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RESEARCH ARTICLE

SERUM LEVELS OF TNF-α AND IL-6 IN PATIENTS WITH DIABETIC NEPHROPATHY

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ABSTRACT

Background: Diabetic nephropathy (DN) is one of the chronic complications of diabetes mellitus (DM) that cause a common end-stage renal disease (ESRD). Cytokines regulate inflammatory processes in response to the degree of inflammations. Overproduction of pro-inflammatory cytokines such as tumor necrosis factoralpha (TNF-α) and interlukin-6 (IL-6) play a role in the development and progression of DN.

Aim: To evaluate the levels of TNF-α and IL-6, and their implication in diagnosis and progression of nephropathy in patients with type 2diabetes mellitus (T2DM).

Subjects and Methods: Sixty subjects (45 patients, 15 normal) were selected from Suez city and general hospital in Suez, with mean age43.3±8.2years. They were classified into four groups, Group I:healthy subjects, Group II:T2DMpatients; GroupIII: Nephropathicpatients identified by high level of creatinine in blood and microalbuminurea; and Group IV: Nephropathic patients with T2DM as identified by high level of creatinine and glucose in blood and microalbuminurea. Blood and urine samples were collected and tested for fasting and postprandial blood glucose (PBG) level, glycosylated hemoglobin (HbA1C), liver and kidney function tests. In addition, serum TNF-α, and IL-6levels were measured by Enzyme Linked Immune Sorbent Assay (ELISA).

Results: The level of IL-6 was significantly increased ($p<0.05$) in nephropathy and DN groups compared to control and T2DM groups with a significant positive correlation between its level and the duration of diabetes in DN group and significant negative correlation between its level and duration of diabetes in T2DM group. On the other side, TNF-α levels were significantly decreased($p<0.05$) in both nephropathy and DN groups compared with control and T2DM groups which was not correlate to the duration of diabetes in either T2DM or DN groups.

Conclusion: Tracking the levels of IL-6 cytokine in T2DM patients could be used as a marker for progression to DN patients.

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INTRODUCTION

Diabetesmellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction, and failure of different organs, especially kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2010). The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In one category, type-1 diabetes, the cause is an absolute de ciency of insulin secretion. In the other, much more prevalent category, T2DM, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (American Diabetes Association, 2010). With T2DM, pancreatic cells do not produce enough insulin or other somatic cells do not respond to insulin normally (insulin resistance), even though the pancreas producesinsulin (Duyff, 2006).T2DM is characterized by hyperglycemia associated with microvascular, macrovascular and neuropathic complications and hyperglycemiadue to lack of endogenous insulin. The explosive increase in number of people diagnosed with diabetes makes this disease epidemic in this century (International Diabetes Federation, 2013). Egypt is one of top

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10 countries for number of people with diabetes, according to the IDF latest estimates, 7.5 million people, or 15.56% of the adult population, have diabetes. This number is set to be 13.1 million by 2035, where diabetes killed more than 86,478 of Egyptian adults in 2013.

