intact proinsullin; proinsullin and C-peptide]; coagulation factor (PAI-1) and oxidative stress marker (ox-LDL). **Results:** Homeostasis model assessment – insulin resistance (HOMA-IR), fasting blood glucose, ox-LDL, insulin, PAI-1, intact proinsullin, proinsullin and C-peptide levels were significantly elevated in diabetic groups compared to control group values at $p \leq 0.001$. Moreover, these values were significantly increased in cardiovascular complicated group when related to diabetic uncomplicated group at $p \leq 0.001$. In addition HbA_{1c} was significantly higher in diabetic groups 1 and 2 compared to control group at $p \leq 0.001$. **Conclusion:** Increased blood glucose level, insulinemia, and elevated levels of ox-LDL and PAI-1 are associated with insulin resistance progression of cardiovascular complications.
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Key Words: Endothelial dysfunction, ox-LDL, type 2 diabetes mellitus, tissue plasminogen activator, plasminogen activator inhibitor-1, cardiovascular diseases.

1. Introduction:

Diabetes mellitus is the most common endocrine disorder that affects 246 million people worldwide. The International Diabetes Federation (IDF) predicts that the number of people with diabetes mellitus will increase up to 380 million within twenty years. Diabetes, mostly type 2 diabetes mellitus (T2DM), now affects 5.9% of the world's adult population with almost 80% of patients from developing countries ***(Sadikot and Mogensen, 2008 and WHO, 2011)***.

Coronary heart disease (CHD) and myocardial infarction (MI) have a significant impact on morbidity and mortality in developed countries ***(Ishihara et al., 2005).*** Thrombosis induced by atherosclerotic plaques rupture and subsequent distal thromboembolism may result in MI progression ***(Alessi and Juhan-Vague, 2006).*** Increased oxidative stress was considered to be a major mechanism involved in the pathogenesis of endothelial cell dysfunction ***(Aird, 2005)***.

Excessive lipid accumulation by macrophages plays a crucial role in the initiation and progression of atherosclerosis. The mononuclear phagocytes in endothelial intima will imbibe modified lipoprotein (ox-LDL) through scavenger receptors to form foam cells ***(Libby and Ridker, 2006)***. Lipid laden macrophage foam cell accumulates in atheromatous plaque and promote inflammation by secreting cytokines that recruit other immune cells to the arterial intima. Foam cells are generated by uncontrolled uptake of modified LDL, especially ox-LDL, and/or impaired cholesterol efflux ***(Kleemann et al., 2008).*** Lipid homeostasis in macrophages is regulated by scavenger receptors, including CD36 and scavenger receptor-A (SR-A), that mediate uptake and specific ATP-binding cassette (ABC) family transporters that mediate cholesterol efflux to apolipoprotein A1 (apoA1) and high density lipoprotein (HDL). Thus, alteration in expression of these molecules in macrophages may affect foam cell formation and progression of atherosclerosis ***(Van Eck et al., 2005)***.

Thrombus formation is regulated by the fibrinolytic system, which prevents luminal occlusion. Tissue plasminogen activator (tPA) is a highly specific serine proteinase that convert plasminogen zymogen to active serine protease plasmin. Plasmin digests fibrin into fibrin degradation products (FDP)

(Hou et al., 2009). Predominance of fibrinolysis inhibitors over its activators and plasminogen deficiency may be considered as risk factors for atherosclerosis and MI. Plasminogen activator inhibitor 1 (PAI-1) plays a significant role in cardiovascular diseases (CVD). Clinical studies have shown a positive correlation between increased levels of PAI-1 and atherothrombosis ***(Lowe et al., 2004)***.

Moreover, high plasma PAI-1 concentration is considered to be a predictor of myocardial infarction ***(Alessi and Juhan-Vague 2006)***. PAI-1 is involved in the insulin reception process as well as in fibrinolysis regulation. PAI-1 also modulates insulin signaling in fibroblasts, preventing the binding of vitronectin to avb3 receptors that, in turn, reduce insulin-induced phosphorylation of protein kinase B ***(Juhan-Vague et al., 2003 and Gonzalez et al., 2012)***.

Insulin resistance (IR) is considered a common feature of T2DM and is regarded as an important mechanism in the pathogenesis of this disease. Cardiovascular risk factors, including hyperglycemia, dyslipo-proteinemia, hypertension, obesity, thrombosis, and smoking are also associated with increased IR risk ***(Huxley et al., 2006 and Vertanen et al., 2013).*** Therefore, the aim of this study was to find the relation between IR, ox-LDL as a marker of inflammation, insullinmia, PAI-1 and cardiovascular complication of T2DM.

