Serum Levels of Interlukin-6, C-Reactive Protein in Diabetic Patients and Their Relation To Progression of Diabetic Retinopathy

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Abstract

Objective: To determine the relationship of serum level changes of IL-6, C-RP with retinopathy in two groups of type 2 diabetic patients (with and without retinopathy) in addition to control groups.

Method and material: The study was conducted from December2012 to March 2013. On well controlled thirty five patients of type 2 diabetic retinopathy (group one) (23 male and 12 female) with age range (41-75) years. Thirty patients of diabetes without clinical retinopathy (group two) (15 male and 15 female) with age range from (40-75) years were included in this study; duration of diabetes is more than five years in both groups. Disposable syringes and needles were used for blood collection. Blood samples (5ml) were obtained from diabetic patients and control groups by vein puncture. About one ml of whole blood put in EDTA tube for estimation of HbA1c, the remaining blood samples were allowed to clot at room temperature, and then centrifuged at 3000 Xg for 10 minutes. Sera were transferred carefully and stored at -17ºC until analysis time in suitable serum tubes.

Statistical analysis: The results were analyzed using Anova and student`s t–test .Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS version 10). A p value less than or equal to 0.05 was considered statistically significant.

Results: The mean sera level of IL-6 was found to be significantly elevated ($p< 0.05$) in DR as compared with the control group. On the other hand, no significant variations were detected in the levels of the IL-6 in the group of DNR with respect to the control group.

C-RP level was found significantly elevated ($p< 0.01$) in patients with DR when compared with control individual, while there was no significant variation of the serum levels of C-RP in DNR ($p = N. S.$) and control group.

Conclusion: Patients with increase serum levels of IL-6 and C-RP need more intensive ophthalmological examination to detect early retinal changes in aim of providing early management of diabetic retinopathy.

Key words: interleukin-6, C - reactive protein, diabetic retinopathy

الخلاصة

الهدف: لتحديد العلاقة بين تغيرات مستوى انتر ليكين -٦ وسي ريئكتف بروتين مع التغيرات الحاصلة في الشبكية لدى مجموعتين من مرضى السكري النوع الثاني (مجموعة مرضى سكري مع تغيرات في الشبكية ومجموعة اخرى بلا تغيرات في الشبكية)

طريقة العمل: استمرت الدراسة من تشرين الثاني ٢٠١٢ الى اذار ٢٠١٣ . قسم المرضى الى مجموعتين , المجموعة الاولى تكونت من ٣٥ مريض مصاب بالسكري مع وجود تغيرات في الشبكية وكانو ٢٣ رجل و١٢ امراة ومعدل الاعمار كان ٤١-٧٥ سنة المجموعة الثانية وتتالف من ٣٠ مريض ، ١٥ رجل و١٥ امراة معدل الاعمار كان ٤٠-٧٥ سنة وبدون تغيرات في الشبكية . فترة الاصابة بالسكري كانت اكثر من خمسة سنوات ، تم اخذ ٥ مل من عينات الدم من المرضى لتحديد نسبة السكر في الدم ولاجراء بقية التحاليل

النتائج: اظهرت النتائج ان مستوى انترليكين ٦ في الدم كان مرتفعا بدرجة مهمة عند مرضى السكري والذين لديهم تغيرات فى الشبكية ، بينما لم يكن هناك تغيرات مهمة بمستوى الانترليكين ٦ عند مرضى السكري الذين لديهم شبكية طبيعية. كما اظهرت النتائج ان مستوى

السي ريئكتف بروتين كان مرتفعا بنسبة مهمة عند مرضى السكري الذين لديهم تغيرات في الشبكية مقارنة بمجموعة المرضى الذين لديهم شبكية طبيعية
الاستنتاج: مرضى السكري الذين لديهم ارتفاع في مستوى النترليكين ٦ والسي ريئكتف بروتين في الدم يحتاجون الى فحص دوري مكثف لاكتشاف اى تغيرات جديدة في الشبكية حتى يتم معالجتها مبكرا.