Diabetic nephropathy (DN) is the major microvascular complication of DM which could progressively develop renal impairment (Libby *et al*., 2002). It is a major cause of end-stage renal disease (ESRD) (Colhoun *et al*., 2001). DN is defined as partial loss of kidney function followed by nephrotic syndrome and glomerulosclerosis. The earliest detectable change is in the thickening of the glomerular basement membrane (GBM) that filters the blood, the damage to the membrane and the cells next to it in the capillary walls causes albumin to leak from the blood into the urine (albuminuria and proteinuria). Depending on the values of microalbuminuria (μalb) and based on the rate of Urinary Albumin Excretion (UAE), DN is divided into 5 stages: the first two stages hyper-filtration and silent phase are normo-albuminuria, μalb is stage 3 and proteinuria in stage 4 and ESRD is in the last stage (Mogensen *et al*., 1983).Hyperglycemia is a crucial factor in the development of DN because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. In vitro studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production(Harris *et al*., 1991) and mesangial cell apoptosis (Mishra *et al*., 2005). Leukocytes, monocytes, and macrophages have all been implicated in the process of DN (Chow *et al*., 2004) and circulating inflammatory markers and pro-inflammatory cytokines are strongly associated with the risk of developing complications(Nguyen, 2006).Cytokines are secreted by cells in response to a stimulus which modulates the behavior of target cells (Dixon and Philips, 1993). Cytokines act as pleiotropic polypeptides regulating inflammatory and immune responses through actions on cells. It is known that, inflammatory cytokines, mainly IL-1, IL-6, and IL-18, as well as TNF-α, are involved in the development and progression of DN (Joseph *et al*., 2002).TNF-α induces a local in ammatory response by initiating a cascade of cytokines and increasing vascular permeability, thereby recruiting macrophage and neutrophils to site of infection (Sugimoto *et al*., 1999; Pamir *et al*., 2009). Urinary TNF-α levels are also elevated in T2DM patients and TNF-α levels rise as DN progresses, suggesting that increased TNF-α levels contribute to the development of renal damage (Kalantarinia *et al*., 2003; Navarro *et al*., 2006). Many studies on T2DM patients demonstrate a significant relationship between IL-6 and glomerular basement membrane thickening, which considered a strong predictor of renal disease progression (Sekizuka *et al*., 1994). In 1994, Sekizuka *et al*. reported that serum levels of IL-6 were significantly elevated in patients with T2DMand its level continue to increase in diabetic nephropathy patient (Sekizuka *et al*., 1994). These data suggest that IL-6 may play a role in the pathogenesis of diabetic nephropathy (Suzuki *et al*., 1995). The aim of the present study is to tracking the level of inflammatory markers as TNF-α and IL-6 as apredictor for diabetic nephropathy.

SUBJECTS AND PATIENTS

This study was done on sixty Egyptian subjects (15 normal, 45 patients) selected from those attending the outpatient clinic of Suez general hospital during the period from 2011 to 2012. The demographic findings (Table 1) showed that 33 (55%) of subjects were females and 27 (45%) were males. Age of subjects ranged from 21 to 70 years with mean of (43.18 ± 10.38) years. The 15 normal subjects were classified as group I: control group (9 females, 6 males). Forty-five adults' patients were classified into three groups, group II: T2DMpatients (11 females, 4 males), group III: nephropathy patients (6 females, 9 males)and group IV: type2 diabetic nephropathy patients (7 females, 8 males). A written consent was taken from every subject included in this study.

METHODS

Five ml of blood were collected from each subject by vein puncture after fasting for at least 8 hrs. The blood samples were divided into three fractions. The first fraction was left without coagulant for separation of serum until used for estimation of TNF-α(Bonavida, 1991) and IL-6(Bauer and Herrmann, 1991) by ELISA technique according to the manufactural instructions (Thermo Fisher Scientific Inc. USA).The second fraction was added to sodium fluoride (NaF) as an anticoagulant to inhibit the glycolysisand plasma fraction used for estimation of fasting glucose (FBG); prandial blood glucose(PBG) by glucose oxidase-peroxidasemethod (Barham and Trinder, 1972) (BioMed diagnostics kit, Egypt;glycated hemoglobin(HbA1c) by ion exchange chromatography (Trivelli *et al*., 1971) (BioMed diagnostics kit, Egypt). The third fraction used as plasma after adding ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and used for liver and kidney function tests with spectrophotometric analysis using commercial kits (BioMed diagnostic, Egypt). Five ml of urine samples were collected from all subjects in the morning, then centrifuged at 3000 rpm (microfuge, UK) for 5 minutes, 1 ml samples were separated and used in microalbuminuria test determined by Immuno-turbidometry latex method(Bernard and Lauwerys,1983) (BioMed diagnostics kit, Egypt).

Statistical analysis

Analysis of the data was performed by using the computer program SPSS (Statistical Package for the Social Science, version 17.0). Results were expressed as mean ± SD or ±SE and analysis was done using one-way ANOVA. Significance was accepted at the level of P values <0.05. Correlation between studied cytokines and other studied parameters were tested using Pearson correlation coefficients (Levesque, 2007).

