

CORRELATION OF INCREASED FIBRINOGEN & OXIDATIVE STRESS IN PROGNOSIS OF DIABETIC FOOT ULCER

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ABSTRACT:BACKGROUND: Diabetic foot ulcer has poor prognosis and is a leading cause of amputation. Oxidative stress is associated with the pathogenesis of chronic wounds. Fibrinogen is a prognostic marker of peripheral vascular disease. **AIMS:** This prospective case-control study was designed to (i) evaluate and compare oxidative stress, protein carbonyl and fibrinogen levels, (ii) assess the correlation of the above parameters with prognosis in diabetic foot ulcer patients and healthy controls and (iii) whether fibrinogen levels can be used as prognostic markers. **MATERIAL AND METHODS:** The study included 100 diabetic patients (40 diabetic without complication, 40 diabetic foot ulcer grade 1 and 20 grade 2 patients) and 60 volunteer healthy controls. Oxidative stress was evaluated by estimating the amount of oxidant load of lipid peroxides by ferrous oxidation products in xylenol orange assay in conjunction with triphenylphosphine version 2 (FOX2 assay) and protein carbonyl. The antioxidant status level was estimated by ferric reducing ability of serum (FRAP assay). Fibrinogen and glycated hemoglobin was measured using commercial kits. SPSS version 17 was used for statistical analysis. **RESULTS:** Oxidative stress was higher in diabetic foot ulcer patients compared to non ulcer ($p < 0.05$) and controls ($p < 0.01$). Increased oxidative stress and plasma fibrinogen correlated with poor prognosis. **CONCLUSION:** Increased oxidative stress and plasma fibrinogen are associated with poor prognosis irrespective of glycemic control.

KEY WORDS: diabetic foot ulcer, fibrinogen, fox2, frap.

INTRODUCTION: Diabetes mellitus type 2 (T2DM) and its vascular complications are a major worldwide health problem. Access to better health care, use of oral hypoglycemic drugs, insulin devices has resulted in increased longevity of diabetic patients and increased prevalence of vascular complications of T2DM (1). The vascular complications of T2DM are either due to microangiopathy or macroangiopathy (2). The macroangiopathy in T2DM is a form of accelerated atherosclerosis affecting carotid, coronary and peripheral arteries. Thus, increasing the incidence and prevalence of diabetic foot ulcer (DFU) (2, 3).

DFU is one of the most common causes of nontraumatic lower extremity amputation and also the the most frequent reason for hospitalisation in T2DM patients (4, 5). In T2DM patients small injuries from shoes and trivial trauma are not perceived owing to neuropathy and these injuries subsequently result in nonhealing DFUs. The etiopathology of DFU includes various causes such as hyperglycemia, neuropathy, endothelial dysfunction and oxidative stress (6). The

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deviant metabolic state in T2DM involves chronic hyperglycemia, dyslipidemia and insulin resistance. This aberrant metabolic condition affects the function of endothelial cell, smooth muscle cells and cells associated with inflammation. The endothelial cells of the vascular compartment secrete various bioactive substances, which regulate vascular function and inflammation (6). In T2DM, the secretion of vascular relaxing factor is impaired and the existing hyperglycemia induces the production of reactive oxygen species (ROS). In T2DM insulin resistance causes excessive release of fatty acids from the adipose tissue, which results in concomitant inhibition of phosphatidylinositol-3 kinase (IP-3) pathway and activation of protein kinase C, thus increasing the production of ROS. Besides an increased production of ROS, the diabetic state induces the generation of many vasoactive substances and vasoconstrictors which lead to vascular smooth muscle hypertrophy. The hyperglycemia, dyslipidemia and increased ROS levels in T2DM enhances atheromatous plaque formation (6,7). During normal health ROS and antioxidant levels remain in balance. In T2DM this balance is disrupted resulting in oxidative stress, as there is an increase in oxidant load and a decrease in the antioxidant level of the serum (8). In addition to oxidative stress, T2DM also increases the coagulability of blood (10). T2DM patients have impaired fibrinolysis and an enhanced production of procoagulants. Thus, an increased oxidative stress, enhanced levels of procoagulants and impaired fibrinolysis nepotises initiation and persistence of atherosclerosis and thromboembolic episodes in T2DM patients (10). Many epidemiological studies have correlated the role of oxidative stress and plasma fibrinogen levels in cardiovascular diseases (CVD) (11, 12). Hence this study was designed to (1) assess the correlation between oxidative stress, plasma fibrinogen levels in DFU patients and (2) to compare the above parameters in T2DM patients with and without foot ulcer with controls and (3) to evaluate the role of these parameters in the prognosis of DFU, using the rate of limb amputation as the basis for assessment.