2. Research design and methods:

Subjects:

This study was performed at Al-Azhar University, Faculty of Pharmacy (boys) between October 2009 and January 2010, 70 male non smoker subjects with T2DM were enrolled in the study under supervision of cardiology stuff member, El-houssin hospital, Cairo, Egypt. T2DM was diagnosed according to the American Diabetes Association Criteria, of these patients, 35 had established cardiovascular disease (CVD) and 35 had no evidence of CVD. Clinical evidence of CVD included myocardial infarction or coronary artery by-pass surgery and peripheral arterial disease. Patients in group without CVD were T2DM patients who had no history of vascular disease and those with normal electrocardiogram (ECG) findings at exercise and normal peripheral artery Doppler ultrasonography findings. The clinical features of the patients are listed in table (1). All patients were receiving antidiabetic and antihypertensive therapies for at least the previous 6 months. Exclusion criteria were the presence of sustained type 1 DM, acute and chronic infections, malignancy, hepatic or renal disease, diabetic retinopathy and nephropathy, and other endocrine dysfunctions.

The control group consisted of 12 healthy male subjects with no history of T2DM, other endocrine dysfunctions, hyperlipidemia, hypertension, or coronary heart diseases. None of the subjects had received any medication (hormone replacement therapy, corticosteroids, vitamin supplements, antioxidant formulations and thiazolidinediones) which may have affected insulin resistance and/or endothelial function and none of these subjects were current smokers and consumers of alcohol. Blood pressure of all subjects was measured twice with a random zero mercury sphygmomanometer after 10 minutes of rest. All subjects were informed in regards to the aim of the study. The study was approved by the Ethics Committee of Faculty of Medicine, Al-Azhar University and by that of the National Organization for Teaching Hospitals and Institutes.

Table (1): Clinical characteristics of studied groups.

Item	Control	Uncomplicated T2DM	Complicated T2DM
Total No.	12	35	35
Gender	Male	Male	Male
Age (M ± SEM)	46.8 ± 1.29	44.8 ± 1.14	45.4 ± 0.89
Duration of DM (years) (M ± SEM)	-	9.00 ± 1.21	9.00 ± 1.24
Fasting blood glucose (mg/dl) (M ± SEM)	86.21 ± 2.81	180.9 ± 3.67	218.7 ± 7.12
SBP (mmHg) (M ± SEM)	122.50 ± 1.26	130.0 ± 1.19	130.0 ± 1.62
DBP (mmHg) (M ± SEM)	80.00 ± 1.43	80.00 ± 1.23	80.00 ± 1.33

Sample collection:

After an overnight fasting, 10 ml of venous blood were drawn from the patients and control subjects between 8.00 and 10.00 a.m. via the venipuncture of an antecubital vein. 2 ml. were taken in heparinized tubes for HbA_{1C} measurements. 1.8 ml. were added into 0.2 ml. citrated tubes and centrifuged at 4000 rpm for 15 minutes, plasma was separated for determination of PAI-1. The remaining blood sample was centrifuged at 4000 rpm for 15 minutes, serum

was collected, fasting blood glucose was measured immediately at the time of separation. Remaining serum specimens were stored at −20°C until analysis of serum ox-LDL-C, proinsullin, intact proinsullin, insulin, and C-peptide levels.

Biochemical Analysis:

Serum glucose was measured by an enzymatic colorimetric method according to commercial available kits of Spinreact, Spain Ref No. 1001191. HbA_{1C} level was measured by a cation-exchange HPLC using D-10 hemoglobin testing system with a gradient mobile phase and spectrophotometric detection.

Serum ox-LDL level was quantitatively determined by ELISA technique using commercial kit supplied by *Biomedica Medizinprodukte GmbH & Co*. Fasting serum proinsulin level was determined by direct ELISA technique using premade kits supplied by *Mercodia AB*, Sweden.

Serum level of intact proinsulin was determined using the TECO human intact proinsulin kit supplied by *TECO medical AG,* Switzerland.

Serum C-peptide was measured by enzyme immunoassay (EIA) using commercially available kits supplied by BioVision, USA.

PAI-1 was determined in plasma using ELISA kit supplied by *Diapharma* group, Inc. West Chester, OH 45069. Serum insulin was determined by direct ELISA technique using premade kits supplied by *Invitrogen* Ltd, UK.