RESULTS

Demographic characteristics of the groups in the present study are shown in Table (1). The collective data showed that the mean of durations of diabetes for T2DM group was (2.6 ±1.3) years and DN group was (14.2±5.9) years.

Table 1. Clinical and demographic characteristics of the four studied groups

Characteristic	group I normal subjects	group II T2DM patients	group III nephropathy patients	group IV DN patients
Sex				
Female (n)	9 (60%)	11 (73.3%)	6 (40%)	7 (46.7%)
Male(n)	6 (40%)	4 (26.7%)	9 (60%)	8 (52.3%)
Age mean ± SD	38.8±11.4	41.0±12.0	43.93±7.04	51.6±7.7
Male mean ± SD	33.33±8.82	40.5±27.58	47.17±7.44	52±9.82
Female mean ± SD	42.56±11.86	39.11±11.39	42.66±7.53	50.86±7.56
Duration of DM mean ± SD	----------	2.6±1.3	----------	14.2±5.9
Systolic Blood Pressure (sBP) mmHg mean ± SD	117±11	122.6±8.8	136.3±8.12	127±7.97
Diastolic Blood Pressure (dBP) mm Hg mean ± SD	74.2±6.4	80±6.5	90.33±6.11	83.33±5.62

SD standard deviation

Table 2. Biochemical markers for the four groups under study

Groups Parameters	Group Inormal subjects (n-15)	Group IIT2DM patients (n-15)	Group III nephropathy patients (n-15)	Group IVDN patients (n-15)
FBG mg/dl	84.4^{a} ± 2.4	213.9^{c} ± 15.9	84.6^{a} ±3.67	181.4^{b} ± 11.54
PBG mg/dl	103.8^{a}±1.8	267.1^{c}±16.8	101.9^{a} ± 3.1	219.2^{b} ± 13.28
HbA1c %	6.2^{a} ± 0.05	9.9^{b} ± 0.23	6.1^{a} ± 0.02	10.1^{b} ± 0.28
AST u/l	26.1^{a}± 0.9	28^{a} ± 2.5	18^{b} ± 1.27	22.07^{a}±1.54
ALT u/l	25.47^{a} ± 1.02	27.6^{a}± 4.3	19.7^{a} ± 1.57	22.2^{a} ±1.9
TP g/l	7.2^{a} ±0.15	7.1^{a} ±0.13	6.6^{b} ± 0.16	6.3^{b} ±0.12
Alb g/dl	3.67^{a} ± 0.06	3.86^{a} ± 0.08	3.65^{a}±0.08	3.92^{a}±0.14
Tbil mg/dl	0.55^{a} ± 0.02	0.72^{b} ± 0.05	0.95^{c}±0.04	0.82^{b} ±0.05
Cr mg/dl	0.78^{a}±0.03	0.87^{a} ±0.05	2.89^{c} ± 0.36	1.49^{b} ± 0.20
Ur mg/dl	24.87^{a}± 0.7	28.93^{a}±1.75	81.07^{c}± 9.77	47.67^{b} ± 4.46
µ-Alb mg/l	12.73^{a}± 0.79	57.07^{b}±3.86	110.67^{c} ± 6.59	107.67^{c} ± 10.87

Results are Mean ± S.E.
Similar characters denote insignificance between groups
The mean difference is significant at p<0.05 between groups.

Table 3. Cytokines levels of the four groups under study

Groups Parameters	Group I normal subjects	Group II T2DM patients	Group III nephropathy patients	Group IV DN patients
TNF-α pg/ml	0.14^{a} ± 0.01	0.14^{a} ± 0.02	0.07^{b} ± 0.01	0.08^{b} ± 0.01
IL-6 pg/ml	0.09^{a} ± 0.01	0.08^{a} ± 0.01	0.15^{b} ± 0.02	0.14^{b} ± 0.01

Results are Mean ± S.E.
Similar characters denote insignificance between groups.
The mean difference is significant at p<0.05 between groups.

