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DISREGULATION OF INFLAMMATORY CYTOKINES FOR IRAQI DIABETICS WITH MODERATE RENAL FAILURE

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ABSTRACT
A study was conducted in the National Diabetes Center (ALmustansiriya University), to evaluate the validity of Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) for early detection of renal failure for diabetics. Renal biomarkers were used to evaluate the renal function for 2 groups of diabetics (total 30 diabetics with nephropathy and the other group 17 diabetics without nephropathy as controls). By estimating glomerular filtration rate (GFR) which represents the best overall index of kidney function. Elevated levels of serum urea nitrogen for diabetics with nephropathy group with mean=48.73 and SD=21 if compared with diabetic controls with mean=23.52 and SD=7. Also elevated levels of serum creatinine of diabetics with nephropathy with mean=1.16 and SD=0.20 if compared with diabetic controls with mean=0.62 and SD=0.17. Estimated glomerular filtration rate (eGFR) by the application of modified diet renal disease equation (MDRD) for creatinine (adjusted for age and sex), the mean of eGFR for diabetics with nephropathy is 58.91 with SD=14.47, while the mean of eGFR for diabetic controls equal to100.58 with SD=18.50. The kidney disease Improving global outcomes (KDIGO) issued the first international guidelines on chronic kidney disease, classify the eGFR of diabetics with nephropathy on stage 2and 3 (mild to moderate reduction of GFR), while the eGFR of diabetic controls on stage1 (normal to elevated GFR). Both IL-6 and TNF-α show an increased serum levels for the diabetics with nephropathy with mean =5.65, SD=3.25 for IL-6 and P value< 0.05(significant) and for TNF-α the mean=28.89, SD=5.85 and P value < 0.01(significant), if compared with diabetic controls with mean=3, SD=1.87 for IL-6 and mean=13.7, SD=5.32 for TNF-α. These results confirm the validity of both IL-6 and TNF-α for early detection 0f nephropathy for diabetics.

KEYWORDS: Interleukine-6, Tumor necrosis factor-alpha, Diabetic Nephropathy, eGFR, Inflammation

INTRODUCTION

Diabetic kidney disease (diabetic nephropathy) is a progressive kidney disease caused by damage to the capillaries in the kidneys glomeruli . It is due to long standing Diabetes mellitus[1] . High levels of blood sugar make the kidneys filter too much blood, this extra work is hard on the filters (glomeruli).The stress of overwork causes the kidneys to lose their filtering ability. Waste products then start to build up in the blood. Later on the kidneys fail. Diabetes causes a number of changes to the body's metabolism and blood circulation that damage the kidneys glomeruli leading to diabetic nephropathy which characterized by persistent increase of blood waste products (urea and creatinine) with a progressive decline in the GFR (glomerular filtration rate) accompanied by elevated arterial blood pressure and this may cause chronic kidney disease. It is also one of the most significant long- term complications of morbidity and mortality for diabetics as a result of kidney failure or irreversible end-stage kidney disease that require dialysis or a kidney transplant. The early diagnosis for kidneys failure keep kidney disease from getting worse by several treatments .In the current study urea and creatinine were estimated for checking kidney function in addition to GFR. Serum urea nitrogen produced during the metabolism of protein in the body, the liver creates ammonia , which is broken down into a by-product urea. Kidneys filter excess urea into the urine and in sweat, but some goes in to the blood stream as serum urea nitrogen, serum urea nitrogen is directly related to the excretory function of the kidneys. It serves as an index for the renal function. A markedly increased urea in the blood is conclusive evidence of severe impairment of glomerular function. A more complete estimation of renal function can be made when interpreting the blood (serum) concentration of creatinine along with that of urea. Creatinine is an important biomolecule because it is the major by-product of energy usage in muscle, it is produced at a fairly constant rate by the body(depending on the muscle mass). Creatinine is chiefly filtered out of the body by the kidneys (glomerular filtration and proximal tubular secretion). If the filtering of the kidney is deficient, creatinine blood levels rise, so the measurement of serum creatinine is an important indicator of renal function [2]. The evaluation of GFR by the application of modified diet renal disease (MDRD) equation for creatinine, in which separate the effects of age and sex from disease[3]. Inflammatory cytokines may contribute to the development and progression of diabetic nephropathy, specifically Interleukin-1, Interleukin-6, Interleukin-8 and tumor

