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DETERMINATION OF ADVANCED OXIDATIVE PROTEIN PRODUCTS LEVELS AND ITS CORRELATION WITH INFLAMMATION IN DIABETIC FOOT PATIENTS

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ABSTRACT : Advanced oxidation protein products (AOPPs) are the marker of protein oxidation through oxidative stress, which represents the overall status of the protein in the cell/tissue. Due to their increased levels of AOPPs were reported during T2DM. The aim of this study was to assess AOPP level in T2DM subjects with foot ulcer (DFU) and explore its correlation with infection. Type 2 diabetic patients (n=108) and healthy subjects (n=25) were enrolled in this study. The T2DM group was subdivided to diabetic patients without complications (n=25) and eighty-three (83) of them have diabetic foot. They were sub- grouped into two groups according to presence Osteomyelitis and abscess, and in reliance on medical analysis of WBC count and CRP. Group of diabetic without superficial or deep ulcer and no osteomyelitis or abscess G(0,1,2) (n =45), which consist patients with grades (0,1,2), and group of diabetic with deep ulcer abscess and osteomyelitis G(3,4,5)(n = 38) that consist patients with grade (3,4,5). Twenty- five (25) non-diabetic apparently healthy subjects serve as control (group C) were enrolled.The Biochemical analysis was done for all participants such as serum glucose, hemoglobin A1C, insulin, lipid profile, WBC, and C-reactive protein (CRP). Serum AOPP concentration was determined using an enzyme-linked immunosorbent assay kit. The oxidative status was assessed by measuring catalase activity, glutathione (GSH), malondialdehyde (MDA) and total thiol levels. AOPP, MDA, WBC, and CRP, were significantly higher in G (0,1,2) and G (3,4,5) groups in comparison to control group, while GSH and total thiol levels were significantly lower as compared with control group. The outcomes of this study reveal the incidence of oxidative stress among DFU patients with higher Wagner grades and the presence of relation between inflammation state in DFU subjects and oxidative stress.

Key words : Advanced oxidation protein products, oxidative stress status, diabetic foot ulcer, inflammation.

INTRODUCTION

The scavenging abilities of antioxidant defenses due to the production of free radicals results in macro- and microvascular dysfunction called oxidative stress. The high level of oxidative stress in subjects with T2DM is a result of a number of abnormalities, including insulin, hyperglycemia, dyslipidemia and hyperinsulinemia resistance (AL-Shammaree, 2017). Oxidation of glucose result to hyperglycemia that leads to create free radicals (Tangvarasittichai *et al*, 2012; Vijay *et al*, 2014).

The diabetic foot ulcer (DFU) is one of microvascular complications of T2DM. It includes a number of pathologies, mostly peripheral neuropathy and diabetic peripheral arterial disease. When wound infection or osteomyelitis is involved may lead finally to amputation (Amin *et al*, 2016). Hyperglycemia lead to inflammatory response in DFU and to assess this response; inflammatory markers were used such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), WBC count and procalcitonin levels (Al-Shammaree *et al*, 2017).

It is well known that Reactive Oxygen Species (ROS) lead to lipid peroxidation and proteins, carbonylation, nitrotyrosine formation, and DNA damage (Marjani *et al*, 2010). The MDA level is one of lipid oxidation markers that assessed oxidative stress in patients. Previous studies confirmed positive correlation between diabetic complications and lipid peroxide levels. MDA was found higher in T2DM than T1DM subjects, and higher serum MDA level was observed in T2DM subjects that have complications (Davì *et al*, 2005; Genet *et al*, 2013). The Advanced oxidation protein products (AOPPs) are produced from protein oxidation by ROS (Pandey *et al*, 2010). AOPPs are produced by reactions among chlorinated oxidants and serum proteins, in chronic oxidative stress. Previous studies found significant positive association between plasma levels of AOPPs and Thiobarbituric acid reactive substances (TBARS) in

T2DM (Savini *et al*, 2013). Total thiols are one of the main antioxidant markers and used to destroy reactive oxygen species by non-enzymatic and enzymatic mechanisms. Thiol groups of proteins are mainly responsible for their antioxidant response and they act as sensitive marker of oxidative stress (Baskol *et al*, 2014). The aim of study was to evaluate the oxidative status in patients with diabetic foot ulcer with and without inflammation in order to explore the relationship between them.

