

Endothelial Dysfunction Induced by Type 2 Diabetes Mellitus and Fibrinolytic Activity

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Abstract: Problem statement: Type 2 diabetes mellitus is the world's largest systemic and metabolic disease. Endothelin (ET) was first described as a peptidergic endothelium-derived constricting factor. Endothelin-1 is one of the endothelial factors that is overexpressed in cases of endothelial dysfunction. The fibrinolytic system is present in plasma with the degradation of fibrin polymers in blood clots. It is a proteolytic mechanism that results in the formation of one main enzyme, plasmin, which cleaves fibrin. **Approach:** A total of 20 normal white albino rats weighing between 180-200 g were used and divided into two groups each of 10 rats, one group served as a control which fed on normal chow diet, while the second group fed on high fat diet for one month, then received streptozocin (25 mg kg^{-1}) intraperitoneally as a single dose and continued feeding on high fat diet. Plasma was separated to determine the levels of tissue Plasminogen Activator (t-PA), Plasminogen Activator Inhibitor-1 (PAI-1), Fibrin Degradation Products (FDPs) and Euglobulin Clot Lysis Time (ECLT). **Results:** Data showed that diabetic rats have inhibited fibrinolytic activity as detected from the significant increase in PAI-1, significant decrease in both t-PA and TDPs with significant prolongation of the ECLT relative to the control group. **Conclusion:** Endothelial dysfunction is associated with a prothrombotic state.

Key words: Type 2 diabetes mellitus, endothelin-1, endothelial dysfunction, fibrinolysis

INTRODUCTION

A balance between blood clot development and blood clot lysis is important for maintaining the integrity of the cardiovascular system and the continuous blood flow. Fibrinolysis serves as the completion step of homeostasis whereby unnecessary fibrin is removed from the vascular system. There are two basic steps that lead to fibrin degradation. The initial step is the activation of plasminogen to plasmin by several important protein/enzymes as tissue Plasminogen Activator (t-PA) and urokinase Plasminogen Activator (u-PA). In the second and final step, the active plasmin that is able to complex, with fibrin, specifically degrades the complexed fibrin into soluble fibrin degradation products (Hajjar, 2003).

Diabetes Mellitus (DM) is a chronic systemic metabolic disease that affects carbohydrates, fat and protein metabolism. It is complicated with increase deaths from coronary heart diseases and cerebro-vascular accidents. Type 2 DM is basically characterized by the presence of peripheral insulin resistance which may be caused by either receptor defect or post receptor defect with an insulin secretory defect. Commonly type 2 DM is caused by overfeeding, obesity with depressed physical activity.

Endothelial dysfunction is characterized by a change of the actions of the endothelium toward reduced vasodilatation and a proinflammatory state. It is associated with most forms of cardiovascular disease, such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes and chronic renal failure. Mechanisms that participate in the reduced vasodilatation in endothelial dysfunction include reduced nitric oxide generation, oxidative excess and reduced production of hyperpolarizing factor. Upregulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1 participate in the inflammatory response state (Verma and Anderson, 2002). Vasoactive peptides such as angiotensin II and endothelin-1; the accumulation of asymmetric dimethylarginine, an endogenous nitric oxide inhibitor; hypercholesterolemia; hyperhomocysteinemia; altered insulin signaling and hyperglycemia can contribute to these different mechanisms. In diabetes, the mechanism that may trigger endothelial dysfunction includes insulin resistance (as in type 2 diabetes). Decreased insulin sensitivity leads to induction of the two major pathways emerging from the insulin receptors; the first pathway is via phosphoinositide 3-kinase, phosphoinositide-dependent kinase-1 and Akt/protein kinase B leading to marked depression of eNOS, whereas the second

pathway is via mitogen activated protein kinases leading to mitogenic effects (Cusi *et al.*, 2000; Montagnani *et al.*, 2002; Osman *et al.*, 2000; Federici *et al.*, 2002). Moreover, hyperglycemia leads to Advanced Glycation End products (AGE), which were shown to quench NO and impair endothelial function (Zhang *et al.*, 2003; Makita *et al.*, 1991). Also, acute hyperglycemia attenuates endothelium-dependent vasodilatation in humans *in vivo* (Giugliano *et al.*, 1997; Williams *et al.*, 1998).