METHODS: The study was approved by the Institute Ethical committee. The study included 100 diabetic patients (40 diabetic without complication, 40 diabetic foot ulcer grade 1 and 20 grade 2 patients) and 60 volunteer healthy controls. The DFU patients were classified as per the University of Texas Diabetic Wound classification for foot ulcer (13). T2DM patients included in the study were selected on the basis of the following criteria: no episodes of diabetic ketoacidosis, no associated CVD, retinopathy, nephropathy; age > 30 years when diagnosed with DM; not on insulin therapy; not undergone lower limb vascular surgery or amputation. In order to avoid confounding factors like preexisting atherosclerosis or nephropathy contributing to an raised plasma fibrinogen level T2DM patients with clinical evidence of CVD and microalbuminuria were excluded. Patients on hormone replacement therapy were also excluded. Group 1 included 40 DFU patients with superficial ulcers not involving tendon, capsule or bone and Group 2 included 20 DFU patients whose ulcer involved the tendon or joint capsule. The biochemical parameters of the DFU patients were compared with 40 T2DM patients without any complications and 60 healthy volunteers. Of the T2DM patients 76 were on oral hypoglycemic drugs (59% on sulphonylurea monotherapy, 12% on metformin and the rest on a combination of sulphonylurea and metformin).

Fasting venous blood sample was collected for estimating biochemical parameters. Plasma fibrinogen was estimated by immunoturbimetric method using kits from Tulip diagnostics adapted to EM360 Erba Transasia Autoanalyser. Glycated hemoglobin was estimated in whole blood by ion exchange chromatography method using kits from Teco

diagnostics, Germany. Lipid profile parameters such as total cholesterol, triglycerides, HDL-Cholesterol were measured using kits from Erba diagnostics, Germany. LDL-Cholesterol was calculated using Freidewalds equation.

The oxidative stress was evaluated by estimating the amount of oxidant load of lipid peroxides was determined by ferrous oxidation products in xylenol orange assay in conjunction with triphenylphosphine version 2 (FOX2 assay) (14). The inter assay and intra assay coefficient of variation for FOX2 were 4.9% and 2.7% respectively. Antioxidant power of serum was measured by ferric reducing ability of serum (FRAP assay) (15). The inter assay and intra assay coefficient of variation for FRAP were 3.0% and 1.0%, respectively. Plasma protein carbonylation (PC) was evaluated by Levine method (its millimolar extinction coefficient is $22.01\text{mmol}^{-1}\text{cm}^{-1}$) (16).

Data are represented as mean \pm standard deviation (SD). The data was analyzed by one-way ANOVA followed by post Hoc Tukey's honestly significant differences test. Correlation coefficient was derived by Pearson's correlation analysis. A p value <0.05 was considered significant. Statistical analysis was done using SPSS version 17 software.

RESULTS: Table 1 depicts baseline clinical data of the diabetic patients. The mean age of the diabetic patients was 58.6 ± 6.37 years and that of the controls 59.8 ± 6.28 years. The mean duration of disease was 7.7 years. Table 2 shows the biochemical data of the study groups and controls. We observed a significant increase in plasma fibrinogen levels in DFU patients than diabetic patients without complications and controls. The DFU patients had increased levels of oxidative stress as observed by increased FOX2 levels, decreased total antioxidant levels, FRAP and increased protein carbonyl levels. DFU patients also exhibited a higher level of total cholesterol, triglycerides, LDL cholesterol and lower levels of HDL cholesterol. The plasma fibrinogen and levels of oxidative stress markers were significantly higher in diabetic patients without foot ulcer. The above parameters were observed to be increased in Grade 2 DFU patients as compared to Grade 1 DFU cases.