Introduction

Diabetic retinopathy (DR) is a sight-threatening micro vascular Complication of diabetes mellitus. Global population– based data indicate that it is the fifth most common cause of blindness in the world, accounting for ~4.8% of global blindness [1]. The lesions that characterize diabetic retinopathy are the results of four main processes : the appearance of micro – aneurysms, increased vascular permeability , capillary occlusion ,fibrous and neovascular proliferation. [2, 3] An increase in the number of micro-aneurysms is considered a risk sign for progression of retinopathy. A further sign that indicates an even greater risk of progression of retinopathy is excessive permeability of the retinal vessels . The lack of pericytes compromise nutritional supply to the endothelial cells and impair the barrier function of these cells . Fluid leakage can range from micro- exudates to the macular edema, which can seriously reduce vision. Capillary occlusion that can appear in diabetic retinopathy gives rise to an area of retinal ischemia. The resulting ischemia leads to the proliferation of vessels that seek out new routes to irrigate the affected area. The vessels are fragile and made up of endothelial cells that proliferate without the benefit of a consistent basement membrane. Sometimes the new vessels are surrounded by fibrous tissue, and the fibro vascular complex adheres to the posterior part of the vitreous cavity .Traction on the vitreous can rupture the weak structure of the new vessels and lead to vitreous hemorrhage or even retinal detachment [2,4] .

Interleukin -6 (IL-6) is a multifunctional cytokine produced by many types of cells [5, 6]. Within the eye , the sources of IL-6 include, retinal pigment and corneal epithelial cells, keratinocytes, the iris and ciliary body and Muller cells [7,8] .Abu el Asrar et al. [9] demonstrated IL-6 in the vitreous of patients with PDR with levels correlating with the severity of disease . In a recent publication Funatsu et al. [10] reported similar findings that showed that IL-6 levels in the aqueous of patients with diabetes were significantly higher than those in non-diabetic subjects and that the levels correlated with the severity of diabetic retinopathy. In addition, it has been reported that IL-6 may induce angiogenesis indirectly by increasing the expression of VEGF [11].

Inflammatory cytokines like interleukin- 6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-*a* (TNF-*a*) have also been studied in the vitreous and serum samples of patients with proliferative diabetic retinopathy [12]. IL-6 and IL-8 were higher in the vitreous of patients with PDR and TNF-*a* was elevated in the serum of PDR patients compared to the patients with non-inflammatory retinopathy. These cytokines form a network and it remains to be resolved to determine the contribution of each component.

Ultra structural studies of blood retinal barrier (BRB) after exposure to IL-1*b* or TNF-*a* show pro-inflammatory effects in experimental animals. The causative factors leading to BRB breakdown are not entirely understood although cytokines have been implicated in the development of diabetic retinopathy. Regulation and inhibition of cytokines may have a potential use to prevent intraocular proliferative diseases. Interleukin-6 (IL-6) is a pleiotropic cytokine with various essential biological activities. It was initially identified as a T

cell factor that induces B cells to differentiate to immunoglobulin-producing cell [13].

A significant relationship between VEGF and IL-6 levels in aqueous humor and in vitreous fluid has been reported [14], thus concluding that these measurements may be useful to analyze the pathogenesis of DR and to predict the progression of the retinal disease. An increased expression of IL- 6 has also been observed in surgically removed epiretinal membranes [15]. Over time, the perturbed neurons die, resulting in permanent visual loss [16]. The leakage may be focal or diffuse. Focal leakage might occur due to leakage from micro aneurysms. These micro-aneurysms resulted from the retinal vessel wall weakening and impairment of the BRB due to hyperglycemia induced pericyte death. In contrast diffuse leakage is related to the microscopic damage of retinal vessels described as BRB breakdown and increased permeability factors like IL-6 and VEGF [17)

