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Original Article

Oxidative Stress Marker and Fibrinogen Level as Indicators of Severity of Diabetic Foot Ulcer

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Abstract

Diabetic foot ulcer (DFU) is the leading cause of lower extremity amputation and is very common in clinical practice. Oxidative stress is important in the pathogenesis of chronic wound and the lipid per oxidation product malondialdehyde (MDA) is toxic molecule which is also associated with pathogenesis of chronic complications of diabetes mellitus. Fibrinogen is a recognized marker in peripheral arterial disease (PAD) and increasing level predict an increased risk of amputation. The aim of this study was to investigate whether the plasma MDA and fibrinogen are associated with severity of DFU. In this study, the plasma MDA and fibrinogen levels were determined in 23 normal subjects, 25 diabetes without ulcer patients, 24 mild DFU patients and 25 severe DFU patients. The results showed that mean plasma MDA levels of normal subjects, diabetes without foot ulcer patients, mild DFU patients and severe DFU patients were0.98± 0.12 µmol/L, 1.3±0.21 µmol/L, 1.61±0.22 µmol/L and 2.3±0.35 µmol/L respectively. Mean plasma fibrinogen levels of normal subjects, diabetes without foot ulcer patients, mild DFU patients and severe DFU patients were 307± 61.5 mg/dl,429±63.8 mg/dl, 513.6±77.8 mg/dl and 643.5±71.3 mg/dl respectively. We found out that mean plasma MDA level of severe DFU patients was significantly higher than that of other groups ($p<0.001$). Similarly, mean plasma fibrinogen level of severe DFU patients was significantly higher than that of normal subjects, diabetic without ulcer patients and mild DFU patients ($p<0.001$). Therefore, we concluded that the higher level of plasma MDA and fibrinogen are significantly associated with severity of DFU.

Introduction

Diabetes is worldwide in distribution and it is extremely common in clinical practice, with a prevalence of 8-10% in the population. There is an epidemic explosion of type 2 diabetes mellitus worldwide and it is estimated to increase from 135 to 300 million over 30 years from 1995 to 2025. In Myanmar, WHO has estimated that the total number of diabetics will rise to 1.5 million during that 30 years period[1]

Diabetic Foot Ulcer (DFU) is defined as "Infection, ulceration and/or destruction of deep tissues associated with neurological abnormalities and various degrees of peripheral vascular disease in the lower limb".[2]DFU are sores on the feet that occur in 15 % of diabetic patients some time during their life time. The risk of lower extremity amputation is increased eight folds in these patients once ulcer develops.[3]Increasing evidence suggests a causal link between hyperglycemia and oxidative stress leading to cellular damage and various diabetes associated complications including DFU.[4]

Malondialdehyde (MDA)

Malondialdehyde (MDA), a lipid per oxidation product, is now considered as toxic molecule and it is important in pathogenesis of chronic wound. Many researchers reported that plasma MDA levels are markedly increased in DM and it is one of the causes of complication in diabetic patients. The reactions of MDA with the side-chains of

protein-bound lysine and arginine of collagen molecules possibly not only affect the functions of collagen by making it less susceptible to degradative enzymes but could result in the formation of adducts that change the charge profile of the collagen molecule and affect collagen-cell interactions. This interfere in the normal process of wound healing.[5]

MDA and atherosclerosis

Plasma MDA concentration is increased in diabetes mellitus and it is found in the atherosclerotic plaque deposits promoted by diabetes. When MDA modifies the protein (apolipoprotein B100) of circulating LDL, it no longer reacts with the normal LDL receptor in hepatic and peripheral cells, but it can react only with scavenger receptors of macrophages. The macrophages ingest these MDA modified LDL and it leads to foam cell formation. This may lead to diabetic macroangiopathy in coronary and peripheral vessels.[6]Several population based-studies showing the prevalence of peripheral vascular disease in diabetics to be 10-40%, as compared with 2.5 – 10 % in non-diabetics.[7]Epidemiological studies in the general population indicate an association between fibrinogen levels and the subsequent development of all the major atherosclerotic events, includingPAD.[8]

Fibrinogen

Plasma fibrinogen is an important component of the coagulation cascade as well as a major determinant of blood viscosity and blood flow. Fibrinogen is a recognized risk factor for macrovascular disease and increased levels may exert effects through a variety of mechanisms including increased blood viscosity, increased size of fibrin clots, increased tissue deposition and stimulation of atherosclerosis. Prolonged poor metabolic control leads to chronic fibrinogen hypersecretion.[9]Fibrinogen is also a cofactor in platelet activation and may directly contribute to plaque formation .[10] In addition, leucocyte-endothelial cell interaction may be important in atherogenesis. Inter cellular adhesion molecule –1 (ICAM-1) is involved in this mechanism. Fibrinogens binding to ICAM-1 on endothelial cells also mediate platelet adhesion.11 these are all mechanisms contribute angiopathy and neuropathy in patient with DM. In Myanmar, Kyaw Kyaw Swe carried out a clinical study [12]of diabetic foot in orthopedic practice at NOGH. The 64% of DFU patients ended with amputation and 36% was managed with wound debridement and daily dressing. These results indicated that although prompt and appropriate antidiabetic and antibiotic treatment, most of the DFU patient undergone amputation. Thus, it can be considered that there might be some intrinsic factors which interferes normal process of wound healing. The aim of this project was to investigate whether plasma MDA and fibrinogen levels can be used as indicators to assess the severity of DFU.

