The relationship between Erythropoietin and adiponectin and the inflammatory cytokines .

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Abstract: Inflammatory cytokines, erythropoietin (EPO) and adipocytokines, including adiponectin, leptin and tumor necrosis factor-alpha (TNF-α) play a role in pathogenesis of type 2 Diabetes mellitus. However, the relationship between erythropoietin (EPO) level and the inflammatory cytokine in type 2 diabetic patients with or without renal complication are not clear. This prospective cohort study is aimed to investigate the relationship between erythropoietin, adipocytokines and inflammatory cytokines in type 2 diabetic patients with or without renal complications based on the serum levels of their urea and creatinine. Patients were classified as group-1 (n = 66), in comparison with those suffering renal complications as group-2 (n = 54). Serum levels of EPO, adiponectin and inflammatory cytokines, TNF- α and IL-6 together with other biochemical parameters were measured in total patients suffering type 2 DM (n=120). Serum levels of EPO, adiponectin and inflammatory cytokines including; TNF- α and IL-6 were measured using enzyme linked immunosorbent assay (ELISA). Our results showed that the typ2 diabetic patients in general, had significantly high interleukin-6 concentrations (mean=25.53±2.36) and no significant difference between the two groups (p>0.05). However, TNF-α were significantly higher in group2 than group1 (p=0.01). On the other hand, serum concentration of EPO, adiponectin and haemoglobin (Hb) levels were significantly lower in group-2 than group-1 (p=0.037, p<0.005, p<0.005 respectively). There were significant positive correlations between EPO and adiponectin in the two groups (r=0.316, p<0.01 and r=0.320, p<0.05 respectively); while no significant correlation was found between EPO and TNF- α or IL-6, in either groups. In conclusion: These results show that the low serum level of EPO may associate with the low serum level of adiponectin in patients with type-2 DM, especially in those with renal complications, but not with TNF- α or IL-6.

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1. Introduction

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycaemia, varying degree of insulin deficiency and/or impaired insulin action with disturbance in protein, lipid and carbohydrate metabolism. Complications of DM are several including; coronary heart disease, cerebrovascular disease, peripheral neuropathy, eye and kidney diseases [1].

Epidemiological studies of type 2 diabetes indicate that the disease is a rapidly growing problem in several countries. It affects large numbers of people from a wide range of ethnic group all over the world [2]. Type 2 diabetes is usually associated with the presence of insulin resistance which may sometime combine with a relative insulin deficiency [3].

Inflammation and activated monocytes will results in increasing the level of inflammatory markers, e.g., C-reactive protein, plasminogen activator inhibitor-1 and inflammatory cytokines including: TNF-α, IL-6, IL-1β and IFN-γ [4-6].

In type 2 diabetes the pancreatic islet β cells failure and reduction of β-cell mass are predominantly associated with the increase in circulating cytokines and free fatty acids as the chronic exposure of β cell to these mediators induces excessive production of reactive oxygen species (ROS) and activation of caspases which inhibit insulin secretion and promote apoptosis of pancreatic β cells [7-9]. Various pro-inflammatory cytokines including TNF-α , , IFN-γ, IL-6, leptin, resistin and adiponectin have been reported to be involved on pancreatic β-cell dysfunction and subsequently, in pathogenesis of type 2 diabetes [7,8].

Human and experimental animal studies of type 2 diabetes revealed the presence of abnormal changes in cytokine profile in both islet β-cell and plasma, where TNF- α, IL-1 beta, VEGF and IL-6 are involved in development of microvasular diabetic complications such as nephropathy and diabetic foot [10-12]. In addition to IL-6 and leptin which has been found to be elevated in newly diagnosed type 2 diabetes [13, 14]. Furthermore IFN-γ gene polymorphism has been detected in patients with type 2 diabetes and latent autoimmune diabetes of adult (LADA) [15].

Recently; there is an extensive work to study the relation and the involvement of erythropoietin (EPO) in diabetes mellitus. These studies indicate the presence of EPO deregulation in patients with type 2 diabetes and the presence of a strong cyto-protective effect of EPO on vascular cells that exposed to elevated glucose [16-19].