Endothelin was first described by Hickey *et al.* (1985) as a peptidergic endothelium-derived constricting factor. Three different endothelin peptides and are known, Endothelin-1,-2 and -3 (ET-1, ET-2 and ET-3, respectively). The most abundant endothelin peptide in circulation is endothelin-1. Under normal physiological conditions, ET-1 is not classified as a circulating hormone, but rather as an autocrine or paracrine factor at many sites of action in the body (Kedzierski and Yanagisawa, 2001). Elevated levels of ET-1 have been reported in numerous disease states including congestive heart failure, obesity and diabetes (Migdalis *et al.*, 2000; Masaoka *et al.*, 1989).

Hyperlipidemia in association with insulin resistance is common in patients with type 2 Diabetes Mellitus (DM) (Zavaroni *et al.*, 1985; Garg and Grundy, 1990). Insulin resistance and the ensuing hyperinsulinemia are associated with hypertriglyceridemia and low serum High-Density Lipoprotein (HDL) cholesterol concentrations.

The lipoprotein abnormalities are related to the severity of the insulin resistance. A study that measured insulin sensitivity using a euglycemic clamp in patients with and without type 2 DM found that greater insulin resistance was associated with larger Very Low Density Lipoprotein (VLDL) particle size, smaller Low Density Lipoprotein (LDL) particle size and smaller HDL particle size (Garvey *et al.*, 2003). Additionally, the number of VLDL, Intermediate Density Lipoprotein (IDL) and LDL particles increased with increasing insulin resistance. Hypertriglyceridemia results both from increased substrate availability (glucose and free fatty acids) and from decreased lipolysis of VLDL triglyceride.

The aim of the present study was to investigate the fibrinolytic activity changes in endothelial dysfunction induced by type 2 diabetes.

MATERIALS AND METHODS

Animals: Rats weighing 180-200 g were supplied from the animal house at College of Medicine at King Khalid University. The rats were divided into 10 rats/cage, the rats were housed in plastic cages at a temperature

regulated (22°C) and humidity (55%) controlled room with a 12 h light/12 h dark cycle. and were allowed free access to normal rat chow (9 % fat, 20% protein, 53% starch and 5% fiber) plus water for 1 week. All studies were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1996).

Experimental design: The animals were then divided randomly into two groups. Normal rats (n = 10) were fed normal rat chow and remained untreated for the duration of the study. Experimental rats (n = 10) were fed a diet enriched in fat (25% fat, 15% protein, 51% starch and 5% fiber) (Jian *et al.*, 1998). After 1 month on their respective diets, experimental rats were injected intraperitoneally with streptozotocin (25 mg kg⁻¹ body weight) and normal rats were injected with vehicle (0.05 mol L⁻¹ citric acid, pH 4.5). Both the low dose of streptozotocin and the high-fat diet are essential elements of the model designed to induce type 2 diabetes with insulin resistance. After 1 month, rats were fasted overnight and given 20% glucose (3 g kg⁻¹ body weight). Blood samples were taken from the tail vein to measure glucose and insulin levels at 2 and 12 h after glucose administration. Only rats with increased postprandial glucose (>200 mg dL⁻¹) were considered diabetic.

Collection of blood plasma: the animals were subjected to overnight fasting and then were anaesthetized by ether and fresh blood samples were immediately withdrawn from the heart between 8:00 and 9:00 am. Blood samples were collected directly into tubes containing 3.8% Trisodium Citrate (TSC) (1:9 TSC/blood, v/v), followed by centrifugation at 3000rpm for 15 min to obtain plasma which was frozen at -20°C for further biochemical analysis.

Biochemical analysis: t-PA antigen, PAI-1 antigen and TDPs were determined by Enzyme Immunoassay (EIA) (IMULYSE) (Koopman *et al.*, 1987; Koppert *et al.*, 1987; 1988). Euglobulin Clot Lysis Time (ECLT) was measured using the method of Lidbury *et al.* (1990). The concentration of ET-1 in the plasma samples was determined by radioimmunoassay by use of commercially available kits (endothelin 1-21 speci@c125 assay system, Amersham International Plc., Buckinghamshire, UK.). The plasma was also analyzed for glucose, insulin and lipid profile. The HOMA -IR was calculated (Turner *et al.*, 1993).

Statistical analysis: Data are expressed as the mean ± SD. Student's t-test was used to determine if the difference observed among groups was significant. Statistical significance was considered at p<0.05.

