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Role of tumour necrosis factor-alpha and interleukin-6 in the development and progression of diabetic retinopathy in type 2 diabetes mellitus patients

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ABSTRACT

Diabetic retinopathy is one of the most serious complications of diabetes mellitus(DM) worldwide. Purpose: To evaluated the role of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in the diabetic retinopathy. Methods: This study included 85 subjects divided into 4 groups: Group A included 25 healthy controls. Group B: 20 diabetic patients without evidence of retinopathy. Group C: 20 non proliferative diabetic retinopathy patients. Group D included 20 proliferative diabetic retinopathy patients. Participants were subjected to full clinical history, clinical and ophthalmologic examination and laboratory investigation including measurements of fasting blood sugar(FBS), glycosylated hemoglobin(HbA1c), lipid profile, serum TNF-α, IL-6 and ultrasensitive C-reactive protein.

Results: This study showed significantly higher values of FBS, HbA1c, lipid profiles, TNF-α, IL-6 and CRP in diabetic patients when compared to healthy controls ($p<0.05$). Groups C and D patients showed significant increase of FBS, HbA1c, lipid profile, TNF-α, IL-6 and CRP when compared to group B. Moreover, high levels of FBS, HbA1c, TNF-α and IL-6 were observed in group D compared to group C but no difference was observed between the 2 groups regarding lipid profile and CRP. Postive correlation between serum levels of TNF-α and HbA1c and between serum levels of IL-6 and CRP was observed in all patient groups. There was a significant positive correlation between severity of diabetic retinopathy and serum levels of TNF-α and HbA1c in addition to the duration of diabetes.

Conclusion: The study provides evidence that TNF-α and IL-6 may play a role in the development and progression of diabetic retinopathy.

Keywords: Diabetic retinopathy; TNF-α; IL-6.

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INTRODUCTION

Diabetic retinopathy (DR) is one of the most serious complications of diabetes mellitus (DM) and is the leading cause of visual impairment and blindness in the world **(1)**. There is growing evidence that immunologic and inflammatory mechanisms are involved in the pathogenesis of diabetic retinopathy and diabetic macular edema **(2).** There are many risk factors for diabetic retinopathy including hyperglycemia, hypertension and dyslipidemia **(3).** These factors have been shown to induce inflammation by a variety of mechanisms including oxidative stress, transcription factor activation, dysregulation of nitric oxide synthase and formation of advanced glycation end products (AGES) **(4, 5).** One of the glucose toxic mechanisms is the protein glycation which is associated with cytokines such as tumor necrosis factor-alpha (TNF-α), interlukin-6 (IL-6) and interleukin-12 (IL-12) that are substantial factors in the development of diabetic microangiopathy **(6).**

TNF-α is a potent proinflammatory multifunctional cytokine produced by macrophages and a variety of cell types **(7).** It consists of 157 amino acid with a molecular weight of 17 KDa and mediates its biological activities through two receptors TNFR1 and TNFR2 and it plays an important role in inflammation, apoptosis, cell migration, proliferation and differentiation **(8).**

Interleukin-6 (IL-6) is a pleotropic cytokine in the body and it has an important regulatory function of immune system and T cell **(9).** IL-6 consists of 184 amino acid with a molecular weight of 26 KDa and many cells reported to produce it **(10).** IL-6 exerts its action via specific cell surface receptor composed of two subunits **(11).**

These cytokines interfer with endothelial cell function, increase capillary vessel wall permeability and promote intravascular clot formation (**12**). They also initiate and sustain the inflammatory process in the vascular wall by inducing the synthesis of acute phase protein, induce expression of adhesion molecules on endothelial cells which serves as chemoattractants for monocytes and other inflammatory cells **(13, 14**) stated that TNF-α and IL-6 may act as local intensification signals in pathological processes associated with chronic eye inflammation.

The aim of this study is to evaluate the possible role of TNF-α and IL-6 in the development and progression of diabetic retinopathy in patients with type-2 diabetes mellitus in an attempt to find therapeutic measures to protect against the occurrence of this complication.