EPO is a 30.4 kDA glycoprotein, It is a hematopoietic growth factor that regulate red blood cell production by stimulating the proliferation and survival of erythroid progenitor. The primary organs of EPO production is the kidney; whereas, the secondary sites involved the liver and the uterus. Several other organs can secret EPO including peripheral endothelial cells (ECs), muscle, and insulin-producing cells [20]. The biological effects of EPO are exerted by its interaction with specific receptor on a target cell. EPO receptors are usually found in erythroid progenitor cells and in a variety of non- hematopoietic cell types including: neurons, endothelial cells, cardiomyocytes, and renal cells [21]. The primary biological function of EPO is to regulate the erythropoiesis; however, EPO has other several functions. It inhibits apoptosis in a wide variety of cell type, plays a potential neuro- and cardio-protective role against ischemia and involved in angiogenesis, neurogenesis. It also plays an important role in reducing inflammation and local edema and it has been reported that EPO enhance cognition in normal and diseased human subjects [22].

Recent interest has been focused upon the inhibitory effect of EPO on pro-inflammatory cytokines. It was found that EPO can directly inhibit several pro-inflammatory cytokines, such as IL-6, TNF-α, and monocyte chemoattractant protein 1 [23]. A number of experimental studies reveal the inhibitory effect of EPO on neuroinflammation in a number of autoimmune diseases [24]. However, a little information is available on the relation between the inflammatory cytokines and EPO in type 2 diabetes. Therefore, in this study we aimed to investigate the relationship between EPO and adiponectin, also between EPO and the pro-inflammatory cytokines, IL-6, TNF-alpha in type 2 diabetic patients with renal failure in comparison with diabetic subject without renal complications.

2. Materials and methods

The Current prospective study was carried out at King Abdulaziz University Hospital (KAUH) in Jeddah, Kingdom of Saudi Arabia between March 2010 and May 2011.

A total of 120 adult male Saudi patients were recruited in this cross sectional study from outpatient diabetic clinic at KAUH. Patients were classified into two groups; group-1 that includes 66 type 2 diabetic patients who have no renal involvement and have no other diabetic complication and group-2 that includes 54 type 2 diabetic patients who represented with renal complication based on high concentrations of serum levels of Blood Urea Nitrogen (BUN) and creatinine.

The diagnosis of type 2 diabetes was made according to the new criteria of American Diabetes Association based on fasting blood glucose ≥126 mg/Dl or glycosylated haemoglobin A1c (HbA1c) ≥6.5%. Type 2 diabetic patients were treated with hypoglycaemic drugs including insulin, metformin and sulfonylurea, lipid lowering drugs.

The following were excluded from the study:1) patients with type 1 diabetes, 2) patients with complicated type 2 diabetes other than renal complication such as patients with cardiovascular or peripheral neuropathy, 3) patients with clinical or laboratory evidence of other hormonal abnormalities or serious systemic disease such as acute/ chronic inflammations or malignancy, 4) patients with connective tissue diseases such as Rheumatoid arthritis.

Blood samples were collected from all participants. Fasting blood samples were obtained.

Red blood cell counts, erythrocyte mean cell volume (MCV) and haemoglobin were measured with automated cell counts on the Beckman-Coulter LH750 machine within 6 hours of phlebotomy. The presence of anemia was defined by Hb<130 g/L on the basis of definition of WHO [25].

BUN and Creatinine were measured by using plasma with automated Dimension-Vista® lab system (Siemens Healthcare Diagnostics Inc.).
Whole blood was used to measure HbA1c and it was assessed with Roche Cobas Integra 400 Plus analyzer (Roche Diagnostics Inc.).

Serum EPO, adiponectin, TNF-α and IL-6 were measured in all samples by using Aliquots of serum which prepared and stored at −80°C and thawed just prior to analysis. Serum EPO, adiponectin, TNF-α and IL-6 levels were measured in duplicate by high sensitivity enzymelinked immuno-absorbent assays (ELISA) kit. EPO was measured by IBL Immuno Biological Laboratories. TNF-α, adiponectin IL-6 were measured by commercial ELISA kit (Assay Max Human ELISA Kit). These assay detected only human cytokines and the minimum detectable concentration were 0.6 mIU/ml for EPO, <10pg/ml for TNF, 0.5ng/ml for adiponectin and<10pg/ml for IL-6. Any sample with level less than these detectable doses reported as 0.
Based on the kit used, the normal reference range of adiponectin, TNF-α and IL-6 are 8.3- 13.9 ug/ml, 5-20 pg/ml and 0-10 pg/ml respectively.

