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Advanced Glycation End Products (AGEs) in Diabetic Patients with Systemic Lupus Erythematosus

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ABSTRACT

Systemic Lupus Erythematosus (SLE), a systemic autoimmune disease has an increased risk for diabetes mellitus (DM) due to accelerated atherosclerosis. The accumulation of advanced glycation end products (AGEs) is recognized as one of the factors contributing to the progression of atherosclerosis. The purpose of this study was to evaluate the levels of AGEs in diabetic patients with SLE. This case-control study included one-hundred and sixteen people with diabetes mellitus (type 1 DM = 19, type 2 DM = 97) divided into two groups namely; diabetic patients with SLE (DSLE group; n = 07) and diabetic patients without SLE (D group; n = 109). Results showed that differences in plasma glucose, HbA_1c, total proteins, non-enzymatic protein glycation and AGEs measurements in diabetic subjects with SLE and those without SLE were not significant ($p > 0.05$). All the patients were thoroughly examined to explore DM and SLE involvements. Arthritis, photosensitivity and nephropathy, retinopathy were most common SLE and DM associated clinical features. Diabetic patients with SLE can experience increased AGEs. The disastrous combination of DM and SLE doubles patient's risk of evolving several complications. Vigilance considerations by ophthalmologists, nephrologists and endocrinologists can reduce patient's uncertainty, allowing them to focus on health and better prospects.

Key Words: Atherosclerosis; Advanced glycation end products; Diabetes mellitus; Systemic lupus erythematosus

INTRODUCTION

Advanced glycation end products (AGEs) are a heterogeneous group of protein and lipids to which sugar residues are covalently bound. AGEs formation is increased in situations with hyperglycemia (e.g., diabetes mellitus: DM) leading to microvascular and macrovascular complications. The microvascular complications of DM particularly affect the eyes, kidneys, liver and nerves (Hussnain *et al*., 2007; Hussain *et al*., 2008a; Ansari & Rasheed, 2010). Although the exact mechanism by which vascular damage occurs in diabetes in not fully understood, numerous studies support the hypothesis of a causal relationship of non-enzymatic glycation and AGEs with vascular complications (Cárdenas-León *et al*., 2009; Schalkwijk & Miyata, 2012). Chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of diabetic complications. Inflammatory cytokines regulate inflammatory, immune responses and provide important signals in the DM pathophysiology (Juan *et al*., 2008).

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with variable clinical signs. SLE frequently coexists with DM and patients with overlapping SLE-DM can have clinical features common to both the disorders. The combination of SLE and DM is uncommon but the predisposition to renal, peripheral neuropathy and retinal disease means that great care must be taken when deciding, which clinical feature is due to which disease, because active SLE requires additional immunosuppression whereas DM requires optimization of the metabolic control (Cortes *et al*., 2008; Heyneman, 2009; Jafri *et al*., 2011). The prevalence of DM and impaired glucose tolerance are quite high in SLE patients (Zeng *et al*., 2010). To date no study at national level has been performed on the status of AGEs in SLE patients to understand disease mechanisms and to effect clinical translation. In this view, present study was designed to evaluate plasma AGEs levels among diabetic patients with SLE.

MATERIALS AND METHODS

Sampling: The research work was conducted at the Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. Before recruitment, ethical approval from institutional Advanced Studies and Research Board (ASRB) was secured. One hundred and nine DM patients without active SLE and seven DM patients with active SLE were included after written consent. Inclusion criteria for recruitment were the following three conditions: (1) be suffering from type 1 or type 2 DM, (2) be diagnosed with DM for at least 1 year, (3)

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having age between 20 – 60 years. The diagnosis of DM was based on WHO criteria (Alberti & Zimmet, 1998). SLE diagnosis and clinical features were defined as per revised classification criteria of the American College of Rheumatology (Tan *et al*., 1982). Baseline demographic data included age, gender, duration of DM and SLE, age at onset, family history of DM and SLE, clinical patterns and associated complications of DM and SLE. Blood samples collected from participants were processed for further analysis.