SUBJECTS AND METHODS

This study carried out on 85 subjects, 60 diabetic patients and 25 healthy individuals served as controls. All subjects signed a written consent to participate in this study. All patients were attendants of the Internal Medicine and Ophthalmology Departments of Menofia University Hospitals. The study cohort was divided into 4 groups:

Group A: It included 25 age and gender matched apparently healthy volunteers They were (16 males and 9 females) with age ranges from 45 – 66 years.

Group B: It comprised of 20 patients with type-2 diabetes mellitus without retinopathy (13 males and 7 females). The duration of diabetes ranged from 7 to 10 years and their ages range from 49 – 64 years.

Group C: It included 20 patients with non proliferative diabetic retinopathy (NPDR) (12 males and 8 females). The duration of diabetes was between 9 -13 years. Their ages were between 52- 67 years.

Group D: It included 20 patients with proliferative diabetic retinopathy (PDR), (12 males and 8 females). The duration of diabetes was between 10 -16 years and their ages range from 53- 69 years.

Type-2 diabetes mellitus was diagnosed according to the American Diabetes Association criteria **(15)** for diagnosis and classification of diabetes mellitus. Diabetic retinopathy was diagnosed by ophthalmologic examination and patients were staged according to the presence and severity of diabetic retinopathy. The staging was performed in accordance with International Clinical Diabetic Retinopathy Disease Severity Scale **(16).**

The exclusion criteria were acute and chronic ophthalmological or general inflammatory disorders, glaucoma, age related macular degeneration, history of malignancy, ischemic disorder and hepatic or renal dysfunction.

All patients and controls were submitted to: i) detailed history taking with special stress on duration of diabetes and type of and adherence to treatment, ii) proper clinical examination with stress on blood pressure measurement and signs of diabetic complications, iii) complete ophthalmological examination including: visual acuity measurement, slit lamp examination, dilated fundus examination, fundus photography and fluorescein angiography, and iv) laboratory investigations including: fasting blood sugar (FBS), glycosylated hemoglobin (HbA_1c), lipid profile (cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL-c) and low density lipoprotein (LDL-c)), serum TNF-α, IL-6 and ultrasensitive C-reactive protein (CRP).

Sample collection and assay

Eight ml venous blood were collected, under complete aseptic precaution in the morning after overnight fast of 12 hours at least, from all subjects and divided into three tubes:

- 2ml blood was transferred into tubes containing sodium fluoride and then centrifuge for 10 minutes at 4000 rpm for determination of serum glucose by enzymatic colorimetric test **(17).**

- 1ml blood was transferred into EDTA containing tube for assay of glycated hemoglobin by quantitative colorimetric measurement as percent of total hemoglobin **(18).**

- The remaining 5 ml blood was transferred into another tube, left to clot, then centrifuged for 10 minutes at 4000 rpm and the serum was stored at -20c^{o} until analysis of the following measurement of (TC) by enzymatic colorimetric method **(19)**, measurement of (TG) by enzymatic colorimetric method **(20)**, determination of (HDL-c) by colorimetric method **(21)**, calculation of (LDL-c), according to **(22**), determination of TNF-α. Serum TNF-α was determined by enzyme linked immunosorbent assay (ELISA) using human TNF-α ELISA Kit **(23)**. TNF-α was determined by quantitative sandwich immunoassay. A murine monoclonal antibody specific for human TNF-α has been pre-coated onto a microplate. TNF-α in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human TNF-α, which is recognized by a streptavidin-peroxidase conjugate, serum IL-6 was determined also by enzyme linked immunosorbant assay (ELISA) using human IL-6 ELISA Kit **(24)** and determination of ultrasensitive (CRP) by using latex enhanced immunoturbidemetric assay **(25)**.