Materials and Methods

The cross-sectional comparative study was carried out and total 97 subjects were selected for this study. The DFU patients selected in this study were from TSH (Thingangyun Sanpya Hospital), NOGH (North Okkalapa General Hospital) and YGH (Yangon General Hospital) all situated in Yangon, Myanmar. The subjects were divided into four groups.

Normal (non-diabetes) subjects (group-1): Twenty-three apparently healthy volunteers (males and females) from Tamwe and South Dagon Townships (age and sex match to cases) were selected as controls.

Diabetic without foot ulcer patients (group-2): Twenty-five diabetic without foot ulcer patients from Tamwe Township, South Dagon Township and OPD of TSH were selected as diabetic controls.

Case (mild and severe DFU) patients (group-3 and group-4): Twenty-four mild DFU patients and Twenty-five severe DFU patients from Orthopedic and Surgical wards of YGH, TSH and NOGH diagnosed by Orthopedic surgeons were selected as cases.

The determination of MDA, fibrinogen and HbA1c were done in Research Lab of Biochemistry Department, University of Medicine (1) and SML lab, advanced medical laboratory Yangon, Myanmar respectively. Mild and severe DFU were classified based on Wagner classification

of DFU.

Grading of diabetic foot was done according to Wagner classification (1987)[13]

Grade 0 – skin intact but "foot at risk", callosities, corn.
Grade 1 – localized superficial ulcer of the skin or subcutaneous tissue
Grade 2 – deep ulcer to tendon, bone, ligament or joint
Grade 3 – deep abscess, osteomyelitis
Grade 4 – gangrene of toes or forefoot
Grade 5 – gangrene of entire foot

Working definition

Mild DFU (group-3) – grade 0, grade 1 and grade 2
Severe DFU (group-4) – grade 3, grade 4 and grade 5

Before sample collection, subjects were explained about the experiments and their written consents were obtained.

Collection of blood sample

About 7 ml of venous blood was taken from all subjects. Then, for fraction A, 3ml of blood was placed into the EDTA vacutainer tube for MDA assay, for fraction B, 1.8 ml of blood was placed into the tri- sodium-citrate containing test tube for fibrinogen assay and for fraction C, the remaining 2 ml of blood was placed into the another EDTA test tube for HbA1c assay. Blood sample for RBG determination was done from finger prick.

Methods

Plasma malondialdehyde level was determined by thiobarbituric assay, spectro photometric method.[14] Plasma fibrinogen level was determined by automated coagulation analyzer by thrombin clotting time method.[15,16] HbA1c level was determined by immune turbid metric method. Determination of random blood glucose (RBG) level by glucose oxidase method (glucometer-Glucocard II GT-1620).

Data processing and statistical analysis

Data analysis was done by using the Statistical Package for Social Sciences (SPSS) software version 16. Standard statistical methods were applied for the calculation of mean, standard deviation and standard error. One Way ANOVA test was done for comparison of MDA, fibrinogen and HbA1c between all 4 groups. Multinomial regression analysis was done to find out significant parameter among MDA, fibrinogen and HbA_1c. Correlations of parameters were tested using Pearson's Correlation Statistics. Significant level was decided if P value of all tests were less than 0.05 and confidence interval was determined to be 95%.

Results

Group-1(normal subjects) were found slightly younger age than other groups but the difference was not statistically significant (p=0.20). Random blood glucose determination was done to detect whether the subjects are diabetic or non-diabetic patients. Table 1 shows baseline data of participants in this study

Plasma MDA level

Mean plasma MDA level in different groups were shown in figure 1. Mean plasma MDA level of normal subjects (group-1) was 0.98 ± 0.12 µmol/L, that of diabetes without foot ulcer patients (group-2) was 1.3 ± 0.21 µmol/L, that of mild DFU patients (group-3) was 1.61 ± 0.22 µmol/L and that of severe DFU patients (group-4) was 2.3 ± 0.35 µmol/L. Mean plasma MDA level of group (4) patients was significantly higher than that of other groups, significant level was ($p<0.001$) each.