RESULTS

Figure 1-4 showed that, the type 2 diabetic rats had a significant decrease in both t-PA from 2.1626 ± 0.381 - 1.101 ± 0.185 ng mL⁻¹ and TDPs from 1.85 ± 0.088 - 1.129 ± 0.142 ng mL⁻¹, with significant increase in PAI-1 from 1.625 ± 0.184 - 5.0345 ± 0.748 ng mL⁻¹. Moreover the ECLT was prolonged in diabetic rats from 63.72 ± 5.169 - 75.71 ± 2.879 min.

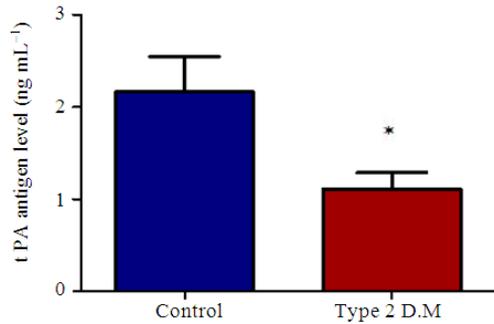


Fig. 1: t-PA antigen level in control and type 2 diabetic rats

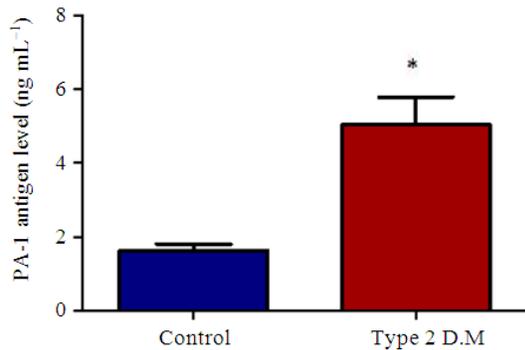


Fig. 2: PAI-1 antigen level in control and type 2 diabetic rats

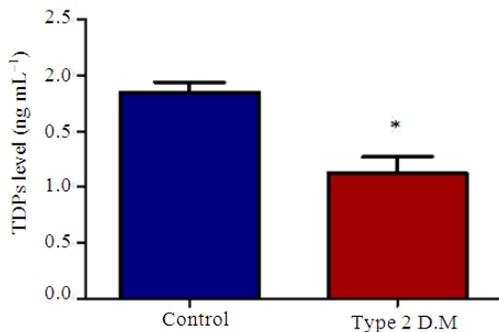


Fig. 3: TDPs level in control and type 2 diabetic rats

Figure 5 showed that the Plasma ET-1 level increased significantly in diabetic rats from 3.165 ± 0.422 - 8.315 ± 1.125 fmol 100 μ⁻¹.

Table 1 showed that the diabetic rats had a significant increase in fasting glucose from 73 ± 6.306 - 155.4 ± 13.68 mg dL⁻¹, fasting insulin level from 3.69 ± 0.356 to 10.637 ± 1.636 μU mL⁻¹ and postprandial glucose from 92.3 ± 7.103 to 282.8 ± 21.632 mg dL⁻¹ when compared to control group. Also HOMA-IR was significantly increased from 0.665 ± 0.086 to 4.101 ± 0.818 , indicating the presence of insulin resistance.

The lipid profile in Table 2 showed significant increase in cholesterol from 82.2 ± 7.983 to 105.8 ± 9.040 mg dL⁻¹, triglycerides from 84.4 ± 3.949 to 107.9 ± 6.505 mg dL⁻¹ and LDL-cholesterol 23.6 ± 3.893 to 40.3 ± 5.417 mg dL⁻¹ with significant decrease in HDL-cholesterol from 43.1 ± 3.60 to 27.7 ± 3.128 mg dL⁻¹ in the diabetic rats as compared to the control group.