Table 3 shows the correlation between glycated hemoglobin levels, oxidative stress markers and plasma fibrinogen. All the diabetic patients plasma fibrinogen correlated significantly with glycated hemoglobin ($r=0.746$; $p<0.01$). A significant positive correlation was observed between oxidant load, protein carbonyl (PC) and plasma fibrinogen (plasma fibrinogen vs FOX2 $r=0.0778$; $p<0.01$, plasma fibrinogen vs PC $r=0.792$; $p<0.01$). we observed a significant negative correlation between plasma fibrinogen and total antioxidant status ($r=-0.702$; $p<0.01$). a significant positive correlation existed between glycated hemoglobin, oxidant load and PC (FOX2 $r=0.812$; $p<0.01$, PC $r=0.0836$; $p<0.01$). A negative correlation was seen between glycated hemoglobin and total antioxidant status ($r=-0.860$; $p<0.01$).

The receiver operating curve (ROC) was calculated to assess whether plasma fibrinogen levels can predict for amputation of foot. The ROC curve (figure 1&2) showed an area under curve 0.976 and $p<0.001$. The optimum cutoff value for plasma fibrinogen to predict for foot amputation was 300.4mg%, with 100% sensitivity and 99.2% specificity.

DISCUSSION: This study was designed with the objective to estimate the association of oxidative stress, plasma fibrinogen and prognosis of DFU. Oxidative stress is considered to instigate the development of insulin resistance, β cell dysfunction and impaired tolerance to glucose in T2DM patients (16). Oxidative stress is also associated with the long term complications of DM such as microvascular and macrovascular complications (17). Various in

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vitro studies have correlated an increase in oxidative stress in cells when exposed to a hyperglycemic environment (18, 19). Oxidative stress occurs as a consequence to an increased oxidant load and decreased antioxidant status (18, 19). Oxidative stress causes cell damage by forming lipid peroxides and protein carbonyl (19). We observed significantly high levels of oxidant load, FOX2 levels, and protein carbonyl in DFU patients than DM. The above parameters were increased in DM without complications as compared to controls. These findings are in concordance with previous studies (16-19). These findings imply a positive correlation of DFU with oxidative stress.

An increased plasma fibrinogen levels was observed in diabetic patients in our study, which is similar to the findings of previous studies (22-24). The higher level of plasma fibrinogen is attributed to increased synthesis and impaired clearance (23-25). We observed higher levels of plasma fibrinogen in DFU grade 2 case as compared to grade 1; higher levels of fibrinogen in DFU cases as compared to diabetics without complication and controls. The level of fibrinogen was increased in diabetics as compared to controls. This implies a positive association of increased plasma fibrinogen with DFU.

An association between oxidative stress and plasma fibrinogen has been observed in diabetics (26, 27). Fibrinogen synthesis is regulated by a feedback mechanism by thrombin activation (28-30). In diabetics thrombin formation is induced by free radicals (31, 32). Thus, oxidative stress is a link between increased fibrinogen levels in diabetics. We observed a positive correlation between glycated hemoglobin, oxidative stress and plasma fibrinogen levels. This is in agreement with previous studies (27-32).

Subsequent to follow up period of 10 months, we observed 18 grade 2 DFU patients underwent lower limb amputation. In these patients the plasma fibrinogen level was ≥ 300.4 mg%. This indicates that increased oxidative stress and high plasma fibrinogen levels are markers of poor prognosis in DFU patients.

The limitations of this research work are it is a prospective non randomized study and the data regarding the vascular status of the patients were not recorded. Nevertheless, the results are interesting and indicate further research in a controlled study to illustrate whether plasma fibrinogen levels and oxidative stress markers can be used as prognostic markers to prevent limb amputation in DFU. The associations with vascular parameters such as ankle brachial pressure indices, toe pressure and tissue oxygen tension should be assessed to provide a better prospect to both the patients and the clinical fraternity.