Statistical analysis was performed using MegaStat, an add-ins Microsoft Excel program. Data from the experiments were presented as mean + Standard error from the mean (SEM).

Differences between groups were analysed by unpaired t-test. Correlation coefficients were determined and a stepwise multiple linear regression analysis were performed to determine relationships between the EPO and other cytokines for each group. The level of significance was set at $p< 0.05$.

The protocol conformed to the ethical guidelines of our institutions, and consent was obtained from each participant.

3. Results

The demographic and biochemical parameters of all patients are detailed in Table 1. Most of the patients in group-2 were presented with anemia (49/54) 90.74%, compared to 25/66 (37.87%) patients in group-1. The mean value for the entire patients in both groups is 11.88+0.22 (60.67%). HbA1c is high in both group and there is no significant difference between the two groups ($p>0.05$).

In table 2 serum levels of EPO and adiponectin were significantly lower in the group-2 than group-1. On the other hand, TNF-α were significantly higher in group-2 than group-1 ($p=0.01$).

The type-2 diabetic patients in general, had significantly high interleukin-6 concentrations (mean=25.53±2.36). Nearly all the patients showed either high our upper limit IL-6 serum levels. Therefore, no significant difference between the two groups ($p>0.05$) were observed. To investigate the influence of renal complication on the levels of the studied cytokines, the percent of patients were calculated in the two groups (fig 1) Figure 1 shows that 31.48% (in group-2) patient with renal complication had low serum EPO and 48.15% had low adiponectin, compared with 7.56% and 3.03% respectively in those without renal nephropathy (group-1). On the other hand, 42.60% patients of group2 had high TNF-α and 57.41% had high IL-6 compare with 22.72% and 56.06% respectively in group-1.

Figure 1a, shows that 31.48% (in group-2) patient with renal complication had low serum EPO and 48.15% had low adiponectin, compared with 7.56% and 3.03% respectively in those without renal nephropathy (group-1). On the other hand, as shown in Figure1b, 42.60% patients of group2 had high TNF-a and 57.41% had high IL-6 compare with 22.72% 56.06% respectively in group-1.

Figure -2 shows significant differences in the two group on the prevalence of abnormal low EPO and adiponectin; and of the abnormal high of TNF-α; as more than the third of the patients in group 2 had low EPO while more than half of these patients had low adiponectin, compared with less than the tenth and less than 3% respectively. in group-1. Nearly 45% compared with 22% of the patients had high level of TNF-a in group2 and group-1 respectively. In comparison, patients with high IL-6 levels were nearly equal in both groups.

Table I: Demographic Variables and General Laboratory Tests in the Studied Groups

	Group-1 (n=66)	Group 2 (n=54)	P value
age	51.92±0.97 (35-67)	49.24±1.08 (29-67)	NS
HbA1c	8. 97± 0.277 (6.5-16)	9.31 ± 0.298 (6.8-16)	NS
urea	4.90 ± 0.155 (2-8.8)	18.96 ± 1.43 (5.6-51.6)	p<0.005***
creatinine	75.08 ± 1.99 (36-114)	626.52 ± 53.53 (125-1884)	p<0.005***
Hb	13.41 ± 0.209 (9-17.7)	10.011 ± 0.250 (6.4-14.6)	P<0.005***

Table II: Laboratory Variables between Group1 and Group 2of , EPO and the cytokines.

	Group-1	Group-2	P value
EPO	13.33 ± 2.19 (0-125.194).	8.34 ± 0.95 (0-34.28)	P<0.05*
Adiponectin	22.37 ± 1.12 (7.01-47.93)	11.19 ± 1.01 (5.89-36.81)	P<0.001***
TNF-alpha	35. 11 ± 5.18 (6-165)	68.11 ±11.72 (10-500)	P<0.01**
IL-6	22.15±2.14 (0-70)	29.74 ± 4.44 (10-170)	NS

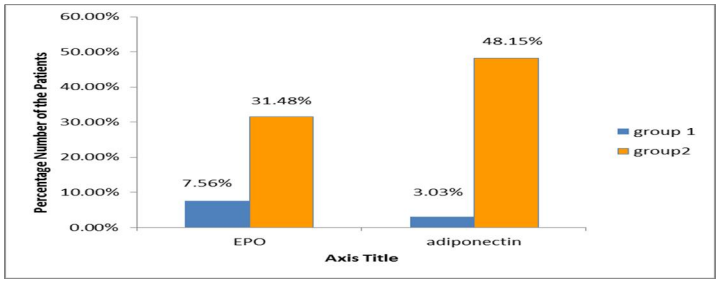


Fig.1a, Comparison in the percentage of patients with abnormally low EPO and *adiponectin in both groups*