MATERIALS AND METHODS

Study population

This study population included T2DM subjects (n=108) and healthy subjects (n=25) serve as control (group C). The patients were selected from the National Diabetes Center, Al-Mustansiyriah University, Al-Yarmuk teaching hospital and Baghdad Teaching Hospital in Baghdad city. The patients group was subdivided to diabetic patients without complications (T2DM group; n=25) and with diabetic foot ulcer (n=83). The DFU group was divided into two groups by the physician according to Wagner classification and acuteness of inflammation in reliance on physical examination and medical analysis of WBC count and CRP. Group with inflammation (G(0,1,2)) that consist patients with Wagner grades (0,1,2) (n=45) and group with acute inflammation (G(3,4,5)) that consist patients with Wagner grade (3,4,5) (n=38).The Wagner classification system assesse ulcer depth and the presence of gangrene using the following grades: grade 0 [post-ulcerative lesion], grade 1 [partial/ full thickness ulcer], grade 2 [probing to tendon or capsule], grade 3 [deep with osteomyelitis], grade 4 [partial foot gangrene] and grade 5 [whole foot gangrene] (PS *et al*, 2010). Demographic data for all participants were collected in the form of height, weight, waist, hip, gender and age, Body mass index (BMI), Waist to hip ratio (WHR) and waist to height ratio (WHtR).

Samples, methods and statistical analysis

Venous blood was taken from subjects, approximately (7-10) milliliters using 10 ml dispensable syringe. Samples were collected after fasting for 8-12 hours. The sample was separated into 2 aliquots; first aliquot was five milliliters of blood were transferred into EDTA tube for measuring HbA1c (using a kit from Stanbio, USA) and CBC (using a kit from Siemens, Germany). The remaining aliquot of blood was transferred into a gel tube, left at room temperature for clotting, and centrifuged at 3000 rpm for (8-10) min in order to collect serum. The serum was used for measuring glucose, lipid profile, MDA, Thiol group and GSH, and AOPP concentration. The serum was stored in Eppendroff tubes at (–40°C) until used. The measurement of fasting serum glucose (FSG) using kit (Spinreact, Spain).C-Reactive Protein (CRP) using kit from (Genrui, china). Lipid profile (triglyceride, high-density-lipoprotein-cholesterol (HDL-C) and total cholesterol) were measured using kits from (Spinreact, Spain). While, Friedewald's equation was using to calculated Very Low-Density Lipoprotein (VLDL) and Low-density lipoprotein-cholesterol (LDL-C) (Ri *et al,* 1972; Hamad and Ali, 2019). The HbA1c level was determined by the colorimetric method (Trivelli *et al,* 1971). Serum insulin level was determined by means of direct sandwich ELISA kit from (Monobind Inc. Company, U.S.A) (Andersen *et al,* 1993).

Serum AOPP concentration was measured using ELISA kit from (MYbiosource, USA), with sensitivity 1.0µmol/L, and range (6.25–200)µmol/L. Level of GSH was estimated by colorimetric method using kit from (MYbiosource, USA). Thiol group level was determined using Ellman's method (Hu *et al*, 1994). MDA level was measured using a procedure based on the reaction of thiobarbituric acid (TBA) with MDA at 95°C (Sochr *et al,* 2014). Statistical analysis was performed using SPSS software version twenty two (USA). Analysis of Variance (ANOVA) was used to compare among groups while, t-teat was used for comparison among two groups. A value of P less than 0.05 was considered significant.

