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Original Article

Biochemistry Section

Serum Erythropoietin Level in Type II Diabetic Nephropathy

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ABSTRACT

Introduction: Diabetes is a disease characterised by poor glycaemic control and development of various complications with age. Diabetic complications include development of nephropathy as well as other complications.

Aim: The study was aimed to elucidate the consequences of diabetic nephropathy on erythropoietin levels and microalbuminuria.

Materials and Methods: A total of 66 subjects with Type II Diabetes Mellitus (T2DM) with and without microalbuminuria and 22 healthy subjects were enrolled in the present study. The following case-control study was completed in Al-Najaf Centre for Diabetes and Endocrinology, Al-Najaf City, Iraq from March 2016 to May 2016. Serum erythropoietin levels and microalbuminuria concentrations were documented in addition to demographic and biochemical data.

Results: Serum erythropoietin concentrations were decreased significantly in patients with T2DM compared to that of healthy control subjects. Microalbuminuria concentrations were increased significantly in patients with T2DM compared to that of healthy control subjects.

Conclusion: Microalbuminuria and erythropoietin levels can be used to assess the occurance of complications in patients with diabetic nephropathy.

Keywords: Chronic kidney disease, End-stage renal disease, Haemoglobin, Hyperglycaemia, Renal function

INTRODUCTION

Diabetes mellitus is a metabolic disorder with unusual aetiology characterised by hyperglycaemia arising from deficiencies in insulin secretion and/or defect in its action. T2DM formerly described as “non insulin dependent diabetes” or “adult-onset diabetes”, comprises of 90-95% of all diabetes. T2DM is a long-term metabolic disorder that is categorized by high blood sugar, insulin resistance, and relative lack of insulin. Common symptoms include increased frequent urination, thirst, and unexplained weight loss [1]. Hyperglycaemia is a risk-factor for complications such as diabetic nephropathy. Diabetic nephropathy is defined as kidney disease that progresses after years of development of diabetes. Diabetic nephropathy is characterized into stages: microalbuminuria (UAE >20 µg/min and ≤199 µg/min) and macroalbuminuria (UAE ≥200 µg/min). Diabetic nephropathy develops from altered blood flow in the small vessels of the glomerular capsule and is a major cause of Chronic Kidney Disease (CKD) and subsequent kidney failure. Nephropathy attributed to increasing blood pressure and impairment of glomerular filtration. It is progressed by hyperglycemic condition that accompany to DM [2].

In the coming 10 years, the number of patients with diabetes and End-Stage Renal Disease (ESRD) is estimated to double [3], thereby aggravating the problem of care for this patient population. Although, the prediction with diabetic nephropathy has developed since early studies, there remains an additional mortality of more than 70 times that of an otherwise matched population [4]. Patients exhibiting diabetic nephropathy usually have a higher degree of anaemia associated with the degree of renal damage than those with other reasons of renal failure, and anaemia progresses earlier in these patients than in those with renal damage from other causes [5]. Recent scientific reports have documented anaemia as a risk-factor for the demand of renal replacement therapy in diabetes; furthermore, lower haemoglobin is significantly connected with a more rapid decline in the glomerular filtration rate. Additionally, treating anaemia early in renal failure has been established to slow the rate of decline of renal function [5,6].

Erythropoietin is a haematopoietic factor with various defensive properties. Erythropoietin treatment enhances renal functions and improve concentrations of Hypoxia Inducible Factor 1-alpha (HIF-1α) in diabetic animals [7]. Erythropoietin is a glycoprotein hormone that controls red blood cell synthesis (erythropoiesis) by linkage to a receptor on the surface of erythroid progenitor cells [8]. The activities of erythropoietin consist of initiation of erythroid progenitor cells and differentiation of normoblasts to promote the red cell mass in response to tissue hypoxia caused by anaemia or haemorrhage [9]. The major site for erythropoietin synthesis is the peritubular fibroblasts of the renal cortex cells in adult humans and hepatocytes in the foetus. A minor quantity of extrarenal erythropoietin production arises in adult liver and there is confirmation that erythropoietin is also synthesised by no less than two other positions: the uterus and the brain. The primary inducement for improved erythropoietin formation is tissue hypoxia [9].