Statistical Analysis

Statistical package for social science for windows, version 9.0, system was used for data analysis, student (t test) and ANOVA tests were used to compare normally distrusted variables, data were represented as mean ±SD. Chi-square test was used to compare gender of studied groups. Pearson correlation coefficient test was used to measure correlation between two continuous variables. P values were significant if <0.05.

RESULTS

This study comprised 3 patients groups and one control group. There was no significant difference among studied groups regarding age and gender however, a significant increase of duration of diabetes mellitus with progression of diabetic retinopathy was identified (Table 1). There was significant increase in the level of glucose, HbA_1c, TC, TG, LDL-c, and significant decrease of HDL-c levels in all patients group when compared with controls. On comparing patients groups with each other, there is significant increase of glucose , HbA_1c , TC , TG , LDL-c in groups C & D in relation to group B with significant decrease in HDL-c levels in group C &D as compared to group B. In addition, there is a significant increase in levels of glucose and HbA_1c in group D as compared to group C but there is no significant difference between group C and D regarding lipid profiles (Table 2).

There was a significant increase in serum levels of TNF-α, IL-6 and CRP in each of the patients groups when compared to controls. On comparing patients groups, there is a significant increase in levels of TNF-α –IL-6 and CRP in groups C and D compared to group B and a significant increase in levels of TNF-α and IL-6 in group D when compared to group C (Table 3).

There was positive correlation between TNF-α and HbA_1c in all patients groups (Table 4). Importantly a positive correlation was identified between the severity of diabetic retinopathy and serum levels of TNF-α, IL-6 and with the duration of DM (Table 5). In addition a positive correlation was found between IL-6 and CRP in patients groups (Table 6).

DISCUSSION

Diabetic retinopathy displays all microscopic signs of inflammation such as vasodilatation, altered flow, fluid exudation and leukocyte migration. Therefore chronic low grade inflammation seems to be an inciting and final common pathway leading to diabetic retinopathy (DR) **(26).**

In agreement with other authors **(27, 28, 29),** the current study showed that diabetic patients with retinopathy demonstrated a longer duration of disease, higher fasting blood glucose levels and glycosylated hemoglobin in relation to diabetic patients without retinopathy and controls. *Katulanda et al* **(30)** reported that HbA_1c has a stronger association with occurrence of DR as it a predictors of both micro and macrovascular complication of diabetes mellitus.

The present study demonstrated a significant increase in serum levels of TC , TG , LDL-c and a significant decrease in serum levels of HDL-c in diabetic patients with or without DR in relation to controls but there was no significant difference with the progression of NPDR into PDR. These results are in concordance with previous studies **(31, 32).** *Kareem et al* **(33**) reported that the reason for these lipid alterations is abnormal lipid and lipoprotein metabolism in diabetes mellitus. Furthermore the activity of the enzyme lipoprotein lipase is low in diabetic patients **(34**).

Jayalakshmi et al **(35**) postulated that the effect of hyperlipidemia in the progression of DR is mainly due to elevation of blood viscosity and alteration in the fibrinolytic system resulting in hard exudates formation, also incorporation of TG into the cell membrane leading to changes in the membrane fluidity and leakage of plasma constituents into the retina which results in hemorrhage and edema in retina. In contrast other authors (**36)** did not find difference in these lipid parameters between DR patients and controls and they explained this by the unrestricted diet and inactive lifestyle in controls.

The current study reported that there was a significant increase in serum levels of TNF-α in diabetic patients with and without retinopathy in comparison with controls and also as the disease advanced from NPDR to PDR. These finding are in consistency with other authors **(37, 38)** and also go with *Koleva-Georgieva et al* **(39)** who reported that serum concentrations of TNF-α have an effect on the development and progression of DR and they correlate with presence and severity of the disease. These results are also matched with those of *Arita et al* **(40)** who found that levels of TNF-α and its soluble receptors were increased in sera of DR patients and correlated with disease severity.

