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Plasma protein glycation in diabetic nephropathy

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Abstract: Non-enzymatic reactions between sugars and the free amino groups on proteins, lipids, and nucleic acids result in molecular dysfunction through the formation of advanced glycation end products (AGE). AGEs have a wide range of chemical, cellular, and tissue effects through changes in charge, solubility, and conformation that characterize molecular senescence. It seems that many of the pathogenic changes that occur in diabetic nephropathy may be induced by non-enzymatic glycation (NEG). For this study, one hundred patients with type 2 diabetes and forty healthy control subjects were recruited. In all subjects, nonfasting plasma glucose, total plasma proteins, ESR, HbA_1c and non-enzymatic glycation were assayed. Diabetic patients with nephropathy (22%) had higher ESR, total proteins and non-enzymatic glycation levels as compared to diabetics without any similar renal complications and controls. Appreciable correlation existed between hyperglycemia and non-enzymatic glycation. Glycation of proteins is a major cause of spontaneous damage to the proteome. Measurement of non-enzymatic glycation shows increasing promise in the assessment and prevention of diabetic nephropathy.

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INTRODUCTION

Diabetic nephropathy is a major cause of morbidity and is associated with increased cardiovascular mortality in diabetic population. The specific pathological changes in the kidney, the clinical course, and the overall risk to develop nephropathy are quite similar in both types of diabetes.[1] An estimated one third of patients with type 1diabetes need renal dialysis after 15–20 years of the disease.[2] One quarter of all patients requiring renal transplants, are diabetics.[3] Nephropathy remains a significant cause of morbidity and mortality in the diabetic population and is the leading cause of end-stage renal failure in the Western World. As a result of the diabetic milieu, increased generation of reactive oxygen species (ROS) is thought to play a key role in the progression of diabetic nephropathy.[4] Recent experimental studies have suggested that the receptor for advanced glycation end products (RAGE), which is central to the advanced glycation pathway, may mediate renal structural and functional damage via oxidative stress.[5] Tubulo-interstitial pathology in diabetic nephropathy is thought to be caused by cell injury that is induced by high ambient glucose levels and increased proportions of glycated proteins. Other mechanistic hypotheses engage glomerular ultrafiltration of proteins and bioactive growth factors and their effects on tubular cells. Some scholars promote tubular ischaemia due to reduced peritubular blood flow as a response to glomerular injury. All of these mechanisms contribute to renal tubulo-interstitial injury in diabetic nephropathy. Dilatation of distal nephron segments is routinely seen in human biopsies or in histological sections from experimental diabetic nephropathy. It is these dilated tubules that are the primary source for pro-inflammatory and pro-fibrogenic cytokines and regulators.[6] As with retinopathy, nephropathy is strongly affected by the degree of hyperglycemia, hypertension and the duration of diabetes.[7] World-wide, today diabetes accounts for 20-50% of patients entering established renal failure programs and absolute numbers increase as greater longevity and western-style living has promoted an 'epidemic' of diabetes at all ages.[8] In addition to the recognized and powerful effects of environmental factors, there is abundant evidence in support of genetic susceptibility to the microvascular complication of nephropathy in individuals with both type 1 and type 2 diabetes. It seems likely that the risk for diabetes-associated kidney disease is magnified by inheriting risk alleles at several susceptibility loci, in the presence of hyperglycemia.[9]In the present study, we compared various diabetes related parameters, especially non-enzymatic glycation levels among type 2 diabetic patients with and without nephropathy as well as with normal controls.

MATERIALS AND METHODS

Physician-diagnosed one hundred type 2 diabetic patients attending District Head Quarter Hospital, Faisalabad, Pakistan were studied along with forty non-diabetic volunteers, taken as control. The Advanced Studies and Research Board of the University of Agriculture, Faisalabad provided ethical approval and informed consent was obtained from all the subjects. Case subjects were further stratified based on the presence or absence of diabetes related renal comorbidities as T2DM group

with nephropathy (type 2 diabetes mellitus patients with nephropathy) and T2DM group without nephropathy (type 2 diabetes mellitus patients without nephropathy) to compare various variables. Baseline demographic information was obtained from medical records. The diagnostic criteria for nephropathy was microalbuminuria [Microalbuminuria defined as either dip stick positive for protein or albumin excretion of > 300 mg in urine over 24 hours].[10] In all subjects, blood samples were taken for the measurement of postprandial glucose, ESR, total proteins, glycated hemoglobin and non-enzymatic glycation.