Table 4. Correlation study between duration of diabetes and cytokines levels

Cytokines	T2 DM Patients (n=15)	DN Patients (n=15)
TNF-α	0.077	0.021
IL-6	-0.661*	0.674*

*: Significant Correlation

Table 5. Correlation study between IL-6 levels and studied parameters among four groups

Parameter	Control group	T2 DM patients	Nephropathypatients	DN patients
HbA1c	0.187	-0.616*	-0.258	-0.455
Creatinine	-0.067	-0.318	0.322	0.562*

*: Significant Correlation

Table (2) illustrates the differences among the studied parameters in the four groups. The results revealed amoderate significant increase in FBG, PPG, and HbAc1 for DN group ($p<0.05$) and highly significant elevation forT2DM group ($p<0.001$) compared with control group. In comparison with control group, kidney function tests showed a significant elevated levels in creatinine (Cr) and urea (Ur), in addition to total bilirubin (Tbil) in nephropathic patients ($p<0.001$) and T2DM &DN ($p<0.05$) patients. Moreover, µAlb was significantly elevated in T2DM ($p<0.05$) and high significant elevation in nephropathic patients and DN patients ($p<0.001$).On the other side, AST and TP showed a moderate significant elevation in nephropathy group ($p<0.05$), while only TP was moderate elevated in DN group ($p<0.05$) compared with control group. The TNF- α and IL-6 profile were examined for all studied groups by testing their plasma levels by using ELISA technique. Table 3 indicated that TNF-

α level was significantly decreased in nephropathy and DN groups ($p<0.05$) compared with control and T2DM group. Meanwhile, IL-6 level was significantly elevated in both nephropathy and DN groups ($p<0.05$) compared with control group.

The correlation coefficient (–r) between studied cytokines and the duration of diabetes amongT2DM and DN patients are represented in Table (4). A correlation was reported between IL-6 levels and duration of diabetes; it was negative significant in T2DM group and positive significant correlation in DN group. On the other side, TNF-α did not show any significant change among any of these groups.

Table 5 represents the significant correlation between IL-6 cytokine with different measured parameters among four studied groups. It is clear that IL-6 showed a negative correlation with HbA1c in T2DM and a positive correlation with creatinine in DN group. While TNF-α did not show any correlation with any of measured parameters.

DISCUSSION

The duration and intensity of high blood glucose level playsan important role in glycosylation of proteins and lead to changes in the shape of the endothelial cells lining the blood vessels; glycoprotein formation and basement membrane become thick and weak (Bajaj, 2002).Kidney damage rarely occurs in the first 10 years of diabetes, where 15 to 25 years will usually pass before kidney failure occurs. Early detection of renal damage may help to delay the process.

Salmela *et al.* in 1984studied the liver function tests of 175 diabetic patients without chronic liver disease, 57% had at least one abnormal liver function test (LFT), 27% had at least two abnormal LFTs. However, these increases in liver function values were rarely more than two times of the upper limit of normal. The results of our study showed thatALT, AST, TP, and albumin were within normal range of T2DM patients compared with control group, while bilirubin was significantly elevated ($p<0.05$), which is in agreement with(Han*et al*., 2012)who reported that 4.9% of diabetic patients had high bilirubin level compared with control group. Albuminuria is a well-known predictor of poor renal outcomes in T2DM patients (Ghazalli and Meng, 2003) therefore patients are usually with normal renal function and microalbuminuria excretion (UAE) rate is (<30 mg/24hr).The first sign of renal involvement in T2DM patients is most often microalbuminuria (µAlb)which is several fold higher (30 to 300 mg/24hr), and these patients were classified as nephropathypatients (Remuzzi *et al*., 2002).In the present study, there were a significant statistically positively increase in µAlb excretions in T2DM ($p<0.05$), nephropathy and DN ($p<0.001$) groups with respect to the normal group. In addition, our results showed a significant increase change in µAlb excretions in DN group with respect to T2DM group by 89%.