The aim of the present study was to measure the levels of erythropoietin in patients with diabetic nephropathy without severe renal function damage and to estimate its correlation with Microalbuminuria levels in patients with diabetic nephropathy.

MATERIALS AND METHODS

Patients

The following case-control study was completed in Al-Najaf Center for Diabetes and Endocrinology, Al-Najaf City, Iraq from March 2016 to May 2016. The inclusion criteria were clinical T2DM (with or without microalbuminuria) with diabetes duration of at least one year. Controls were selected from healthy, non diabetic adult volunteers. Informed written consent was received from each subject.

Both in cases and controls, exclusion criteria were set as having history of cigarette smoking, heart failure and hyper or hypothyroidism, severe renal dysfunction.

A total of 66 subjects with T2DM and 22 healthy subjects were included. Sample size was estimated according to Kadam P and Bhalerao S method [10]. Weight, height, waist girth, duration of

diabetes (in patients), vital signs, including systolic and diastolic blood pressure were documented for each subject. Diabetic patients were classified into three groups: diabetic patients without complication, diabetic patients with hypertension and diabetic patients with nephropathy and hypertension.

Ten millilitres venous blood sample was taken from each subject subsequent to a fasting period of 10 hours. After centrifugation at 400 x g for 10 minutes, serum samples were supported and were stored at -40°C until investigation.

This study was completed in accordance with the ethical standards set by the Declaration of Helsinki and was approved by the ethics committee of Chemistry Department/University of Babylon-Iraq.

Analytical Methods

Erythropoietin (EPO) determination: EPO was measured using an Enzyme Linked Immunosorbent Assay (ELISA) based on the double-antibody sandwich method {Human EPO (Erythropoietin) ELISA Kit; Elabscience; UK}. The procedure included the addition of 100 µL of biotin conjugated detection antibody, standards and test samples to the wells of micro plate. The second step involved washing with 10 mM phosphate buffer solution pH 7.4. The third step comprised of Horseradish Peroxidase (HRP)-Streptavidin addition; then unbound conjugates were washed away with wash buffer. The fourth step comprised of 3,3', 5,5'-Tetramethylbenzidine (TMB) substrates to visualise HRP enzymatic reaction. HRP catalysed TMB to form a blue coloured product that changed into yellow after adding acidic stop solution. Finally, micro plate reader was used to measure the absorbance at 450 nm, and then the concentration of EPO was calculated [11].

Microalbuminuria (MAU) determination: MAU was measured using ELISA based on the double-antibody sandwich method {Human MAU (Microalbuminuria) ELISA Kit; Elabscience; UK}. The procedure included addition of 100 µL of biotin conjugated detection antibody, standards and test samples to the wells of micro plate. The second step was washing with 10 mM phosphate buffer solution at pH 7.4. The third step comprised HRP-Streptavidin addition; then unbound conjugates were washed away with wash buffer. The fourth step comprised TMB substrates to visualise HRP enzymatic reaction. HRP was catalysed TMB to form a blue color product that changed into yellow after adding acidic stop solution. Finally, micro plate reader was used to measure the absorbance at 450 nm, and then the concentration of MAU was calculated [12].

STATISTICAL ANALYSIS

The statistical analysis was done using Statistical Package for the Social Sciences (SPSS) Version 21 (SPSS Inc., Chicago, USA). The results are presented as mean±SD. Comparisons between groups were done using one-way Analysis of Variance (ANOVA). Differences between the groups with respect to the distribution of categorical variables were examined using the t-test; p-values ≤0.05 were considered to be statistically significant.