Biochemical analysis

Post-prandial glucose

Enzymatic Kinetic Colorimetric test was used. Appropriate amounts of test sample and reagent were mixed. Incubated for 20 minutes at 37 ^{o}C and noted the absorbance at 546 nm against the blank within 60 minutes.

Total proteins

Biuret test[11] 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 minutes at 37^{o} C. The tubes were cooled and absorbance at 540 nm was noted against an incubated blank. The standard curve was constructed by using bovine serum albumin (1.0 mg of bovine serum albumin (BSA) / mL).

Erythrocyte sedimentation rate

The Erythrocyte Sedimentation Rate (ESR) was measured by Westergren's method.[12] The blood diluted with heparin was drawn up into a Westergren's tube to 0 mark by means of a teat. The tube was placed in exactly vertical position and left it undisturbed for 60 minutes free from vibration and draughts. Then the height of clear plasma above the upper limit of the column of the sedimented cells in the nearest mm was read. The ESR measurement was expressed in mm/ 1st hour.

Glycated Hemoglobin (GHb) or HbA_1c

For the detection of glycated hemoglobin in plasma, A_1c Kit (Biosystem, Spain) was used.

Hemolysate preparation

Pipetted 500 µL lysing reagent into labeled tubes. Added 100 µL blood sample into the tubes and mixed well. Left for 5 minutes at room temperature.

Glycohemoglobin separation A_1c

To the tubes with pre-pipetted 2.5 mL ion exchange resin, added 100 µL of hemolysate from step A. Positioned the resin separators in the tubes approximately 1 cm above the ion exchange resin. Mixed the tubes for 5 minutes. Pushed the separators into the tube until the resin is firmly packed. Poured the supernatant into a cuvette and noted the absorbance against water at 415 nm.

Total Hemoglobin A_t

Pipetted 20 µL of the hemolysate from step A into labelled tubes. Dispensed 5 mL distilled water to each tube and mixed well. Read the absorbance against water at 415 nm.

Non-enzymatic glycation (NEG)

Thiobarbituric Acid (TBA) colorimetric technique used for the determination of both enzymatic and non-enzymatic glycation[13] is based upon the reaction between fructose – amino acids and weak acid, yielding 5 – hydroxymethylfurfural (HMF).

Non-enzymatic and enzymatic glycation (collectively)

One mL dialyzed plasma whose total protein is already estimated (10 mg/mL) was used. Arranged three test tubes for reduced and three for non-reduced samples. Added 0.1 mL of $NaBH_4$ in reduced and 0.1 mL of 0.01N NaOH in non-reduced labeled samples. Leave the tube for 30 minutes at 37 ^{O} C. After half an hour, added 1 drop of 1N HCl in each test tube, followed by 0.5 mL addition of oxalic acid. Capped the tubes and autoclaved for half an hour at 124 ^{O} C for a pressure of 115 Lb /inch^2. Cooled the tubes to room temperature and put in ice. In each tube, added 0.5 mL chilled 40% Trichloroacetic Acid (TCA). Centrifuged the samples for 15 minutes at 15000 rpm. Supernatant (1.5 mL) was taken and 0.5 mL of freshly prepared Thiobarbituric Acid (TBA) was added. The samples were incubated the samples at 37 ^{O} C in water bath for 15 minutes 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 NaBH4 was used. After reduction, the glycation level was determined by the same process mentioned above.

Non-enzymatic glycation was determined as follows:

NE glycation = (NE + E Glycation) – E Glycation

EN = Non-enzymatic

E = Enzymatic

Statistical analysis

Results are expressed as means ± SD, number (n) or percent (%) as appropriate. Comparison and correlations between variables were done using student t-test and Pearson's correlation coefficient (r) with significance level set at $p \leq 0.05$. Multivariant regression by SPSS (version 14) was used to assess the relation between nephropathy and different biochemical parameters.