Calculation:

In order to determine insulin resistance, HOMA-IR index (Homeostasis model assessment) was calculated as: HOMA = [(fasting blood glucose x fasting insulin /22.5] ***(Wallace et al., 2004)***.

Statistical Analysis

All data were expressed as mean ± standard error of mean ($\bar{\chi}$ ± SEM). Descriptive statistics were performed using Microsoft Excel 2007. All analysis and graphics were performed using **Graphpad prism (windows version 7; Graphpad software 2007)**.

Difference between means were assessed by one way analysis of variance (ANOVA) followed by tukey's procedure. Differences were considered statistically significant at $P < 0.05$.

3. Results:

The results of our study clarifies a significant increase in blood glucose level (mg/dl), serum insulin (µIU/ml), serum proinsulin (Pmol/L), serum intact proinsulin (Pmol/L), serum C-peptide (ng/ml) and HOMA-IR in diabetic group compared to control group at $p \leq 0.001$ with significant elevation in complicated group compared with diabetic uncomplicated group at $P \leq 0.001$; table 2 demonstrate these data. Regarding to blood HbA_{1c} the data in table (2) demonstrate a significant elevation in both diabetic groups when related to normal control group.

Table (2): Mean ± SEM measured biochemical parameters in different studied groups.

Biochemical Parameter	Control (M ± SEM)	Uncomplicated T2DM (M ± SEM)	Complicated T2DM (M ± SEM)
Fasting blood glucose (mg/dl)	86.21 ± 2.81	180.9 ± 3.67[a]	218.7 ± 7.12[a b]
HbA_{1c} (%)	4.96 ± 0.22	9.98 ± 0.29 [a]	10.57 ± 0.42 [a]
Insulin (µIU/ml)	6.34 ± 0.51	12.68 ± 0.52 [a]	19.39 ± 0.36 [a b]
HOMA-IR	1.34 ± 0.11	5.67 ± 0.28 [a]	10.51 ± 0.42 [a b]
Proinsullin (Pmol/L)	7.88 ± 0.38	20.05 ± 0.39 [a]	33.24 ± 0.51 [a b]
Intact proinsullin (Pmol/L)	6.02 ± 0.46	13.95 ± 0.56 [a]	20.51 ± 0.32 [a b]
C-peptide (ng/ml)	0.52 ± 0.03	0.84 ± 0.04 [a]	0.98 ± 0.03 [a b]

a: significantly different from control group.
b: significantly different from uncomplicated T2DM group

Concerning serum ox-LDL level, they were found to be significantly elevated at $P \leq 0.001$ in both diabetic uncomplicated T2DM (47.43 ± 0.53) and complicated T2DM (81.03 ± 0.75) when compared to control group (40.38 ± 0.57), also they were found to be significantly elevated at $P \leq 0.001$ in complicated T2DM compared to uncomplicated T2DM group figure (1) represent these data.

Regarding to serum PAI-1 level, they were found to be significantly elevated at $P \leq 0.001$ in both diabetic uncomplicated T2DM (2.67 ± 0.08) and complicated T2DM (4.62 ± 0.17) when compared to control group (1.29 ± 0.10). Moreover, they were found to be significantly elevated at $P \leq 0.001$ in complicated T2DM compared to uncomplicated T2DM group figure (2) illustrate these data.

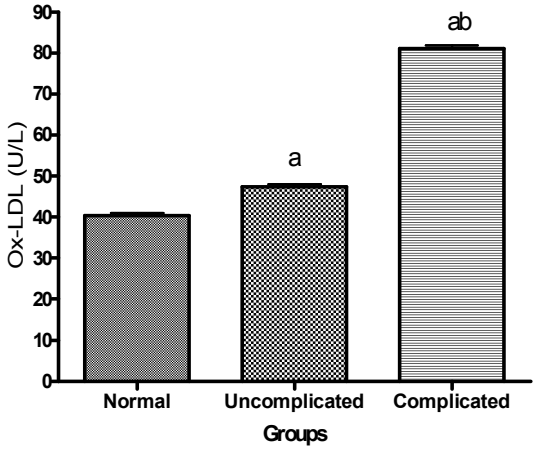

Figure (1): M ± SEM of serum ox-LDL (U/L) in different studied groups.
a: significantly different from control group.
b: significantly different from uncomplicated T2DM group

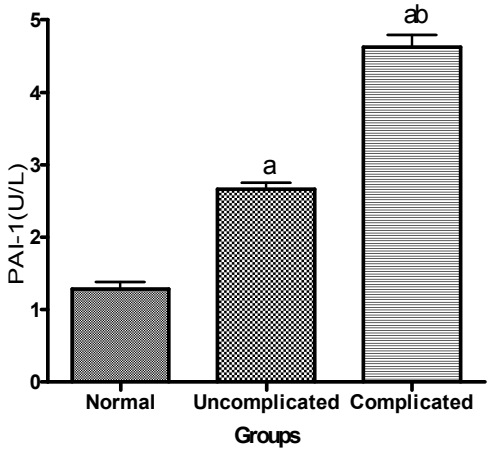

Figure (2): M ± SEM of plasma PAI-1 (U/L) in different studied groups.