Glucose: For the estimation of plasma glucose levels, spectrophotometric kit method was used as per manufacturer's instructions. Appropriate amounts of test sample and reagent were mixed. Incubated for 20 min at 37^{o}C and the absorbance at 546 nm against the blank was used to calculate glucose concentration.

HbA_1c: A_1c Kit (Biosystem, Spain) was used for the detection of HbA_1c. Hemolysate was prepared by pipetted 500 µL lysing reagent with 100 µL blood samples and mixed well. Tubes were left for 5 min at room temperature. To the tubes with pre-pipetted 2.5 mL ion exchange resin, 100 µL of hemolysate was added. Positioned the resin separators in the tubes approximately 1 cm above the ion exchange resin. After mixing the tubes for 5 min, pushed the separators into the tube until the resin is firmly packed. Poured the supernatant (Glycohemoglobin A_1c) into a cuvette and noted the absorbance at 415 nm. To determine total Hemoglobin A_t, pipetted 20 µL of the hemolysate into labeled tubes. Dispensed 5 mL distilled water to each tube and mixed well, and absorbance taken at 415 nm.

Total proteins: Biuret method (Gornell *et al*., 1949) was used to measure the level of total protein in heparized plasma samples. To 1 mL of appropriately diluted plasma sample, 1 mL of biuret reagent was added and incubated for 15 min at 37^{o}C. The tubes were cooled and absorbance at 540 nm was noted against an incubated blank. The standard curve was constructed using bovine serum albumin as standard.

Non-enzymatic glycation: Thiobarbituric acid (TBA) colorimetry test was used for the determination of both enzymatic and non-enzymatic glycation (Furth, 1988).

Non-enzymatic and enzymatic glycation (combined): One mL dialyzed plasma whose total protein is already estimated (10 mg/mL) was used. 0.1 mL of $NaBH_4$ was added in reduced and 0.1 mL of 0.01N NaOH in non-reduced labeled samples. Tubes were left for 30 min at 37^{O}C. After half an hour, 1 drop of 1N HCl was added in each test tube, followed by 0.5 mL addition of oxalic acid. All the tubes were capped and autoclaved for half an hour at 124^{O}C for a pressure of ll5 Lb /inch^2. After cooling, the tubes were placed in ice. In each tube, 0.5 mL chilled 40% trichloroacetic acid was mixed, centrifuged the samples for 15 min at 15000 rpm and in supernatant (1.5 mL), 0.5 mL of freshly prepared TBA was added. The samples were incubated at 37^{o}C in water bath for 15 min and absorbance was noted at 443 nm.

Enzymatic glycation: For determination of enzymatic glycation, 0.1 ml NaOH (0.01N) containing 400 molar excess of $NaBH_4$ was used. After reduction, the glycation level was determined by the same process as mentioned above. Standards solutions (100 to 500 nmol /mL) were prepared by using Fructose (0.9 g/100 mL).

Advanced glycation end products (AGEs): AGEs were detection by enzyme-linked immunosorbent assay (ELISA). Anti-AGE immunoglobulins (Antibodies) were purchased commercially from Sigma. AGEs-BSA was prepared by using bovine serum albumin (BSA) according to Zhang *et al*. (2005).