RESULTS AND DISCUSSION

Thirty-eight men (48.71%) and forty women (51.28%) were type 2 diabetic persons without nephropathy having mean age 57±8.12 years. Age at onset of diabetes was 46±5.19 years (duration of diabetes 10.8±5.1 years).

Of one hundred diabetic patients, 22 had renal impairment. Our results were in compliance with the findings of Diabetic Association of Pakistan that indicated 20% prevalence of diabetic nephropathy.[14]This group had mean age 65.0 ± 8.45 years. Consisted of 10 women (45.45%) and 12 men (54.54%), age at onset of diabetes was 48.6 ± 5.76 years (duration of diabetes 16.3 ± 6.61 years).

Third group of normal controls (52.6 ± 9.58 years mean age) had 45% men and 55% women (Figures 1, 2). All the subjects under study had mean age in the range of 50 -70 years. Diabetic patients with and without nephropathy indicated almost comparable mean age, age at the onset of diabetes and male/female ratio. Given the limited generalizability of this hospital-based sampling, these results were found in a population diagnosed with type 2 diabetes and we do not know whether the proportion of undiagnosed diabetes varies for mean age, age at onset of diabetes and gender distribution or not.

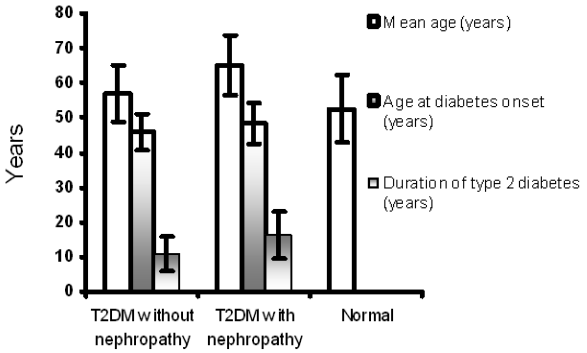

Figure 1: Mean age (years), age at diabetes onset (years) and diabetes duration (years) in type 2 diabetes mellitus patients (T2DM) with and without nephropathy and in normal subjects

Duration of diabetes was significantly higher in T2DM group with nephropathy as compared to that without nephropathy. Duration of diabetes is a very important factor in the development of diabetic nephropathy and the cumulative risk of renal failure increase with diabetes duration as demonstrated in several studies,[15, 16] This has also been confirmed in our study, that longer the duration of diabetes, higher the frequency of diabetic nephropathy.

Clinical characteristics of case and normal persons are summarized in table 1. Postprandial plasma glucose, ESR, total proteins, HbA_1c and NEG showed variations among diabetic patients with and without nephropathy and controls. The erythrocyte sedimentation rate has been used for predicting disease severity to assess general sickness index.

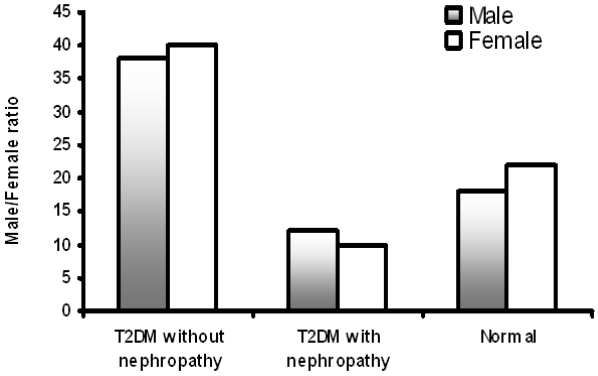

Figure 2: Male and female (n) in type 2 diabetes mellitus patients (T2DM) with and without nephropathy and in normal subjects

Table 1: Clinical characteristics of type 2 diabetics (with and without nephropathy) and normal participants.