4. Discussion:

Hyperinsulinemia was first suggested as a risk factor for atherosclerosis more than 30 years ago, based on the observation that insulin levels are higher in patients with ischemic heart disease ***(Stout and Vallance-Owen 1969 and Reddy et al., 2010)***. However, the role of hyperinsulinemia as an independent risk factor for atherosclerotic coronary disease has been controversial. Mostly, hyperinsulinemia occurs with insulin resistance in type 2 diabetes and is regarded as a compensatory mechanism of insulin resistance.

Macrophages perform a crucial role in the atherogenic process by generating lipid laden foam cells. Macrophages are known to express most insulin signaling molecules except insulin receptor substrate 1 (IRS1) and glucose transporter type 4 (GLUT-4) ***(Malide et al., 1998 and Haka et al., 2013)***.

In the present study, serum ox-LDL was measured as inflammatory marker in normal and diabetic subjects. This study demonstrates that uncomplicated and complicated T2DM subjects have elevated serum ox-LDL in comparison with control subjects. In addition it revealed significant elevation of serum ox-LDL in complicated group compared with uncomplicated group. This results may be due to that hyperglycemia are associated by increased inflammatory burden and increased lipid peroxidation, all leading to enhanced macrophage foam cell formation, low density lipoprotein (LDL) oxidation by macrophages was increased due to the activation of several pro-oxidant systems, as well as the depletion of antioxidants ***(Hsu et al., 2002 and Nakhjavani et al., 2009)***. Moreover, hyperglycemia may lead to intracellular changes in redox state resulting in depletion of cellular NADPH+H pool, leading to increased tendency for oxidative stress and high level of oxidized lipoprotein, especially LDL ***(Grover-Paez and Zavalza-Gomez 2009)***. In addition, the macrophages express scavenger receptors for modified lipoprotein (ox-LDL) permitting them to ingest lipids and become foam cells, the activated macrophage can generate reactive oxygen species that augment oxidant stress ***(Libby and Ridker 2006)***.

On the other hand, ***Hamed et al., 2010*** found that plasma ox-LDL levels in T2DM without coronary artery disease were not significantly different when compared with healthy control. Higher ox-LDL level in our study associated with cardiovascular complications of T2DM was in agreement with ***Yan et al., 2011*** who stated that, a receptor for ox-LDL, plays critical roles in multiple signal transduction pathways and is involved in the process of oxidative stress and inflammation that lead to the development and progression of diabetic vasculopathy which is the underlying mechanism of diabetic complications.

PAI-1, insulin, proinsullin, intact proinsullin, HOMA-IR and C-peptide were elevated in T2DM complicated and uncomplicated subjects with significantly higher in complicated than uncomplicated ones.

Enhancement PAI-1 expression was associated with high ox-LDL levels mediated through the generation of reactive oxygen species and phosphorylation of extracellular signal-regulated kinase. Furthermore, lysophosphatidylcholine, a major lipid component of ox-LDL, was responsible for the enhanced expression of PAI-1 as phospholipase A(2)-treated acetyl LDL, which generates lysophosphatidylcholine, strongly stimulated PAI-

1 expression, whereas acetyl LDL itself had no such activity. These data demonstrate that the uptake of ox-LDL and, in particular, its lipid component lysophosphatidylcholine into adipocytes triggers aberrant ROS-mediated PAI-1 expression, which may be involved in the pathogenesis of metabolic syndrome ***(Hamed et al., 2010 and David et al., 2013)***.

In current study, higher PAI-1 level were associated with greater level of ox-LDL, and HOMA-IR, was in agreement with ***Kraml et al., 2013 and Vertanen et al., 2013*** who's found positive correlation between markers of insulin resistance, ox-LDL and PAI-1 and suggest PAI-1 as an early marker of inflammation and atherosclerosis initiation. In conclusion, ox-LDL and PAI-1 were closely related with one another in cardiovascular complicated diabetic patients.

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