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Table 1 baseline clinical data of diabetic patients

Number	100
Age (years)	58.6 ±6.37
Sex (M/F)	93/7
Duration of Diabetes (years)	7.7 years
Body Mass Index (BMI) (Kg/m ²)	27.4 ±4.3
Systolic blood pressure (mm Hg)	124±14
Diastolic blood pressure (mm Hg)	76±10
Smoking (n)	12
Site of ulcer	
Forefoot	26
Mid-foot	4
Hind foot	30
Grade of ulcer	
Grade 1	40
Grade 2	20

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Table 2 comparison of biochemical parameters in controls, diabetic foot ulcer patients without foot ulcer and diabetic foot ulcer patients (DFU)

Biochemical parameters	Controls	Diabetic patients without foot ulcer	DFU Grade 1	DFU Grade 2
Glycated hemoglobin (%)	6.53±0.22	7.46±0.54 ^b	8.22±0.56 ^b	8.76±0.48 ^{b,d}
Total cholesterol (mg/dl)	141.26±5.51	189.36±5.92 ^c	190.78±4.98 ^b	244.42±21.6 ^{b,d}
Triglycerides (mg/dl)	61.22±5.55	100.40±6.08 ^c	106.4±8.0 ^b	116.92±8.92 ^{b,d}
HDL cholesterol (mg/dl)	56.76±7.77	48.12±6.22 ^c	50.02±6.66 ^b	43.44±4.54 ^{b,d}
LDL cholesterol (mg/dl)	100.4±6.06	122.36±5.12 ^b	116.58±5.56 ^b	131.68±4.4 ^{b,d}
Fibrinogen (mg/dl)	230.72±5.14	250.48±6.48 ^b	274.88±14.2 ^{b,e}	313.2±5.68 ^{b,d,e}
FOX2 (µmol/L)	4.33±1.7	8.44±2.2 ^b	10.96±4.4 ^{b,e}	18.0±4.9 ^{b,d,e}
FRAP (µmol/L)	423±15.23	388.2±10.22 ^b	112.66±8.8 ^{b,e}	99.87±7.48 ^{b,d,e}
Protein carbonylation (nmol/mg of protein)	0.74±0.008	1.86±0.62 ^b	2.36±0.14 ^{b,e}	2.81±0.20 ^{b,d,e}

HDL=high density lipoprotein; LDL= low density lipoprotein
All data is represented as mean ± Standard deviation.

b p<0.001 compared to controls

c p<0.05 compared to controls

d p<0.01 compared to controls

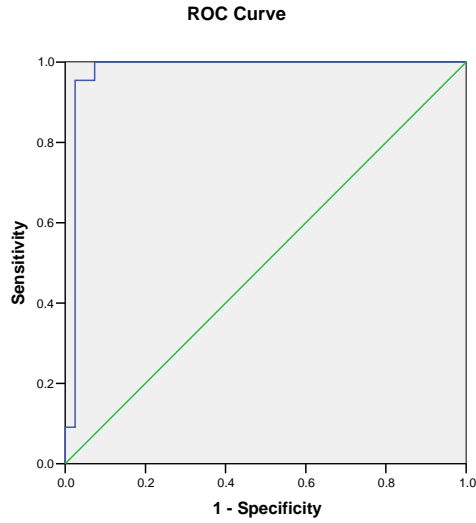
e p<0.005 when mean of DFU Grade 1 and DFU Grade 2 compared with diabetics without complication.

Table 3 Correlation of Plasma Fibrinogen with Glycated Hemoglobin and Oxidative Stress Markers in Diabetic foot Ulcer patients

	FOX2 r value	FRAP r value	PC r value	fibrinogen r value
Glycated hemoglobin	0.812*	-0.860*	0.836*	0.746*
Plasma fibrinogen	0.778*	-0.702*	0.792*	

r = pearson's correlation coefficient; * p<0.01

Figure-1: Receiver Operating Curve of plasma fibrinogen



¹ Area under the curve
 Test Result Variable(s): Fibrinogen

Area	Std. Error(a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.976	.022	.000	.932	1.019

a: Under the nonparametric assumption
 b: Null hypothesis: true area = 0.5