ELISA: ELISA was performed by using alkaline phosphatase enzyme and para-nitrophenyl phosphate as a substrate, following the procedures of Turk *et al*. (1998) and Zhang *et al*. (2005) with slight changes according to laboratory conditions. Briefly, the antigen was diluted to a final concentration of 20 µg/mL in phosphate buffer saline (PBS), coated the wells of the PVC micro-titer plate with the antigen by pipetting 50 µL of the antigen dilution per well and covered the plate with an adhesive plastic and incubated for 2 h at room temperature. The coating solution was removed and washed the plate twice by filling the wells with 300 µL PBS. The solutions or the washes were removed by flicking the plates over a sink. The remaining drops were removed by patting the plate over a paper towel. Blocked the remaining protein-binding sites in the coated wells by adding 300 µL of blocking buffer, 5% non-fat dry milk PBS, per well, covered the plate with an adhesive plastic and incubated for at least 2 h at room temperature or, if more convenient, overnight at 4^{o}C. Plates were washed twice with PBS. Made 10-fold dilutions (1:100, 1:1000, 1:10,000, 1:100,000 & 1:1000, 000) of plasma in blocking buffer. Added 50 µL of each dilution to an antigen coated the well, covered the plate with an adhesive plastic and incubated for 2 h at room temperature. After washing the plate four times with PBS, added 50 µL of secondary anti-specie antibody conjugated to alkaline phosphatase, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use. Plate was covered with an adhesive plastic and incubated for 2 h at room temperature. Again washed the plate four times with PBS, dissolved p-nitrophenyl phosphate at a concentration of 1 mg/mL in substrate buffer (1 M Di-ethanolamine, 0.5 mM $MgCl_2$, pH 9.8) and added 50 µl of the substrate solution per well. Absorbance was taken at 405 nm, using a micro-titer plate spectrophotometer and then performed an end-point measurement after 1 h.

Data were expressed as number (n), mean ± standard deviation or % ± standard deviation of triplicate measurements. Student's t-test was performed to compare variables among DSLE and D groups. Pearson's correlation coefficient (*r*) was used to identify the associations among parameters within the group. The data were analyzed using SPSS for Windows (version 14.0, ® SPSS Inc.) with significance level set at $p \leq 0.05$.

RESULTS

Diabetes mellitus and Systemic lupus erythematosus can affect any organ. However, the impact of the SLE will be different for each person. The results are summarized in Table I. This case-control study included one-hundred and sixteen people with DM (type 1 DM = 19, type 2 DM = 97) divided into two groups namely; diabetic patients with SLE (DSLE group; n = 07) and diabetic patients without SLE (D group; n = 109). Mean age of D group was slightly higher than that of DSLE group. The overall prevalence of type 2 DM was 83.62% (97 of 116). In DSLE group, most of the patients were male (85.71%) and onset of SLE occurred above 50 years of age. Contrary to that, about 34% patients in D group were female. The duration of DM was comparable in both DSLE and D groups. About 14% patients in DSLE group had family history of DM, an observation quite similar with that of D group with 16% DM history. Only one patient in each group had past SLE occurrence within the family. Mean age, gender wise DM distribution, age at onset of DM and DM duration did not differ significantly ($p > 0.05$) between DSLE and D groups. Within the DSLE group, mean age, type of DM, duration of DM or SLE, and family history of DM or SLE had no significant relation ($p > 0.05$).

Glucose and HbA_1c concentrations in both groups namely; DSLE and D groups were parallel ($p > 0.05$). Similarly, total proteins, and non-enzymatic plasma protein glycation, AGEs measurements in diabetic subjects with SLE and those without SLE were not significant ($p > 0.05$). All the patients were thoroughly examined to explore DM and SLE involvements. Arthritis and photosensitivity were most common SLE associated observations in DSLE and D groups. Nephropathy and retinopathy were the relevant DM involvements. D group had lesser associated complications (Table II).

DISCUSSION

Disturbed glucose metabolism is an important factor leading to advanced glycation end products formation. Many dermal manifestations including SLE are recognized as diabetes mellitus markers. Some of these skin disorders occur as direct sequelae of diabetes mellitus or of its major complications involving well-demonstrated risk of progressive liver ailment. Neuropathy, decreased large vessel perfusion and increased susceptibility to infection all play a role (Perez & Kohn, 1994; Hussain *et al*., 2008b; Hussain *et al*., 2009). SLE is characterized by unpredictable remissions of clinical manifestations involving the joints, skin, kidney, brain, serosa, lung, heart, gastrointestinal tract (Johnson *et al*., 1996; Gourley *et al*., 1997).