Plasma fibrinogen level

In figure 2, mean plasma fibrinogen level of normal subjects was 307 ± 61.5 mg/dl, that of diabetes without foot ulcer patients was 429 ± 63.8 mg/dl, that of mild DFU patients was 513.6 ± 77.8 mg/dl and that of severe DFU patients was 643.5 ± 71.3mg/dl.Mean plasma fibrinogen level of group (4) patients was significantly higher than that of other groups, significant level was ($p<0.001$) each.

Hemoglobin A 1 c Level

In figure 3, mean blood HbA_1c level of normal subjects was 4.57 ± 0.19 % that of diabetic without foot ulcer patients was 7.10 ± 0.97 %, that of mild DFU patients was 8.47 ± 1.89 % and that of severe DFU patients was 10.69 ± 2.9 %.Mean HbA_1c level of group (4) patients was significantly higher than that of other groups, significant level was ($p<0.001$)

each. Mean HbA_1c level of group (3) patients was also significantly higher than that of group (2) and group (1) and significant level was ($p<0.001$) each. In addition, the comparison between mean HbA_1c level of group (2) and group (1) subjects, group (2) have significantly higher HbA_1c level than that of group (1) ($p<0.001$).

Table 2 indicates that there was no correlation between plasma MDA and fibrinogen level in all four groups. But there was positive correlation between MDA and HbA_1c in diabetic without ulcer patients ($r=0.45$, $p<0.05$) and severe DFU patients ($r=0.624$, $p<0.001$).

Table 3 indicated that there was no correlation between fibrinogen and other parameters (MDA and HbA_1c) in all four groups. Multinomial Regression analysis was conducted to be able to identify significant predictor variables among MDA, fibrinogen and HbA_1c for severity of foot ulcer. Table 4 indicated multinomial regression analysis of three parameters between the 2 groups- diabetics without ulcer and mild DFU patients. Table 5 showed multinomial regression analysis of three parameters between the 2 groups- diabetics without ulcer and severe DFU patients. Multinomial regression showed plasma fibrinogen concentration only could increase the risk of having mild DFU comparing to diabetics without foot ulcer ($P<0.05$).Plasma MDA and fibrinogen levels could increase risk of having severe DFU comparing to diabetics without ulcer ($P<0.01$).

Table1 : Baseline data of participants in this study.
(Data are expressed as Mean +/- SD)

Groups	Group – 1 Normal person		Group – 2 Without Ulcers		Group – 3 Mild DFU		Group – 4 Severe DFU	
Age(years)	52.91±3.17		55.96±5.42		54.88±6.77		55.28±6.99	
Sex	M	F	M	F	M	F	M	F
	9	14	6	19	6	18	8	17
RBS (mg%)	96.3±18		180±47		257±63		334±83	

Table 2 : showing correlation between plasma MDA with fibrinogen and HbA_1c in all four groups.

Group	Group-1		Group-2		Group-3		Group-4	
parameter	r	p	r	p	r	p	r	p
Fibrinogen	0.021	0.92	0.11	0.602	0.157	0.464	0.109	0.603
HbA_1c	0.363	0.074	0.45	0.024	0.072	0.739	0.624	0.001

Table 3 : showing correlation between plasma fibrinogen with MDA and HbA_1c in all four groups.

Group	Group-1		Group-2		Group-3		Group-4	
parameter	r	p	r	p	r	p	r	p
MDA	0.021	0.92	0.11	0.602	0.157	0.464	0.109	0.603
HbA_1c	0.037	0.86	0.387	0.056	0.081	0.707	0.280	0.175

Table 4 : showing multinomial regression analysis of three parameters between the 2 groups- diabetics without ulcer and mild DFU patients.

Parameters	Odds ratio (95% Confidence Interval)	P value
MDA	163.95(0.998-1.043)	0.092
Fibrinogen	1.025(1.003-1.047)	0.024
HbA_1c	1.661(0.517-5.333)	0.394

Table 5 : Multinomial regression analysis of three parameters between the 2 groups - diabetics without ulcer and severe DFU patients.

Parameters	Odds ratio (95% Confidence Interval)	P value
MDA	1.66 (1.005-1.066)	0.005
Fibrinogen	1.042 (1.015-1.070)	0.002
HbA_1c	1.755 (0.482-6.394)	0.394

Discussion

In the present study, the lipid per oxidation product, plasma MDA level of DFU patients was significantly higher than diabetics without ulcer and non-diabetics controls. Furthermore, the higher plasma MDA level was also found in DFU patients than diabetic without foot ulcer patients. Many investigators have found that the lipid per oxidation product, plasma MDA, was higher in diabetic patients than normal subjects.[17-19]This finding indicates that DFU patients had higher oxidative stress and increased lipid per oxidation than diabetic controls. This increased in lipid per oxidation in DFU patients is due to excessive formation of free radicals and increased glycation of proteins in these patients. The glycated protein might themselves act as a source of free radicals. The highest plasma MDA level in severe DFU patients may be due to the maximal production of free radicals in these patients. Free radicals interact in arachidonic acid metabolism. The lipid peroxide formed stimulates the cyclo oxygenase and prostaglandin and thromboxane synthesis. This will cause increased platelets aggregation, leading to vascular complications and tissue damage in severe DFU patients. All the above finding

Figure 1 : Plasma MDA level in all four groups.