RESULTS

Baseline demographic and biochemical characteristics of the patients and control groups are shown in Table 1. No significant difference was observed regarding duration of disease, BMI, WHR, LDL-C and SBP, between control and patients groups. The mean value of CRP and WBC in G (3,4,5) group was significantly higher than other groups ($P < 0.01$), whereas, non-significant difference was found among T2DM, G(0,1,2) and control groups ($P > 0.05$).

Serum AOPPs and MDA concentrations in patients with G(3,4,5) were higher significantly than in patients with G(0,1,2), T2DM, and C groups ($P < 0.01$). The results in Table 2 revealed that the mean level of AOPPs for $G_{(0,1,2)}$ and $G_{(3,4,5)}$ was highly significant ($P<0.01$) when compared with C group, also the mean level of $G_{(3,4,5)}$ was highly significant ($P<0.01$) when compared with T2DM group. Serum CAT spicific activity in patients with G (3,4,5) was higher significantly than that in patients with T2DM, G(0,1,2) and control groups($P <0.01$) (Table 2). A significant lower GSH levels ($P<0.01$) in G(0,1,2) and G(3,4,5) was observed when compared with that of T2DM and C groups ($P<0.01$). However, GSH level was

Table 1 : Demographic and biochemical analysis.

Parameters	C group	T2DM group	DFUG(0,1,2)	DFUG(3,4,5)	P value post hok test
Number	25	25	45	38	-
Gender Male/ female	15\10	17\8	28/17	27\11	-
Age (year)	50.92±9.02	58.04±8.87*a	53.8±10.29	57.7±6.95*a	0.003
Duration of disease(year)	-	12.18±9.6	12.6±6.13	12.26±6.9	0.32
BMI (kg/m^2)	28±3.31	28.67±5.91	28.57±5.52	26.58±6.56	0.65
WHR	0.99±0.07	1.027±0.07	1.03±0.11	1.06±0.18	0.43
WHtR	1.278±0.19	1.30±0.14	0.77±0.3**a, b	0.82±0.31**a, b	<0.0001
SBP(mmHg)	8.31±0.92	8.6±6.1	1.76± 9.24	8.62±1.51	0.073
DBP(mmHg)	12.02±0.91	13.72±1.76**a	14.02±1.8**a	13.6±2.4 **a	0.02
FSG (mg/dL)	85.18 ±16.34	203.04±81.08 **a	216.0 ±87.17**a	222.0 ± 76.3**a	<0.0001
HbA1c(%)	4.82 ±0.56	7.88±1.46**a	9.15 ±1.85**a b	10.01±1.66**a b	<0.0001
Insulin (ìIU/ml)	8.8 ± 3.4	11.1 ± 3.7	15.47±4.8**a	24.39±10.42**(a,b,c)	0.02
TC(mg/L)	147.02± 38.4	176.12±44.52	182.72±52.99*a	192.1 ± 50.46*a	0.05
TG (mg/L)	124.87 ± 46.43	170.06 ± 65.22	184.68±76.10*a	201.48±72.88**a	<0.0001
HDL-C (mg/L)	42.24± 14.95	34.58± 11.99	32.61± 10.94*a	33.45± 11.04*a	0.05
LDL-C (mg/L)	75.78 ± 35.25	97.41± 50.23	101± 51.14	85.18±41.17	0.064
VLDL-C (mg/L)	25.97± 9.67	35.81 ±14.03	36.14 ±15.26*a	40.36± 17.7*a	0.004
CRP(mg/L)	2.68 ± 1.7	8.95± 4.61	12.46 ± 6.93	103.11 ± 68.35**abc	<0.0001
WBC(10^3/ ìl)	6.64 ±1.24	8.38 ± 1.6	9.01 ± 1.82**b	13.9 ± 6.23**abc	<0.0001