necrosis factor, by affecting the disease via multiple mechanisms.These cytokines act as pleiotropic polypeptides regulating inflammatory and immune responses through actions on cells, providing important signals in the pathophysiology of a range of diseases including diabetes mellitus[4]. Among diverse factors that could interact actively in pathogenesis and progression of diabetic nephropathy poor control of blood glucose, the age, gender, smoking, hypertension and hyperuricemia. All these factors show correlation with diabetic renal diseases[5]. Interleukins are produced by many cells in different tissues. According to their physiologic actions, they classified as anti-inflammatory and pro-inflammatory molecules[6]. Inflammation play an important role in the pathogenesis of diabetic nephropathy[7]. Multiple pathways that joint inflammation with diabetic complications. Interleukine-6 (IL-6) is a pleiotropic cytokine, produced by endothelial cells, leukocytes, adipocytes and mesangial cells[4] .act as both a pro-inflammatory cytokine and an anti-inflammatory myokine in human ,mainly is known as a chief regulator of acute phase inflammatory response[8], it is encoded by the IL-6 gene[9]. It has a direct effect in glomerular and infiltrating cells, this effect modified extracellular matrix dynamics affecting membrane thickening in renal glomeruli[7], as a result increase endothelial permeability and mesangial cell proliferation. Tumor necrosis factor-alpha (TNF-α) an inflammatory cytokine with many determinant actions. It is produced by infiltrating cells as monocytes, macrophages and T lymphocytes as well as kidney cells, including mesangial, glomerular, endothelial, dendritic and renal tubular cells [10]. TNF-α alters glomerular hemodynamics and promotes increased vascular endothelium permeability infiltration by inflammatory cells, neo-formation of extracellular matrix, production of reactive oxygen species and blood flow disturb are other recognized effects in renal structures[7]. A comparison study was established for serum IL-6 and TNF-α of both diabetic nephropathy patients and diabetic controls. Different statistic studies were applied using SAS (Statistical Analysis System). Normality for data confirmed by Shapiro test, $p<0.05$ was considered statistically significant. Results also confirmed the validation of the two cytokines IL-6 and TNF-α for early detection of diabetic nephropathy.

PATIENTS & METHODS

The study was conducted in the National Diabetes Center (Al – Mustansiriya University), a total 30 diabetics type 2 with mild to moderate nephropathy (19 males, 11 females), age range (40 –72) years and 17 diabetics type2 without nephropathy (7 males, 10 females), age range (39 – 55) years (controls). Enzymatic method was applied for determination of glucose (SPINREACT com). Glucose oxidase (GOD) catalysis the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H_2O_2) is detected by a chromogenic acceptor, phenol-aminophenazone in the presence of peroxidase (POD). The intensity of the color formed is proportional to the glucose concentration in the sample[11]. One ml working reagent which is a mixture of TRIS PH7.4 with phenol and enzymes (Glucose oxidase, peroxidase with 4-aminophenazone), is added to 10μl of sample and standard, incubate for 20 minutes at room temperature. Then, reading the absorbance of both sample and standard against the blank. Urease Modified Berthelot Reaction (BIOMERIEUX com) is applied for the determination of urea concentrations. Urease hydrolyzes urea by producing ammonium. In an alkaline medium,the ammonium ions react with the salicylate and hypochlorite to form a green colored indophenols (2-dicarboxylindophenol). The reaction catalyzed by the sodium nitroprusside. The color intensity is proportional to the urea concentration in the sample[12]. One ml of the working reagent was prepared by the addition of urease enzyme to a mixture of (phosphate buffer pH8, sodium salicylate, sodium nitroprusside and EDTA), then added to 10μl of sample and standard, incubate for 5 minutes at room temperature, after that 200μl of alkaline reagent (sodium hydroxide and sodium hypochlorite) is added to the reactant and incubate for additional 10 minutes at room temperature. Then reading the absorbance of both the sample and standard. Creatinine JAFFE Reaction: For the determination of serum creatinine, in an alkaline solution, creatinine reacts with picric acid to form a colored complex. The rate of formation of the complex is measured and it is directly proportional to the amount of creatinine in the sample[13]. One ml of working reagent (alkaline buffer with picric acid) is added to 10μl of both sample and standard, gently mix and incubate for 20 minutes at 25ºC, measure the absorbance of sample and standard against blank. Glomerular Filtration Rate (GFR) was estimated by modified diet renal disease (MDRD) equation for Creatinine[3], and the equation was adjusted for age and sex. The kidney disease Improving Global Outcomes (KDIGO) in 2004 issued the first international guidelines on chronic kidney disease (CKD), including a definition and classification of kidney disease (KD) in comparison with eGFR, as shown in table (1).