Gross *et al.* (2005) reported that, the progression to µAlb or overt nephropathy occurs in 20% to 40% of patients over a period of 15 years after the onset of diabetes (Remuzzi *et al*., 2002; United States Renal Data System USRDS, 2007). In addition, there is an accumulating evidence to prove that immunologic and inflammatory mechanisms play a significant role in development and progression of DN (Chow *et al*., 2004; Nguyen, 2006).Moreover, increases in TNF-α levels and its excretion precede the increase in albuminuria in diabetes, and its level risesin DN, suggesting that increased TNF-α levels contribute to the development of renal damage (Kalantarinia *et al*., 2003; Navarro *et al*., 2006). Therefore, inflammatory cytokines level such as TNF-α and IL-6 may be used as predictor markers of DN and open the possibility of new potential therapeutic targets.

Navarro *et al.* (2003) studiedT2DM patients with microalbuminuria (µAlb), and proved that serum and urine TNF-α were significantly higher in diabetic than in control subjects. These results are in agreement with previous data(Navarro *et al*., 2003), who observed that serum TNF-α level in diabetic patients with microalbuminuria were significantly increased as compared to those without albuminuria. In the present study, no significant difference in TNF-α level was recorded in T2DM group and a significant decrease ($p<0.05$) in its level in both nephropathy or complicated DN patients with µ Albcompared with control group. Our data may indicate that the level of µ Albreflects the pathogenicity of kidney but the level of TNF-α still low. Therefore, in contrast with previous data, TNF-α level may not reflect exactly the status of kidney at mild injuries, and therefore, it is not a suitable cytokine marker for kidney damage.

Moriwaki *et al.* (2003) reported that IL-6 level was same in T2DM patients without albuminuria as control, but significantly increased in patients with DN and clinical albuminuria. The results of present study were in agreement with previous data (Moriwaki *et al*., 2003; Choudhary and Ahlawat, 2008) in case of T2DM and its progression to DN patientwith µAlb where a significant elevation ($p<0.05$)in IL-6 level among nephropathy and DN groups compared with control group. Also IL-6 level was higher in other study(Choudhary and Ahlawat, 2008)inT2DM patients with µAlb and had higher values of serum creatinine. Thus, IL-6 cytokines may be a good marker for indication of progression T2DM to DN. Duration of time in diabetic patients with µAlb is a key parameter for progression of T2DM to DN for early diagnosis of kidney injury. Asignificant negative correlation was reported between IL-6 levels and the duration of diabetes in T2DM group, which changed to a significant positive correlation in DN group. This finding is very important which indicated thatIL-6 level increased during progression of T2DM to DN patients with µAlb. This data reflect that IL-6 level is a predictor cytokine in case of T2DM and gradually leading to progression to DN disease.

The correlation between different parameters in current study and IL-6 level showed that, serum creatinine reported a significant positive correlation with IL-6 in the patients with DN. In addition to a significant positive correlation in DN group with duration of diabetes reflecting the concept that IL-6 is a perfect diagnostic. This finding may support the use of IL-6 level as diagnostic tool for suspected nephropathy patients among diabetic patients in Egyptian population. However,

convincing evidence has been reported that renal damage rarely occurs in patients with T2DM when PBG levels are < 200 mg/dl and HbA1C is < 7.5% to 8.0% (Nosadini and Tonolo, 2004). In the present study, the glucose profile for DN group showed that PBG level is 219.2 ± 13.28 mg/dl, where HbA1C was exceeds 8.0% (10.13 ± 1.08) compared with control group.

Conclusion and prospective

Pro-inflammatory cytokines IL-6 may play a role in the development and progression of DN among T2DM patients. The present study reveals that with the development of T2DM patients to DN patients the IL-6 level was significantly increased, and it showed a significant positive correlation with demographic parameters such as (duration of diabetes and creatinine). So tracking the level of IL-6 in T2DM patients may be used as early diagnostic tool for suspected kidney injury in Egyptian population, and may be used as a marker to prevent more complications or turned to DN patients. Hence, more studies may be necessary to understand the pathophysiology and new therapeutic interventions of diabetic nephropathy.

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