C-reactive protein (CRP) is an objective and sensitive index of overall inflammatory activity in the body [18]. Diagnostic tests for CRP are broadly applicable for monitoring inflammation and response to treatment for inflammatory and autoimmune diseases such as rheumatoid arthritis. In addition, a CRP measurement has diagnostic value when acute viral or bacterial infection is suspected [19]. In another clinical study, the prevalence of diabetic retinopathy was reported to be increased with higher tertiles of CRP (highest tertile range,3-35 g/mL).[20]

Aim of the study:

To determine the relationship of serum level changes of IL-6, C-RP and HbA1c% with retinopathy in two groups of type 2 diabetic patients (with and without retinopathy) in addition to control groups.

Patient and method

The study was conducted during the period from December 2012 to March 2013. Thirty five patients of type 2 diabetic retinopathy (group one) (23 male and 12 female) with age range (41-75) years, thirty patients of diabetic without clinical retinopathy (group two) (15 male and 15 female) with age range from (40-75) years were included in this study; duration of diabetes is more than five years in both groups. The samples were collected from ophthalmology and laser center in AL- Hakeem general hospital in AL-Najaf city.

Exclusion criteria included Patients with Diabetic nephropathy, Diabetic neuropathy, Type one diabetes mellitus; Rheumatoid arthritis, malignancy, I.H.D (e.g. MI, angina, CHF) and Pregnency were excluded from the current study.

The diagnosis of diabetic retinopathy is carried out by fundus examination using slit lamp bio-microscope after dilatation of the pupil with Topicarmide 1% eye drop. Full detailed history and full informed written consent was obtained from all patients included in the study.

Thirty five healthy volunteers were included in this study (control group). They were matched in their sex and age with patient groups. They were collected from the same centre in Al-Hakeem general hospital, 15 of them were female and 20 were male, with age range from (40–78) years.

Collection of Samples:

Disposable syringes and needles were used for blood collection. Blood samples (5ml) were obtained from diabetic patients and control groups by vein puncture. About one ml of whole blood put in EDTA tube for estimation of HbA1c, the remaining blood samples were allowed to clot at room temperature, and then centrifuged at 3000 Xg for 10 minutes. Sera were transferred carefully and stored at -17ºC until analysis time in suitable serum tubes.

The chemicals and kits that were used in this study were of the highest purity and are listed in the table (1) with their suppliers.

Table (1): Chemicals.

Supplier	Chemical
CUSABIO BIOTECH CO., LTD. USA	Human Interleukin 6 (IL-6) ELISA kit
STANBIO Laboratory. USA	Glycohemoglobin (HbA1c) Kit.
Plasmatic , France	Glucose kit.

Materials provided:

Table(2): Reagents and their quantities in IL-6 kit.

Reagent	Quantity
Assay plate	1
Standard	2
Sample Diluent	1× 20 ml
Biotin-antibodyDiluent	1× 10 ml
HRP-avidin Diluent	1× 10 ml
Biotin-antibody	1× 120 µL
HRP-avidin	1× 120 µL
Wash Buffer	1× 20 ml (25× concentrate)
TMB Substrate	1× 10 ml
Stop Solution	1× 10 ml

Results

A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was draw through the points on the graph as shown in (Figure 1).The data may be linearized by plotting the log of the IL-6 concentrations versus the log of the optical density (O.D.) and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less précis fit of the data.