TABLE 1: GFR (Glomerular Filtration Rate)

Stage	GFR(ml ⁄ min ⁄ 1.73m2)	Description
1	≥ 90	Normal or elevated GFR
2	60--89	Mild GFR reduction
3	30--59	Moderate GFR reduction
4	15--29	Severe GFR reduction
5	< 15	Renal failure

GFR determination provides the basis for detection and classification of CKD. The GFR usually expressed in ml ⁄min ⁄1.73m^2 and provides the volume of blood that is cleared per minute by the kidneys, standardized for the body surface, which is 1.73m^2 for the average person. According to the (KDIGO) staging which depends on GFR, the estimated GFR of the patients included in the study (diabetics type2) is categorized in stage 2 and 3 (mild to moderate GFR reduction). The eGFR of the diabetics (controls) is categorized in stage 1 (normal GFR). Human Interleukin-6 (IL-6) ELISA Kit[14], for quantitative determination of (IL-6) concentrations in serum was used, The micro-titer plate provided has been pre-coated with an antibody specific to IL-6, 100μl of standards or samples are then added to the micro-titer plate wells with a biotin-conjugated polyclonal antibody preparation specific for IL-6 and avidin conjugated to Horseradish peroxidase (HRP) is added to each micro plate well and incubated. Then a TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution is added to each well. Only those wells that contain IL-6, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at wavelength of 450nm. The concentration of IL-6 in the sample is then determined by comparing the OD. of the samples to the standard curve. Tumor necrosis factor-alpha (TNF-α)[15] enzyme linked immunosorbent assay(ELISA) applies a technique called a quantitative sandwich immunoassay. During the first incubation period TNF-α in samples are captured by the monoclonal antibody to human TNF-α coated on the wall of the micro titer wells. After washing away the unbound components from samples, a peroxidase-labeled second monoclonal antibody conjugated is added to each well and then incubated. After a second washing step, the bound enzymatic activity is detected by addition of tetramethyl benzidine (TMB) chromogen-substrate, Finally, the reaction is terminated with an acidic stop solution and the optical density is measured with a photometer at 450nm.The intensity of the color is directly proportional to the concentration of human TNF-α in sample. The result is expressed as pg⁄ml.

Statistical Analysis

After collection and categorization of data for 30 patients (diabetics with mild to moderate renal failure), compared with 17 diabetics without nephropathy (controls). Statistical analysis was performed using SAS (Statistical Analysis System. Version 9.1[16]. Data were tested for normality by shapiro test.

RESULTS

The biochemical parameters for the patients and controls were established in table 2, which showed the mean, standard deviation, minimum and maximum values for these parameters and p value. In comparison with normal values that listed by the National Diabetes Center, except IL-6 and TNF-α normal values from other references [17].

TABLE 2

Biochemical parameters	Mean	SD	Min	Max	P value	Normal value
Fasting blood glucosemg⁄dl						75—109 mg⁄dl
Diabetics with nephropathy	179.3	70.25	104	385		
Diabetic controls	99.17	9.75	79	118	< 0.05	
Serum Urea mg⁄dl						20--45mg⁄dl
Diabetics with nephropathy	48.73	21	22	106	< 0.05	
Diabetic controls	23.52	7	19	47		
Serum Creatinine mg⁄dl						0.7--1.3mg⁄dl
Diabetics with nephropathy	1.16	0.20	0.6	2.2	< 0.05	
Diabetic controls	0.62	0.17	0.5	1.2		
eGFR ml⁄min⁄1.73m^2						≥90ml⁄min⁄1.73m^2
Diabetics with nephropathy	58.91	14.47	25.5	83.4	< 0.05	
Diabetic controls	100.58	18.50	82	136		
Interleukin-6(IL-6)pg⁄ml						2.6--15pg⁄ml
Diabetics with nephropathy	5.65	3.25	1.3	42.3	< 0.05	
Diabetic controls	3	1.87	0.69	8.2		
TNF-α pg⁄ml						0--16pg⁄ml
Diabetics with nephropathy	28.89	5.85	14	37.4	< 0.01	
Diabetic controls	13.7	5.32	4.2	25.5		

eGFR by modified diet disease (MDRD) equation for creatinine that adjusted for age and sex. Calculations shows the mean of eGFR for diabetics with nephropathy is 58.91 with a range of (25.5—83.41), according to the classification of (KDIGO) this range represent mild to moderate GFR reduction. The mean of eGFR for controls (diabetics without nephropathy) is 100.58 with a range of (82—136), which is mostly within the normal GFR values. Both IL-6 and TNF-α shows a significant increased levels for diabetics with nephropathy,($p< 0.05$) for IL-6 and ($p< 0.01$) for TNF-α (table-2-).