Both the groups under study had mean age in the range of 55–60 years and age at onset of DM between 46–51 years. The expression of the SLE is not dependent on age (Voulgari *et al*., 2002). The D group had higher averaged age and age at onset of DM than that of DSLE group. Conversely, DSLE group had higher duration of DM as compared to D group. DSLE group had 50 years or older age at onset patients with more than three years of SLE duration. Cervera *et al*. (1993) also observed 50 years or older age at onset patients. Type 1 DM is the only major organ-specific autoimmune disorder without a strong female bias. Populations with the highest DM incidence show male dominance. Most of non-European origin people show a female biasness. Type 2 DM is equally prevalent among men and women in most populations. Men seem more susceptible than women to the consequences of indolence and obesity, possibly due to differences in insulin sensitivity and regional fat deposition (Gale & Gillespie, 2001). In diabetic groups without and with SLE, 66-85% patients were male and 94% had type 2 DM. The reason for male biased population in our study groups may be the use of convenience sampling method. SLE was more prevalent in males than females in DSLE group. However, no link was identified between SLE and gender. Controversial results are reported about gender wise distribution of SLE. Gender does influence the clinical expression but not the overall damage score in SLE (Voulgari *et al*., 2002). As reported by Wasef (2004), SLE is known to be much more common in females than in males but the cause of this sexual predilection is not established. Sometimes, initial symptoms of SLE occur in males more often than females (Cervera *et al*., 1993). Similarly, present study observed more male patients with SLE than females.

Type 1 DM is the most common heritable disease (Polychronakos & Li, 2011). Great advances have recently occurred in our understanding of the genetics of type 2 DM, but much remains to be learned about the disease etiology (Ahlqvist *et al*., 2011). Negative family history of diabetes may reduce the risk of developing hyperglycaemia and type 2 DM in some ethnic groups (Xu *et al*., 2011). According to some studies (Bo *et al*., 2000), maternal and paternal diabetes do not influence clinical characteristics of type 2 diabetic patients, while there is evidence that parental diabetes brings to an earlier onset of the disease. Genetic factor of SLE has been proposed in some studies (Shai *et al*., 1999). History of SLE in parents or siblings was associated with an increased risk for the development of SLE (Cooper *et al*., 2002). Most of the patients in present study were type 2 diabetics and majority reported negative family history of DM and SLE.

The concentrations of plasma glucose and HbA_1c were almost similar in both the groups. HbA_1c is the first example of *in vivo* non-enzymatic glycation of proteins and its discovery opened new and still-growing avenues of research on maillard reactions in biological systems, including the concept of AGEs. Disturbed glucose metabolism is more prominent in DM (Monnier *et al*., 2003). Total plasma proteins in both groups namely; DSLE and D groups were analogous. There is a general agreement that the total plasma protein level may be normal in SLE.

Table I: Baseline demographic and clinical characteristics of study participants

Characteristics	DSLE Group	D Group
Patients (n)	7	109
Age (years)	57.35 ± 1.54	58.63 ± 1.62
Types of DM (type 1 / type2)	02/05	17/92
Gender (male/female)	06/01	72/37
DM duration (years)	8.66 ± 1.31	7.5 ± 2.41
SLE duration (years)	3.27 ± 0.78	-
Age at onset of DM (years)	47.62 ± 1.28	48.93 ± 1.71
Age at onset of SLE (years)	51.25 ± 0.54	-
Family history of DM (n)	01	17
Family history of SLE (n)	1	1
Glucose (mmol L^{-1})	11.78 ± 1.36	10.95 ± 2.41
HbA_1c (%)	11 ± 0.5	10 ± 1.1
Total proteins (g/dL)	12.89 ± 1.75	13.61 ± 0.67
NEG (mol /mol)	1.27 ± 0.55	1.35 ± 0.35
AGEs (u/mL)	2.12 ± 0.37	1.9 ± 0.23