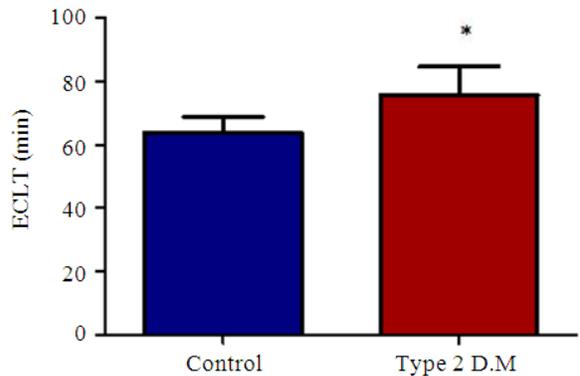


Fig. 4: ECLT in control and type 2 diabetic rats

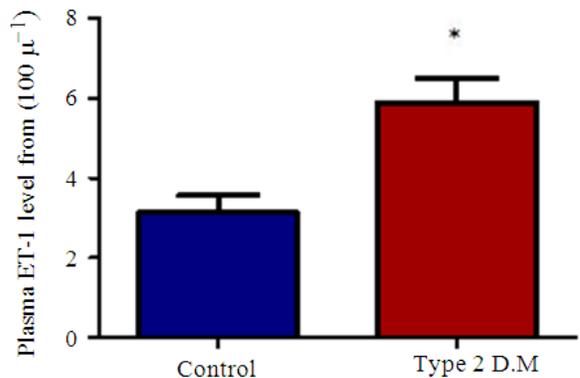


Fig. 5: ET-1 level in control and type 2 diabetic rats

Table 1: Fasting glucose, insulin, post-prandial glucose levels in the plasma and HOMA-IR calculations for the control and the experimental groups of rats

	Fasting GLU	PP glucose	Insulin	HOMA-IR
Control group	73.0±06.306	92.3±7.103	3.690±0.356	0.665±0.086
Type 2 diabetic rats	155.4±13.680*	282.8±21.632*	10.637±1.636*	4.101±0.818*

Values are given as mean ± SD for groups of ten rats each; Values are statistically significant at p* < 0.05 as compared to control group

Table 2: Cholesterol, triglycerides, LDL and HDL-cholesterol levels in the plasma of the control and the experimental groups of rats

	Cholesterol	Triglycerides	LDL	HDL
Control group	82.2±7.983	84.4±3.949	23.6±3.893	43.1±3.60
Type 2 diabetic rats	105.8±9.040*	107.9±6.505*	40.3±5.417*	27.7±3.128*

Values are given as mean ± SD for groups of ten rats each; Values are statistically significant at p* < 0.05 as compared to control group

DISCUSSION

The present study was designated to study the effect of endothelial dysfunction induced by type 2 DM on the fibrinolytic activity in rats. The endothelial dysfunction in type 2 DM is caused by different mechanisms as described previously mainly due to insulin resistance and the uncontrolled hyperglycemia. The endothelial dysfunction is defined as decrease in the endothelial release of the vasodilator substances, with increased synthesis and expression of the vasoconstrictor substances like ET-1 with increasing the pro-inflammatory substances than the anti-inflammatory substances. The biological markers of endothelial dysfunction include von-Willibrand factor, E-selectin and endothelin-1, so in the present study endothelin-1 was measured to prove the presence of endothelial dysfunction. In agreement to our data, ET-1 was significantly increased in the diabetic rats concluding the presence of endothelial dysfunction as compared with the control group (Fig. 5) which is caused possibly by the hyperglycemia, hyperinsulinemia and altered insulin signaling. In agreement to our data, plasma levels of ET-1 are elevated in diabetes mellitus (Kakizawa *et al.*, 2004). The possible factors which increase ET-1 expression include leptin, insulin and TNF- α . Levels of these factors are elevated in obesity and diabetes and their effects on ET-1 production have investigated by several laboratories. Interestingly, leptin increased ET-1 mRNA and protein release from the human umbilical vein endothelial cells in a dose and time dependent manner (Quehenberger *et al.*, 2002). In the presence of γ -interferon, TNF- α increased ET-1 expression and release from human vascular smooth muscle cells (Woods *et al.*, 1999). Glucose induced insulin secretion from pancreatic B-cells resulted in a moderate but significant rise in circulating ET-1 levels (Ferri *et al.*, 1996).

Moreover, elevated levels of ET-1 in obese and diabetic patients may contribute to endothelial dysfunction as well as impaired insulin signaling in

these patients. Studies suggest that ET-1 mediated vasoconstriction is increased in insulin resistant states such as obesity and diabetes. Ishibashi *et al.* (2001) demonstrated that chronic exposure to ET-1 led to decrease insulin and ET-1 stimulated glucose uptake.