RESULTS

Patient characteristics and research laboratory results are shown in [Table/Fig-1]. There was no significant difference in the age of T2DM patients and controls (p-value=0.89). Patients with T2DM had significantly higher HbA1c compared to healthy subjects (p-value=0.031), as shown in [Table/Fig-1]. Serum EPO concentrations were decreased in T2DM compared to controls and/or other groups, as shown in [Table/Fig-2]. EPO levels correlate with microalbuminuria in diabetic nephropathy groups, as shown in [Table/Fig-3]. The results of the present study show a significantly

Group Parameter	Healthy subjects	All diabetic subjects	Diabetic without complication	Diabetic with hypertension	Diabetic nephropathy with hypertension
Number	22	66	22	22	22
Age (years)	46.31±7.434	54.60±7.094 (p-value=0.89)	56.32±4.84 (p-value=0.87)	52.53±5.58 (p-value=0.83)	53.60±7.28 (p-value=0.84)
BMI (kg/m^2)	27.95±3.79	29.41±4.12 (p-value=0.92)	27.42±3.18 (p-value=0.94)	30.08±5.51 (p-value=0.89)	29.70±3.47 (p-value=0.91)
HbA1c	5.69±1.16	8.45±2.46 (p-value=0.031)	7.47±2.52 (p-value=0.036)	7.93±2.24 (p-value=0.038)	7.41±1.67 (p-value=0.043)

[Table/Fig-1]: Age, body mass index and glycated haemoglobin of the diabetic patients and controls; Quantitative variables are expressed as mean±SD.
*The mean difference is significant at the 0.05 level.

Clinical parameter	Group Duration of Diabetes (year)	Control	Total study Diabetic Population	Diabetic without complication	Diabetic with hypertension	Diabetic nephropathy with hypertension
Erythropoietin (pg/mL)	< 5 years	1600±366.6	1667±500.6	1618±535.6	1739.00±360.05	1719.0±344.57
	(5-10) years		1550.4±205.8	1549.66±196.78	1650.40±166.8	1675.0±172.67
	>10 years		1604.52±531.63	1630.66±498.10	1159.12±710.84	1514.66±325.8
Microalbuminuria (mg/L)	<5 years	11.90±4.47	84.4±55.25	43.33±29.43	16.66±11.54	80.00±62.6
	(5-10) years		98.0±48.74	63.0±51.25	103.33±36.14	122.6±38.34
	>10 years		92.22±57.55	84.38±46.55	90.00±69.28	136.66±36.14

[Table/Fig-2]: Comparison of microalbuminuria and erythropoietin concentration in healthy controls and diabetic patient.

Clinical parameter	Group Duration of Diabetes (year)	Control	Total study Diabetic Population	Diabetic without complication	Diabetic with hypertension	Diabetic nephropathy with hypertension
		p-value (t-test)	p-value (t-test)	p-value (t-test)	p-value (t-test)	p-value (t-test)
Erythropoietin (pg/mL)	< 5 years	----	0.305	0.318	0.177	0.123
	(5-10) years		0.187	0.179	0.344	0.127
	> 10 years		0.233	0.188	0.033*	0.211
Microalbuminuria (mg/L)	< 5 years	----	0.015*	0.009*	0.028*	0.008*
	(5-10) years		0.009*	0.119*	0.004*	0.005*
	> 10 years		0.004*	0.019*	0.005*	0.007*

[Table/Fig-3]: Statistical analyses of microalbuminuria and erythropoietin concentration in the groups of diabetic patients compared with healthy control group. A p-value was obtained by using student t-test. Groups presented in same format as in [Table/Fig-2].
*The mean difference is significant at the 0.05 level

decrement (p=0.033) of serum EPO concentration of diabetic patients with hypertension compared to the levels of control subject; on the other hand, microalbuminuria levels were increased significantly (p=0.015) in diabetic patients compared to the levels of control subject.