**P< 0.01, *: P <0.05 using ANOVA. a : significant between C& T2DM, G(0,1,2), G(3,4,5); b: T2DM & G(0,1,2), G(3,4,5); c: significant between G(0,1,2) & G(3,4,5). BMI: Body Mass Index, WHR: Waist to Hip Ratio, WHtR: Waist to Height Ratio, SBP: Systolic Blood Pressure, DBP: diastolic blood pressure, TC: Cholesterol, TG: Triglyceride, LDL-C: Low Density Lipoprotein, HDL-C: High Density Lipoprotein, VLDL-C: Very Low Density Lipoprotein cholesterol, HbA1c: Hemoglobin A1c, FSG : Fasting Serum Glucose, CRP: C-Reactive protein , WBC: white blood cell.

Table 2 : Mean (±SD) level of oxidant and antioxidant levels in the studied groups.

Parameters	C group	T2DM group	G(0,1,2)	G(3,4,5)	P post hok test
AOPP(ìmol/L)	77.05±15.5	111± 18.1*a	131.26± 33.4**a	162.0± 35.34.17**(a ,b, c)	<0.0001
MDA(nmol/L)	8.42 ± 3.3	15.69 ± 5.86**a	17.07 ± 6.63**a	20.69 ± 6.31**a *(b ,c)	<0.0001
GSH(mg GSH/L)	26.59±5.34	22.58± 3.76*a	17.08 ± 5.10**(a,b)	11.89± 7.17**(a ,b) *c	<0.0001
Catalase activity (KU/L)	8.53±3.05	6.69 ±3.33	11.06± 5.73*b	15.21 ± 6..75**(a,b), *c	<0.0001
Thiol group(nmol/L)	0.5 ± 0.12	0.49 ± 0.17	0.33 ± 0.14 **(a,b)	0.19 ±0.077**(a,b,,c)	<0.0001

**P< 0.01, *: P <0.05 using ANOVA. a: significant between C& T2DM, G(0,1,2), G(3,4,5). b: T2DM & G(0,1,2), G(3,4,5), c: significant between G(0,1,2) & G(3,4,5). AOPP: advance oxidative protein products, GSH: glutathione, MDA: Malondialdehyde.

higher significantly (P<0.05) in G(0,1,2) when compared with G(3,4,5). A significantly lower GSH level was found in T2DM group when compared with C group.The concentration of thiol group showed non-significant difference (P>0.05) between T2DM and control groups. The mean level of thiol group in G(,1,2), and G(3,4,5) was lower significantly (P<0.01) when compared with C group. While, in patients of G(0,1,2) group; the thiol level was higher significantly (P<0.01) when compared that of G(3,4,5). A significant increase was observed in AOPPs concentration as Wagner grade increase, as it was higher in G(3,4,5) when compared with that of G(0,1,2) ($P < 0.01$).

DISCUSSION

The level of AOPP in this study is comparable with results of KarKaushik *et al* (2014) and Kalousova *et al* (2002) studies that showed AOPP levels were higher in

Table 3 : The AOPP values among different types of diabetic foot grades according to Wagner classification.

DFU groups	Wagner grade	Patients No. (%)	AOPP (ìmol/L) for each Wagner grade	AOPP(ìmol/L) between two groups	P-value using T-test between groups
G(0,1,2) n=28	0	9 (32)	110.56 ± 38.82	130.75 ± 36.71	*<0.01
	1	10 (35)	138.2 ± 33.75		
	2	9 (33)	141.92 ± 32.56		
G(3,4,5) n=32	3	13 (40)	154.36 ± 39.7	162.06 ± 35.48	
	4	10 (31)	171.32 ± 38.97		
	5	9 (29)	163.54 ± 27.45		