From our data, the significant increase of ET-1 in the diabetic rats could contribute in inhibiting the fibrinolytic activity either by decreasing t-PA or increasing PAI-1. ET-1 seems to be an important modulator of smooth muscle cell function. It is a potent vasoconstrictor peptide released from endothelial cells which also regulates smooth muscle cell proliferation. Thus, it is thought to be implicated in several pathophysiological conditions of the cardiovascular system associated with endothelial cell damage and/or smooth muscle cell proliferation such as myocardial infarction and atherosclerosis. On the other hand, these pathological states have also been shown to be associated with elevated PAI-1 activity in blood and the expression of PAI-1 in atherosclerotic.

ET-1 and ET-3 seem to be implicated in the regulation of the fibrinolytic system of increasing the expression of PAI-1 and simultaneously decreasing the expression of t-PA in cultured smooth muscle cells via activation of endothelin-B receptors. Changes in the fibrinolytic system of smooth muscle cells correlate with proliferation and migration of these cells. ET-1 which then would act in a paracrine way to activate the underlying smooth muscle cells. Such activation of the smooth muscle cells would not only result in vasoconstriction and smooth muscle cell proliferation through activation of endothelin-A receptors, but might also contribute to an antifibrinolytic and antiproteolytic environment. An autocrine role for ET-1 in this latter process has been suggested because ET-1 has been shown to decrease basal and thrombin-stimulated t-PA release from cultured endothelial cells. One could also speculate on an autocrine/paracrine effect in which ET-1 in smooth muscle cells and endothelial cells, would contribute to the upregulation of PAI-1 expression in these cells (Kaji *et al.*, 1992).

Plasminogen is synthesized as a single chain molecule which is cleaved by t-PA into two chain disulfide linked plasmin. The t-PA is a serine protease which converts the proenzyme plasminogen to plasmin, a fibrinolytic enzyme. This enzyme plays a role in cell migration and tissue remodeling. Increased enzymatic activity causes hyperfibrinolysis, which manifests as excessive bleeding; while decreased activity leads to hypofibrinolysis which can result in thrombosis or embolism (Levin *et al.*, 1998).

PAI-1 is the principal inhibitor of tissue Plasminogen Activator (t-PA) and urokinase (u-PA), the activators of plasminogen and hence fibrinolysis (the physiological breakdown of blood clots). It is a serine protease inhibitor (serpin) which is secreted by different cell types including the endothelium, vascular smooth muscle cells, hepatocytes, platelets and the adipocyte. The main role of PAI-1 is probably to limit the fibrinolytic activity of a haemostatic plug. The increase in the plasma level of PAI-1 is associated with increased clearance of t-PA. Complexes between tissue-type Plasminogen Activator (t-PA) and its rapidly acting inhibitor Plasminogen Activator Inhibitor type 1 (PAI-1) are bound, internalized and degraded by HepG2 cells. The mechanism involves endocytosis mediated by a specific high-affinity receptor (Owensby *et al.*, 1989). PAI-1 is the major fibrinolytic determinant that strongly influences both basal and exercise-induced fibrinolytic activity (Hamsten *et al.*, 1985).

The data obtained from this study concluded that the fibrinolytic activity was inhibited in the diabetic rats as detected by the significant decrease in t-PA antigen level with the significant increase in the PAI-1 antigen level. Also, the ECLT was significantly prolonged in the diabetic group versus the control and the TDPs of fibrinogen and fibrin was significantly reduced.

Several possible mechanisms describe the prothrombotic state in the diabetics. The first mechanism of this prothrombotic condition is the endothelial dysfunction induced by the hyperglycemia and the insulin resistances seen in type 2 D.M. Patients with diabetes invariably show an impairment of endothelium-dependent vasodilation. This is partly due to the frequent association of the disease with other cardiovascular risk factors, including hypertension, obesity and dyslipidemia. Moreover diabetic as well as obese patients usually consume a high-calorie diet rich in macronutrients that per se is able to induce vascular abnormalities. Indeed, protein, lipid (Mohanty *et al.*, 2002) and glucose (Mohanty *et al.*, 2000) loads are associated with a marked production in ROS and high-fat meals are associated with an impaired endothelial-dependent vasodilation (Vogel *et al.*, 1997). A crucial

negative effect is particularly attributable to high levels of circulating free fatty acids, which are able to induce ROS production and impair endothelial function (Tripathy *et al.*, 2003). Mechanisms of endothelial damage in diabetes, independently from other cardiovascular risk factors, include insulin resistance, hyperglycemia and low-grade systemic inflammation (Calles-Escandon and Cipolla, 2001).