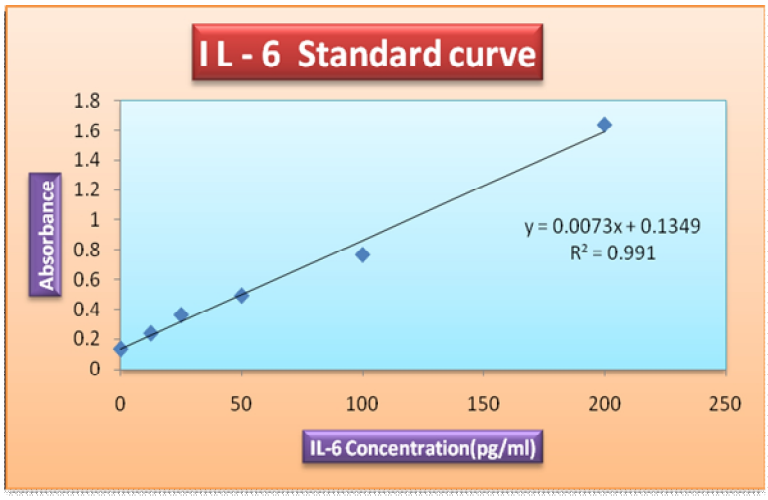

Figure (1): The standard curve for determination of IL-6 concentration.

The characteristics of the participants are shown in Table (3). It includes the data of three groups: group one the diabetic retinopathy (DR), group two the diabetic without {no} retinopathy (DNR) and group three, the healthy control subjects (Control), number of patients, sex and the mean ± SD of age, duration of the diabetes, serum levels of blood glucose and glycosylated hemoglobin (Hb_A1C) were tabulated .The results were analyzed using Anova and student`s t–test statistical analysis. This analysis was carried out by using the SPSS program.

Table (3): Clinical and biochemical characteristics of the diabetic patients (DR&DNR) and the control group included in the study.

Parameter	Subject	Mean ±SD.	Range	P-value
NO.	**DR**	٣٥	----------	-------
	DNR	٣٠	----------	---------
	Controls	35	----------	----------
Sex(M/F)	**DR**	23/12	----------	-------
	DNR	١٥/١٥	----------	----------
	Controls	20/15	----------	----------
Age(year)	**DR**	58.2±8.6	41-75	**N.S**
	DNR	٥٩.١±9.8	٤٠-٧٥	-------
	Controls	53.5±10.5	40-78	----------

Duration of diabetes(year)	**DR**	16±8.6	5-32	-------
	DNR	9.8±٤.٤	٥-٢٢	**p<0.001**
	Controls	-------	-------	----------
RBS(mg/dl)	**DR**	225±58.8	128-350	
	DNR	٢٢٢.٦±٦٩.1	١١١-٤٧٤	**p<0.001**
	Controls	113.3±21	75-156	
HbA1C(%)	**DR**	9.2±2.5	14-51	
	DNR	٩.١±3	٤.٨-١٥	**p<0.001**
	Controls	5.6±0.6	4.2-6.4	

DR= diabetic retinopathy, DNR= diabetic with no retinopathy ,NS= no significant

Interleukin- 6 (IL-6) levels in sera were measured for patients with DR and DNR in addition to that for control group. Table (4) summarized the mean ± SD values for concentrations of IL-6 for all groups. The mean sera level of IL-6 was found to be significantly elevated ($p< 0.05$) in patients with DR (group1) as compared with the control group. On the other hand, no significant variations were indicated in the levels of the IL-6 in patients with DNR (group2) with respect to the control group.

Table (4): Serum IL-6levels in diabetic patients (DR , DNR) and control group.

Groups	**Number**	**IL -6 (pg/ ml)**		**P- values**
		Mean ±SD	**Range**	
DR	35	64.8 ± 63.8	178.3- 0	P<0.05
DNR	30	48.8 ± 58.6	197.6- 0	N.S.
Controls	45	40.7 ± 42	144- 0	

N.S. = Non-significant.

Table (4) shows a significant elevation of serum levels of Interleukin-6 (IL-6) in DR in compared with control group (p<0.05).

The results of serum IL-6 and C-RP in diabetic retinopathy patients in comparison with diabetic without retinopathy group are presented in Table (5).