Biochemical Parameters	T2DM with nephropathy	T2DM without nephropathy	Normal
ESR (mm/1st hour)	55.33±24.68	46.88±23.95	12.73±2.34
Glucose (mmol./L)	13.8±3.17	12.91±3.28	6.22±0.71
Total plasma proteins (g/dL)	15.71±4	14.01±4	6.18±1.16
HbA_1c (%)	11±2	10±2	6±1.11
NEG (mol./mol.)	1.73±0.48	1.47±0.58	0.48±0.18

The erythrocyte sedimentation rate is a simple, inexpensive laboratory test that clinicians have used for decision-making.[17] Despite all merits and demerits, ESR testing is still part of clinical chemistry in Pakistan. ESR values in our case patients with nephropathy and without nephropathy were statistically higher ($P < 0.05$) from normal controls. While diabetics with nephropathy also showed elevated ESR than diabetics without nephropathy ($P < 0.05$). Existing data suggest that an elevated ESR may occur in many different clinical settings such as serious underlying disease, most often infection, collagen vascular disease or metastatic malignancy and inflammation may play a role in the etiology of diabetes mellitus.[18] Our study provides limited support to the hypothesis that inflammation is an etiologic factor for diabetes.

Nonetheless, higher measurements of ESR among type 2 diabetic patients were extraneous.

Postprandial plasma glucose levels were analogous (P>0.05) between both groups of case participants but appreciably higher (P<0.05) than that of control participants. Due to the tendency to rapid variations of hyperglycemia constant in the life of diabetic patients (above all in the postprandial phase), it is proper to think that postprandial glucose may exert an influence on the onset of complications. Postprandial glucose level is the major determinant of HbA_1c level after mean daily blood glucose.

One question that remains unanswered is whether postprandial hyperglycemia has a unique role in the pathogenesis of diabetic vascular complications or not and should be a specific target of therapy. Previously postprandial glucose has been linked to the progression of complications in type 2 diabetics,[19] but we did not find any such association of postprandial glucose with nephropathy in regression analysis.

People with diabetes whether suffering from nephropathy or not had noticeably higher plasma proteins concentrations (P<0.05) than that of controls. This is attributed mainly to intracellular protein degradation in target tissues of insulin in diabetes mellitus. Degradation of serum proteins is also affected in diabetes and starvation. In normal conditions, a general correlation exists between isoelectric points of serum proteins and their degradative rates. This relationship is abolished in diabetes.[20] Anabolic processes like protein synthesis are sacrificed to catabolic activity such as gluconeogenesis.[21, 22]

Factor of proven or suspected efficacy in attenuating renal disease progression is hyperglycemia. Plasma haemoglobin A_1c reflects ambient mean glycaemia over a 2–3 months period. Considerably elevated (P < 0.05) HbA_1c values among type 2 diabetic participants with and without renal complication than of controls but insignificant among both diabetic groups (P > 0.05) were in agreement with Kalia *et al*.[23] Huraib *et al*.[24]

The thiobarbituric acid method, used for the estimation of non-enzymatic glycation in present study is less frequently used in diagnostic laboratories in Pakistan. It is quite economical to replace costly chromatographical techniques. International studies using TBA method have indicated that glycated serum proteins are sensitive indicator of the degree of hyperglycemia in diabetes.[25] Diabetic patients exhibited higher NEG levels (P<0.05) in comparison to healthy controls. Within the diabetic groups, those with nephropathy showed drastic increase in NEG (P<0.05) than those without nephropathy. It was demonstrated by Piwowar *et al*.[26] that diabetic patients had significantly higher levels of glycation products in comparison to healthy people. AGEs were increasing progressively from normoalbuminuria, through microalbuminuria to macroalbuminuria. Plasma AGE correlated significantly with urinary albumin/creatinine ratio.

Diabetic nephropathy is a leading cause of end-stage renal failure, which could account for disabilities and high mortality rates in patients with diabetes. Multivariate analyses revealed no association between nephropathy and various variables. Appreciable correlation (r=0.735) existed between hyperglycemia and non-enzymatic glycation. In diabetes mellitus, hyperglycaemia accelerates non-enzymatic glycation and oxidative stress leading to damage of macromolecules, among others proteins. This manifests in the increased levels of advanced glycation end products (AGE) and advanced oxidation protein products (AOPP). The chronic hyperglycemic status also favors glycation reactions (irreversible glucose binding on protein amino groups), thereby leading to advanced glycation endproducts.

CONCLUSION

The clinical consequences of non-enzymatic glycation of circulating proteins remain ambiguous. In non-diabetics the effects are probably negligible. In diabetic patients, however, extensively glycated species could exhibit significant alterations in function. Measurement of non-enzymatic glycation shows increasing promise in the assessment and prevention of diabetic nephropathy.

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