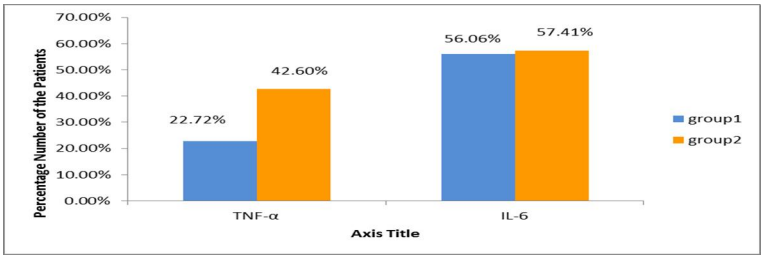
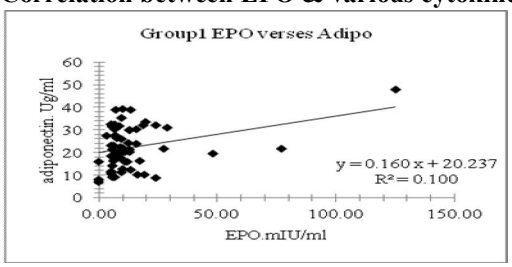
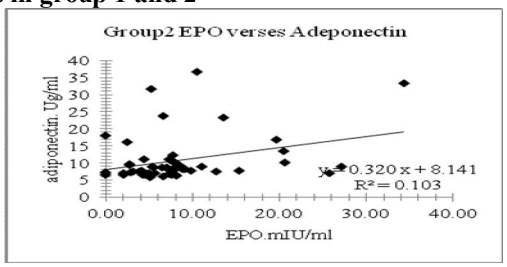


Fig. 1b, Comparison in the percentage of patients with abnormally high TNF-α and IL-6 in both groups

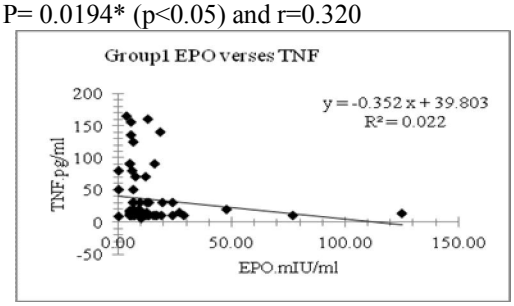
Figure 2, Correlation between EPO & various cytokines in group 1 and 2



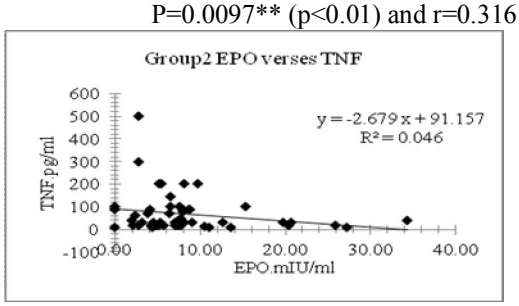
P= 0.0194* (p<0.05) and r=0.320



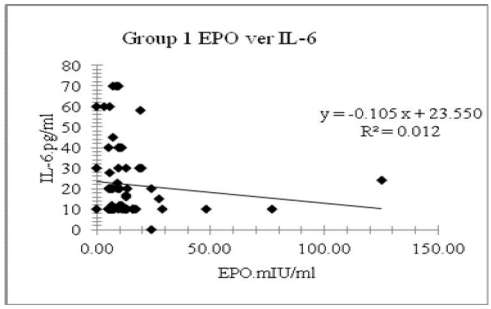
P=0.0097** (p<0.01) and r=0.316



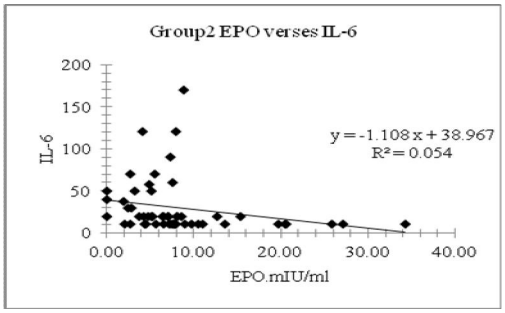
P=0.321 and r=-0.15



P=0.125 and r=-0.213



P=0.3897 and r=-0.108



P=0.937 and r=-0.233

4. Discussion.

Diabetic nephropathy (DN) is a common complication in patients with diabetes mellitus. In Saudi Arabia like several other countries; there are tremendously increasing trends in the incidence of diabetes and diabetic nephropathy. It was reported