**P< 0.01, *: P <0.05 using t-test analysis.

diabetic patients (T2DM 157.5 ìmol/l *vs* controls subjects 79.8 ìmol/l, p<0.001). Also, study of Baskol *et al* (2008) found higher AOPP levels in DM patients with (222.7 ìmol/L) or without diabetic retinopathy (210.9 ìmol/L) when compared to C group (152.4 ìmol/L). Previous studies observed higher AOPP level and it was more pronounced in T2DM than T1DM and it is a better parameter than AGE (KarKaushik *et al,* 2014). Previous study also observed that AOPP is a good marker for progression of diabetic nephropathy (Piwowar *et al*, 2008). The findings in our study keep in tract with the observations found by Piwowar *et al* (2010), who described that AOPP formation is induced by intensified glycol-oxidation process, oxidant/ antioxidant imbalance and coexisting inflammation. Similar findings were also observed in Gil del valy *et al* (2005), Fathy *et al* (2009), Pan *et al* (2010) studies. Furthermore, Fathy *et al* (2010) observed the existence of a correlation between AOPP levels with T2DM microvascular complications. Baskol *et al* (2008) found higher AOPP and lower antioxidants in diabetes patient. The sources of oxidative stress in DM include auto-oxidation of glucose, shifts in oxidation-reduction balances and impaired activities of antioxidant defense enzymes such as (superoxide dismutase (SOD) and catalase), decreased tissue concentrations of antioxidants such as reduced vitamin E and glutation (GSH). Oxidative stress include damage to biological macromolecules such as proteins, DNA, carbohydrates and lipids (Baynes, 1999). The result about level of GSH in this study was in agreement with previous study by Khan *et al* (2014).

The role of GSH is protecting cells from oxidants like peroxides. Through its action; it is converted to the oxidized form (GSSG), which represent ten percent from GSH pool in the normal cells and the remaining 90% is still in its reduced form. However, this ratio is subjected to imbalance in the presence of oxidative stress. In a study on diabetic patients, a reduction in GSH levels was found in comparison to that of non-diabetic patients. Also, a study by Sekhar *et al* (2011) found similar results where they found lower GSH synthesis in uncontroled DM patients that may increase their risk to diabetic complications. concluded from their studies in uncontrolled diabetes that glutathione synthesis is decreased in these patients. Decreased levels of reduced glutathione levels aggravate diabetic complications (Sekhar *et al*, 2011). While, an oppesit results were found by Srivatsan *et al* (2009). They suggested the presence of DM increase the oxidants level that may lead to a defencive response. Therfore, they suggest that supplementation with dietary antioxidant vitamins with change in lifestyle may help to decrase damage resulted from radical toxicity in DM. Lower serum GSH levels were observed in our study on DFU patients. Low GSH level may be responsible for some of the subsequent events in diabetic complication and metabolic disturbances. Increased lipid peroxidation in DM and DM with complication had been reported in various studies (Vairamon *et al*, 2009; Jabeen *et al*, 2013; Vijay Kumar *et al,* 2014). The enhanced production of MDA has been proposed as one of the mechanism that fosters rapid progression of diabetic vasculopathy. The most possible causes for the high MDA level in T2DM are abnormal lipid metabolism and higher lipoprotein levels makes them at risk of oxidation by ROS (Ri *et al,* 1972). High levels of MDA in T2DM perhaps due to the change of erythrocytes membrane function. This change may leading to inhibit the activity of SOD enzyme that cease to accumulation of superoxide radicals, which case maximum lipid peroxidation and the tissue damage in diabetes. Elevated lipid peroxide may be the cause of the increased glycation of protein in DM. The glycated protein might themselves perform a source of free radicals. There is a clear association between lipid peroxide and glucose concentration. A low antioxidant activity of catalase had been related to higher concentration of peroxide that may be lead to imbalance between scavenging and production of free radical (Trivelli *et al*, 1972).

CONCLUSION

The outcomes of this study revealed the increase in AOPP, MDA, and catalase levels as well decrease GSH, and thiol grouplevels as the degree of Wagner grade increase; the incidence of oxidative stress among DFU with higher Wagner grades and the presence of relation between inflammation state in DFU patients and oxidative stress.

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