DISCUSSION

Anaemia is a usual conclusion in diabetes, particularly in patients with nephropathy. The results of the current study show a significant decrement of EPO concentrations of diabetic patients with hypertension after >10 years of duration of diabetes (p-value = 0.033), as shown in [table/Fig-2]. The negative correlation between erythropoitin levels and micro albuminuria in T2DM patients with nephropathy was obtained (p-value = 0.042; r = -63). Other groups showed non-significant change in EPO levels. The most significant associations of EPO are indicators of renal function. On the other hand, the microalbuminuria is closely related to this change (p-value=0.015), as shown in [table/Fig-3]. The findings of the current study are compatible with that of the previous studies, which indicated EPO deficiency in patients with T2DM and unrecognised anaemia in diabetic patients. In spite of scientific conclusions that specified plasma EPO concentrations are generally low in diabetic subjects, anaemia and EPO are not routinely assessed in diabetic subjects. Erythropoietin deficiency is owes to efferent sensitive interruption of the kidney that resulted from diabetic nephropathy. The subclinical inflammation is indicating the functional iron insufficiency through increased hepcidin concentration [13], increased non selective proteinuria excretion, transferrin and EPO loss, improved red blood cell destruction and advanced glycation end products [14].

A function of chronic inflammation concerning anaemia in diabetes mellitus is also expected. Recent conclusion recommend that diabetic patients have higher ferritin and hepcidin concentrations than matched non diabetic subjects [15]. Concentrations of ferritin as an indicator of inflammation and hepcidin were shown to associate effectively in numerous populations comprising patients with diabetes mellitus [15,16].

While the previous studies [17-19] have indicated that serum EPO concentrations were lower in T2DM patients compared to control subjects; there were no significant alterations in haemoglobin concentrations between the groups. Low EPO concentrations with normal haemoglobin have been documented in normo albuminuric and micro-albuminuric T2DM patients. Conversely, the previous reports [20,21] in normo-albuminuric T2DM were restricted by non characterisation of patient groups in terms of glomerular filtration rate and albuminuria and/or absence of a control group, and lack of documentation on haematinic concentrations.

Microalbuminuria has been estimated to be the first significant indicator of diabetic nephropathy [22]. Around 30% of T2DM patients may have microalbuminuria or proteinuria at analysis [23]. Primary microalbuminuria may arise from glomerular and proximal tubular dysfunction [24]. On the other hand, in diabetic patients with normal renal function, intensified urinary excretion of N-acetyl-b-D-glucosaminidase (NAG) and Retinol-Binding Protein (RBP) may specify proximal tubular injury and possibly help in recognising patients at high-risk of developing diabetic nephropathy [25].

The most essential correlations of EPO are markers of renal function. Conversely, the most significant determining factor of EPO deficiency is the estimation of the degree of albuminuria [26]. The results of the current study are compatible with previous documents that showed EPO deficiency in patients with T2DM and undiagnosed anaemia in diabetic patients [5,27].

It is often supposed that in patients with diabetes, anaemia may be due to reduced EPO production as a result of declining kidney function, but the results of the applied studies have showed that high number of normoalbuminuric patients have EPO deficiency. These documents suggest that progress of diabetic kidney disease is not a complete originator of low EPO [28]. A previous study described a similar conclusion and established that low EPO and insufficient response to anaemia could be indicators of diabetic kidney disease in the existence or absence of overt renal damage [29]. Progress of EPO deficiency in diabetic patients is prospective gradual development that initiates early during the progress of diabetic nephropathy, interstitial damage decreasing the number of EPO producing fibroblasts and causing EPO deficiency [19].

LIMITATION

The current study was a case-control study which estimated the parameters at one point of time. Other limitations comprise limited sample size with less number of controls.

CONCLUSION

This study confirmed that EPO deficiency is common in diabetic nephropathy patients and increases steadily with advancing duration of diabetes. EPO levels were linked directly with Microalbuminuria concentrations in T2DM patients with nephropathy. Furthermore, the prevalence of EPO deficiency is higher in diabetic patients compared to matched non diabetic subjects.

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