that 20% of end stage renal failure in Saudi Arabia is due to diabetes mellitus [26].

In the current study about 45% (54/120) of our diabetic patients were presented with renal involvement in compare with 55% (66/120).

Prevalence of anemia is very common in our patients 60.67% of our diabetic patients present with anemia , particularly in those patients in group 2 with renal involvement as 90.74% of them present with anemia in compared with 37.87% patients in group 1. This results in agreement of previous studies these results which indicate the high preference of anaemia in type2 DM patients particularly in diabetics nephropathy patients and this may be attributed to the effect of diabetic nephropathy on vascular compartment of renal tissues [26-28].

The aetiology of anemia in diabetes is multifactorial including deficiency in erythropoietin synthesis and its release, systemic inflammation, iron deficiency, erythrocyte abnormalities, shortened erythrocyte half-life, increased osmotic stress, diminished sodium-potassium ATPaseactivity and reduced membrane fluidity and probably iatrogenic factors, e.g., angiotensin converting enzyme (ACE) inhibitors[29]. It has been reported that anemia is an independent risk factor for progression of diabetic retinopathy and renal failure and it not only has its own consequences, but also accelerate micro and macrovascular damage in diabetes mellitus[30].

Our results show that EPO concentration is significantly lower in group-2 than group-1 and this result is in agreement with previous studies which suggested that low EPO concentration could be a major factor in the genesis of anemia in diabetic patients, especially in patient with renal diseases [31].). However, another study indicated that plasma EPO concentration is often low in diabetic individuals even in absence of anemia [32]. It is hypothesized that chronic inflammation which associated dysregulation of adipocyte and excess of inflammatory cytokines have been have been implicated in the progression of diabetes and chronic kidney disease in both animal models and in human[33], In the current study; the serum adiponectin was significantly lower in group-2 in comparison to group-1.

Our results in agreement with other previous studies which indicate the antidiabetic effect of adiponectin, the patients who have high concentration of adiponectin are less likely to develop type-2 diabetes mellitus [34] and reduced levels of adiponectin have been reported in obese and type-2 diabetic patients [35].

The relation between adiponectin and renal impaired renal function has been extensively studied. Report of these studies are controversial; some reports indicate presence of high level of serum adiponectin concentrations in type 2 diabetic patients with renal impairment [36,37], In our study, we found a positive relation between EPO and adiponectin particularly in those patients with renal involvement in group-2, whereas many patients in group-2 have low level of both adiponectin and EPO. Although the study reflects a negative correlation between EPO and inflammatory cytokines, however; there are statistical significances in the association of EPO with TNF-α and EPO with IL-6 between the two groups, in type-1 diabetic patients with or without end-stage renal disease [38,39] and in nondiabetic patients with different degrees of renal dysfunction [40]. Whereas, another study indicated only weak relation between serum adiponectin and renal functions [41]. Moreover, the presence of a low blood adiponectin concentration in renal patients is associated with insulin resistant [42]. On other hand other studies reflect the presence of only high serum HMW adiponectin concentrations in type-2 diabetic patients with renal insufficiency and nephropathy [42].

The limitation of the present study is in the use of serum levels urea and creatinin as evidence of renal dysfunction, whereas, the estimation of glomerular filtration rate would be a better indicator.

In conclusion, the decreased serum level of EPO were observed in patients with type2 DM which was correlated with low serum level of Adiponectin especially in those with renal complications; however, this correlation was not observed between EPO and TNF-α or IL-6, in either group. Therefore, our study may suggest that the decreased level of EPO and adiponectin may directly be involved with impairment of kidney function in patients with type2 DM.

Further studies are needed to clarify the effects of the clinical use of both EPO and adiponectin in type 2 DM as a therapeutic agent.

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