Parameter	DR (mean ± SD)	DNR (mean ± SD)	P-value
IL-6 (pg/ ml)	64.8±63.8	48.8±58.6	N.S.
C-RP (mg/L)	5.1±2.7	3.4±3.1	P< 0.05

N.S.:- No significant.

Table (5): Comparison between DR and DNR in serum levels of IL-6 and C-RP.

Discussion

Interleukin-6(IL-6) is one of several pro-inflammatory cytokines that have been associated with insulin resistance and type II diabetes [21]. IL-6 is a multifunctional cytokine and it is synthesized by a variety of cells including fibroblasts, macrophages, epidermal cells, vascular endothelium and within the eye, the sources of IL-6 include, the retinal pigment epithelial cells, corneal epithelial cells, keratocytes, iris and ciliary body [10]. IL-6 was reported to be related to hyperglycemia and diabetic nephropathy [22]. Also, IL-6 is considered to be an inducer of angiogenesis that exerts its activity through the induction of VEGF [23]. The mean sera level of IL-6 was found to be significantly elevated ($p< 0.05$) in patients with DR (group1) as compared with the control group. On the other hand, no significant variations were indicated in the levels of the IL-6 in patients with DNR (group2) with respect to the control group.

It has also been demonstrated that IL-6 can also induce the expression of VEGF by various cell lines [11] and increase in vitro endothelial permeability [23]. These findings, in combination with the results of the present study, suggest that IL-6 may have an important pathogenic role in the early stages of diabetic retinopathy.

Our study reveals a statistically significant elevation was observed in the mean C-RP between diabetic retinopathy and diabetic without retinopathy. C-RP was found significantly elevated ($p< 0.01$) in patients with DR when compared with control individual, while there was no significant variation of the serum levels of C-RP in DNR ($p = N. S.$) and control group.

It has been demonstrated that inflammatory reactions are possibly involved in the pathogenesis of diabetic retinopathy [24, 25]. Jones *et al.* [26] reported that C-reactive protein (CRP) and that biologically relevant C-RP derived peptides stimulated sIL-6R production by human neutrophils. Memis¸oğullari *et al.* [27]consistent to the results of our study have found increased level of Ceruloplasmin(Cp) as well as CRP in diabetic patients, when compared with control, and also studied parameters were significantly higher in group of patients with diabetic complications than compared with group without complications. Targher *et al.* [28] and van Hecke *et al.* [20] also have found that in diabetic patients with the presence of micro vascular complication (i.e. DR.), plasma biomarkers of inflammation (CRP) and endothelial dysfunction (sICAM-1) were significantly increased in those with more advanced disease compared

with those with early complications or without complications [29]. Hoom study (20) a large population-based cohort study of 625 adults, higher CRP was associated with the prevalence of any DR which is consistent with our present and earlier studies. While Spijkerman et al. (30) reported that CRP levels were not associated with DR progression over 10 years in a prospective clinic-based study of subjects with type 2 diabetes, and Le et al.(31) also did not find an association between CRP levels and the severity of DR with early-onset type 2 diabetes. Reports from the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) and the Multi-ethnic Study of Atherosclerosis (MESA) also did not find any associations between CRP and DR (32, 33), this controversy of the results about the significant association between serum level of c-reactive protein and diabetic retinopathy because C-reactive protein (CRP) is an objective and sensitive index of overall inflammatory activity in the body [18] . Diagnostic tests for CRP are broadly applicable for monitoring inflammation and response to treatment for inflammatory and autoimmune diseases such as rheumatoid arthritis. In addition, a CRP measurement has diagnostic value when acute viral or bacterial infection is suspected [19].

Conclusions

Increased serum levels of IL-6 may act as a key regulator of DR in type 2 diabetics when compared with diabetics without complication, may be involved in patho physiology of DR. Patients with increase serum levels of IL-6 and C-RP need more intensive ophthalmological examination to detect early retinal changes in aim of providing early management of diabetic retinopathy.

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