Endothelial dysfunction is characterized by increased the prothrombotic markers like PAI-1, von Willibrand factor and tissue factor with depression of the fibrinolytic parameters particularly t-PA. The inhibition in fibrinolysis in endothelial dysfunction, this was reported by several previous studies.

Other possible mechanisms of inhibition of the fibrinolytic activity in diabetic rats include insulin resistance present in the diabetic rats which leads to induction of fatty liver and by the accumulation of white adipose tissue specially, in the central region. This is in agreement with Bastard *et al.* (2000) and Jackson *et al.* (2005) who found that white adipose tissue is the main tissue source of elevated plasma PAI-1 in obesity, with a major participation from visceral fat.

Also, Esposito *et al.* (2002) and Lopez-Garcia *et al.* (2005) found that insulin resistance and endothelial dysfunction detected in obesity is associated with decrease t-PA secretion from the endothelial cells as a result from endothelial dysfunction.

TNF- α , a pro-inflammatory cytokine that is chronically elevated in adipose tissue in obese and insulin resistance states like type 2 D.M. and that is produced by adipose tissue, stimulates the production of PAI-1 by adipose tissue *in vitro*, *ex vivo* in human adipose tissue explants and *in vivo* in mice. Hence, adipose tissue itself may regulate PAI-1 expression by paracrine and autocrine effects. Moreover, TNF- α neutralizing antibodies blocked induction of PAI-1 production. TNF- α may also influence PAI-1 production by modulating the TGF- β pathway. The TNF- α and TGF- β pathways are interrelated, as deletion of TNF- α receptors resulted in a major decrease of TGF- β . The multifunctional cytokine TGF- β is capable of increasing plasma PAI-1 activity and PAI-1 expression and it up-regulates PAI-1 production specifically in adipose tissue. Furthermore, analogous to TNF α , TGF- β mRNA expression is increased in adipose tissue in obesity; thus, TGF- β may locally regulate PAI-1 expression. In human cultured isolated adipocytes substantial increases in PAI-1 production were found after stimulation with TGF- β (Loskutoff and Samad, 1998; Samad *et al.*, 1999).

Hypertiglyceridemia which is one of the most important features of insulin resistance detected in type 2 DM is associated with inhibition of the fibrinolytic activity due to increased secretion of PAI-1 (Oishi *et al.*, 2006). High levels of VLDL stimulate increased PAI-1 synthesis and secretion by cultured human endothelial cells. This transcriptional regulation of PAI-1 expression by VLDL has been shown to be genotype-specific. Also, Tabengwa *et al.* (2000) suggested that the repression of t-PA gene expression by VLDL may contribute to the impaired fibrinolysis.

Several mechanisms for PAI-1 overexpression in adipose tissue have been proposed. Evidence from mouse studies, using lean insulin sensitive and obese insulin resistant mice, indicated that hyperinsulinemia leads to increased PAI-1 plasma levels and increased PAI-1 gene expression in adipose tissue, which confirmed the role of adipose tissue in PAI-1 induction. It was shown that the PAI-1 gene remains sensitive to the action of insulin in the presence of insulin resistance, because several insulin signaling pathways remain functional in insulin resistance. Apparently, some consequences of obesity such as up-regulation of PAI-1 are not due to insulin resistance but rather caused by compensatory hyperinsulinemia. In human adipocytes and human adipose tissue explants, it was also demonstrated that insulin induces PAI-1 production in adipocytes. In human adipose tissue explants, however, insulin-stimulated PAI-1 production was restricted to adipocytes, indicating that mainly the adipocyte fraction of adipose tissue is sensitive to increased insulin levels. The increased PAI-1 synthesis by visceral fat compared to subcutaneous fat may be explained by the greater and more rapid response of visceral adipocytes to insulin than the response of subcutaneous adipocytes (Shoelson *et al.*, 2006).

Also, one of the possible mechanisms of hypofibrinolysis is the hyperglycemia and hyperinsulinemia detected in diabetic rats. These was also concluded by Pandolfi *et al.* (2001) who concluded that acute hyperglycemia and acute hyperinsulinemia can decrease plasma fibrinolytic potential and that this is due to increased plasma PAI-1 and decreased free t-PA activities.

CONCLUSION

From our data, we conclude that endothelial dysfunction caused by type 2 diabetes mellitus is associated with a prothrombotic state characterized by inhibition of the fibrinolytic activity that may increase the incidence of thrombo-embolic accidents.

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