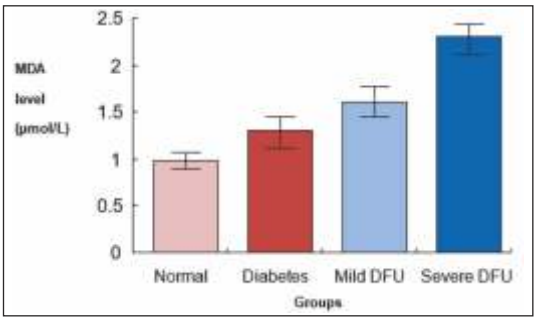


Figure 2 : Mean plasma fibrinogen level of all four groups

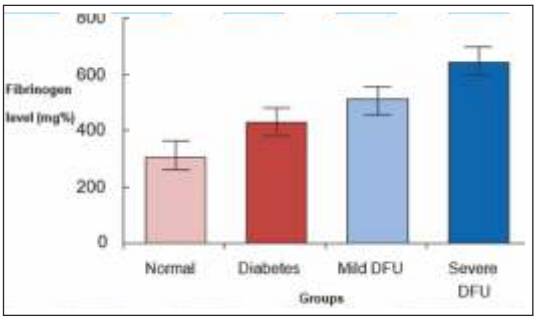
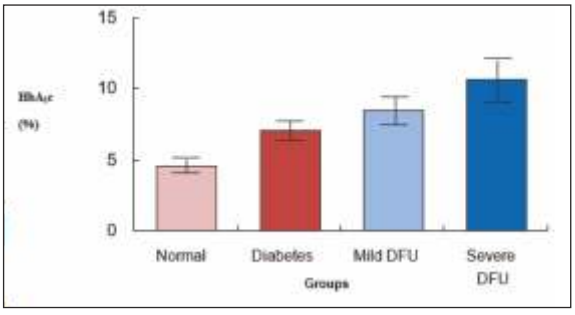


Figure 3 : Hemoglobin A_1c (HbA_1c) levels in all four groups.



reflects a positive association between DFU with oxidative stress, lipid per oxidation and MDA formation.

In the present study, the highest level of plasma fibrinogen in severe DFU patients was found in comparison to mild DFU patients, diabetic and non-diabetic controls. It may be due to poorly controlled of blood glucose in these patients. The higher the blood glucose, the greater the production of free radical and then it may lead to increased endothelial injury and then to fibrinogen production. In addition, plasma fibrinogen is an acute phase protein and is increased with inflammation and tissue necrosis. The inflammatory response may promote the production of cytokines which have major role in the regulation of the synthesis in the liver of acute phase proteins including fibrinogen. DFU patients have infection and inflammatory reaction and therefore, it may be one of the causes of increased plasma fibrinogen than diabetic without ulcer patients. Severe DFU patients have more extensive inflammatory reaction and tissue necrosis than mild DFU patients. Therefore, fibrinogen production may increase more in severe DFU patients. The findings are consistent with the studies carried out by various researchers.[19,20]

Furthermore, severe DFU patients had the highest glycated hemoglobin level than other groups. This indicated that DFU patients had poor glycemic control than other groups and so hyperglycemia induced free radical production in DFU patients was highest in these patients. In the present study, significant positive correlation between plasma MDA and HbA_1c was a found in diabetic patients and severe DFU patients. It reflects the higher the HbA_1c level, the greater the production of ROS and then to MDA formation. This indicates that MDA is markedly increased in uncontrolled diabetic patients. Many other researchers reported similar results. [19, 21]Multinomial regression analysis showed that plasma MDA and fibrinogen increase the risk of severity of diabetic foot.

Conclusion

The results of this study indicated that a high plasma fibrinogen level and increased lipid peroxidation are associated with the severity of DFU. It can be concluded that the higher level of plasma MDA and fibrinogen are associated with severity of DFU. Thus, better glycemic control to reduce hyperglycemia induced oxidative stress and reduction in fibrinogen from the plasma of DFU patients may be helpful in the management of DFU.

Recommendation

We recommend that future research should involve investigation of the relationship with fibrinogen and commonly expressed vascular parameters such as ankle brachial pressure indices and tissue oxygen tension in DFU patients. Meanwhile, plasma fibrinogen levels could potentially be considered for screening programs to identify people at high risk of vascular events.

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