DISCUSSION

Inflammation may be a key factor which is activated by the metabolic, biochemical and hemodynamic derangement known to exist in the diabetic kidney. Diabetes mellitus is the leading cause of chronic renal failure and is increasing as a cause of morbidity and mortality, diabetic nephropathy is one of the most important disorder that should be followed[17]. Impairment of renal function in patients with diabetes is evident[18]. The sooner kidney dysfunction is diagnosed and treated the greater odds of preserving remaining nephrons and preventing the need for dialysis. Both type 1 and type 2 diabetes causes renal dysfunction but principally type2 because of the impact of its complications[5], moreover the onset of diabetes is not usually well established with diabetes. The small blood vessels in the body are injured and when the blood vessels in the kidneys are injured the kidneys cannot clean the blood properly, and the body will retain more water and salt, also waste materials will build up in blood. Diabetes also may cause damage to nerves, so this can cause difficulty in emptying bladder and the pressure resulting from full bladder can back up and injure the kidneys. Also, if urine remains in bladder for a long time leading to development of an infection from the rapid growth of bacteria in urine that has a high sugar level. Different renal biomarkers were used for the diagnosis of the kidney function. Blood urea is directly related to the excretory function of the kidneys, it serves as an index for its function. A markedly increased blood urea is conclusive evidence of severe impairment of glomerular function. Creatinine is most widely used biomarker of kidney function by estimating how much blood the glomeruli filter in a minute. The calculation of eGFR is based on the amount of Creatinine (waste product) found in a blood sample. As the level of Creatinine goes up, the eGFR goes down. Kidney diseases are present when eGFR is less than 60ml ⁄ min ⁄ 1.73m^2. It has been found that despite all pharmacologic therapies available for diabetic nephropathy treatments, some patients develop kidney damage that is why the need of complete understanding of molecular, metabolic and environmental factors that leads to diabetic nephropathy and their interaction between them. Many mechanisms were investigated and these are divided in mechanisms of immune cell infiltration of kidney, molecules involved in progression and intracellular pathways activated in diabetic nephropathy. Activation of the immune system and chronic inflammation are both involved in pathogenesis of diabetes and as a result diabetic nephropathy, studies have demonstrated that cytokines, chemokines, growth factors, adhesion molecules, nuclear factors as well as immune cells as monocytes, lymphocytes and macrophages are all involved in diabetic pathogenesis and of course play an important role in diabetes complications[5]. Cytokines act as pleiotropic polypeptides regulating inflammatory and immune responses through actions on cells. They provide important signals in the pathophysiology of a range of diseases, including diabetes mellitus .Chronic low –grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of diabetes and its micro vascular complications. Inflammatory cytokines, mainlyIL-1, IL-6 and IL-8 as well as TNF-α, are involved in the development and progression of diabetic nephropathy[4] . In current study it was found that IL-6 serum levels are significantly higher in diabetic type 2 nephropathy than the levels that observed in diabetic patients without nephropathy (controls). IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relation with increased endothelial permeability and mesangial cell proliferation. It has in addition to its immuno-regulatory actions been proposed to affect glucose homeostasis and metabolism directly and indirectly by action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic β cells, and neuroendocrine cells. So IL-6 act in nonimmune events in most cell types and tissues outside the immune system[19]. Thus inappropriate regulation of IL-6 may play a direct protective or deleterious role in diseases where IL-6 or other inflammatory factors cause a low-grade inflammation (like in type2 diabetes)[20]. Also this study shows that the TNF-α serum levels for the diabetic patients with nephropathy are significantly higher than the levels of diabetic (controls). TNF-α is an inflammatory cytokine with many determinant actions in inflammatory response by several tissues and pleiotropic-effects, it is produced by infiltrating cells as monocytes, macrophages and T lymphocytes as well as kidney cells, its actions are widely known as systemic and in many cases direct cytotoxic effect in kidney cells principally. When TNF-α binds to the receptors several signaling pathways are activated and a cascade of molecules begins their expression in renal cells, many of these actions results in apoptosis and necrosis [20]. Also TNF-α alters glomerular hemodynamics and promotes increased vascular endothelium permeability infiltration by inflammatory cells, neo-formation of extracellular matrix and blood flow disturbs are other recognized effects of TNF-α in renal structure[7].

CONCLUSION

Certainly, inflammation plays an important role in the pathogenesis and development of diabetic nephropathy. Multiple pathways that joint inflammation with diabetic complications. The blockade of the principle mediators could be useful in the prevention of these complications; several studies have been designed in order to identify therapeutic targets. And better understanding of the inflammatory response in diabetic kidneys is expected to identify novel anti-inflammatory strategies for the potential treatment of human diabetic nephropathy.

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