Data as number (n), mean ± SD or % ± SD of triplicate measurements
DM: Diabetes Mellitus, SLE: Systemic Lupus Erythematosus, NEG: Non-Enzymatic Glycation, AGEs: Advanced Glycation Endproducts

Table II: Clinical features of study participants

Type of DM	DSLE group		D group	
	SLE Influence	DM Influence	SLE Influence	DM Influence
Type 1 DM	Arthritis	Retinopathy	Photosensitivity	Retinopathy
	Photosensitivity	Peripheral Neuropathy		Peripheral Neuropathy
Type 2 DM	Arthritis	Retinopathy	Nose/Mouth ulcerations	
	Photosensitivity	Peripheral Neuropathy		Nephropathy
	Nose/Mouth ulcerations	Diabetic foot ulcers		Diabetic foot ulcers

DM: Diabetes Mellitus, SLE: Systemic Lupus Erythematosus

While metabolic derangements in DM results in tissue protein break down leading to increased plasma protein levels (Robbinson & Kumar, 1989; Chatterjee & Shind, 2002). In case of SLE, disease course and nephropathy may increase proteinuria (Rees & Wilkinson, 1959). The total plasma proteins in SLE have received comparatively scant attention in the literature and probably have minute prognostic value.

Elevated tissue and plasma AGEs in DM are due to chronic hyperglycemia. But the status of these AGEs in DM patients with SLE is still a baffling puzzle. A study by Rodríguez-García *et al*. (1998) indicated AGEs accumulation in DM, but not in SLE. Meanwhile, Nienhuis *et al*. (2008) found increased Skin AGEs levels in SLE patients. In present study, diabetic patients with SLE (DSLE group) had slightly higher circulating AGEs levels compared to that of diabetic patients without SLE (D group). In D group, AGEs levels were associated with the diabetes duration. Though, AGEs in DSLE patients were not associated with DM or SLE duration. de Leeuw *et al*. (2007) pointed towards elevated AGEs in SLE patients having strong association with SLE duration. Several mechanisms may explain increased accumulation of AGEs in DSLE group. Firstly, inflammation and activation of the innate immune system are closely involved in the pathogenesis of DM and SLE (Juan *et al*., 2008). Secondly, the augmented oxidative stress may be speculated to contribute to the pathogenesis of DM and SLE (Avalos *et al*., 2007; Pan *et al*., 2008). In the presence of active inflammation and oxidative stress, AGEs might be even further increased in DSLE patients.

Clinical features of participants are presented in table II. Diabetes associated retinopathy and neuropathy were more prevalent. Serious retinal involvement is a frequent and troublesome DM complication and a major cause of blindness in Pakistan. It is a consequence of the microangiopathy affecting the retinal pre-capillary arterioles, capillaries and venules (Hussain *et al*., 2011). SLE affects the eye as part of the disease or due to the drugs used in therapy. Ocular involvement is seen in one third of the patients with SLE. SLE-induced macular infarction is rare and has poor visual prognosis. Serious ocular complications of SLE can be silent (Rao *et al*., 2010). Peripheral neuropathy also complicates diabetes and SLE with a predilection to lower extremities amputations especially foot ulcerations (Florica *et al*., 2011). In the present study, most relevant clinical and immunologic features of SLE were arthritis, photosensitivity and nose/mouth ulcers as reported previously (Cervera *et al*., 1993). These findings may have important implications regarding the choice of outcome measures in DM and SLE clinical trials.

In conclusion, patients with SLE can experience increased AGEs. Though ambiguity and prospects coexist, DSLE patients may well be at increased risk of developing neuropathy, retinopathy and lower extremities amputations. This disastrous combination of DM and SLE doubles patient's risk of evolving several complications. Vigilance

considerations by ophthalmologists, nephrologists and endocrinologists can reduce patient's ambiguity, allowing them to focus on health and better prospects.

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