There is accumulating evidence that TNF-α plays an important role in endothelial dysfunction in both micro and macrovascular diseases by impairment of nitric oxide-mediated vasodilatation, increased production of reactive oxygen species, activation of transcription factors and subsequent inflammation and in the regulation of both proliferation and apoptosis **(41).** These factors are important in diabetic retinopathy as it causes pericyte and endothelial cell death as well as uncontrolled endothelial cell proliferation **(37).** In contrary with these results some authors **(27)** did not find significant difference in serum levels of TNF-α and IL-6 between DR patients when compared with controls and they stated that serum concentration of cytokines are influenced by many factors and several types of cells could produce cytokines.

The present study showed that a significant positive correlation between TNF-α and HbA_1c in patients groups. These results correlates with that reported by other authors **(42, 39, 38).**

There was a significant increase in the serum levels of IL-6 in diabetic patients with and without retinopathy in relation to controls and also as the disease progress from NPDR to PDR. These results are consistent with the results of previous studies **(43, 39).** *Duncan et al* **(44)** demonstrated that in individuals with impaired fasting blood glucose, circulating levels of inflammatory markers such as CRP, sialic acid and IL-6 are independent predictors of the future development of diabetes.

In agreement with other authors **(45)** there was a significant elevation in CRP levels in diabetic patients with and without DR as compared to controls but there was no significant difference as the disease changes from NPDR to PDR. *Kaut et al* **(46**) stated that CRP is not only an inflammatory marker but also contributes to vascular pathogenesis and exacerbates tissue damage by triggering complement activation. It is one of the markers which shows significant rise when diabetes starts developing vascular complication. Contrasting this some authors **(47**) have reported that the levels of CRP were lower in diabetic retinopathy in comparison with control and explained this by some humoral factors related to retinopathy such as insulin growth factor-1 which negatively modulates the CRP production.

The current study showed a positive correlation between IL-6 and CRP in diabetic group this result is in consistency with that reported by Mirza et al **(38)**. *Lau et al* **(48)** reported that CRP is produce by hepatocytes under transcriptional control by cytokine IL-6 as a part of non specific acute phase response to most forms of inflammation, infection and tissue damage.

The present study also demonstrated a significant positive correlation between presence and severity of DR and serum levels of TNF-α, IL-6 and also with the duration of diabetes mellitus. These results are in agreement with other authors (**39, 49)**.

In conclusion, the results of this study indicate that dyslipidemia and inflammation as well as bad glycemic control are risk factors for DR. Serum concentration of TNF-α and IL-6 are increased and correlated with severity of DR in patients with type-2 diabetes mellitus, suggesting the possible role of these inflammatory markers in pathogenesis and progression of diabetic retinopathy and could be an important target for developing new drugs in the treatment of DR and also these cytokines can be used as a diagnostic and prognostic factor in this disease.

Statement of Human and Animal Rights

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Statement of Informed Consent

Informed consent was obtained from all patients for being included in the study.

Conflict of interest:

None

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Tables

Table (1): Comparison of the demographic criteria among the studied groups:

	group A (n=25)		group B (n=20)		group C (n=20)		group D (n=20)	
	Mean±SD		Mean±SD		Mean±SD		Mean±SD	
Age (years)	55.5 ± 4.8		55.4 ± 5.0		58.2 ± 3.9		58.0 ± 4.6	
Duration of diabetes (years)	-		8.7 ± 1.0#.		10.9 ± 1.6^		13.7 ± 1.5	
Gender	no	%	no	%	no	%	no	%
Male	16	64	13	65	12	60	12	60
Female	9	36	7	35	8	40	8	40

# p < 0.05 (Group B versus C)

. p < 0.05 (Group B versus D)

^ p < 0.05 (Group C versus D)

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Table (2): Comparison of the studied group as regarding blood glucose, glycated hemoglobin and lipid profiles

	group A (n=25)	group B (n=20)	group C (n=20)	group D (n=20)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Glucose level (mg/ dl)	89.0 ± 5.5	167.0 ± 53.5*#.	184.6 ± 8.3**^	204.1 ± 13.0***
HbA_1c %	4.9 ± 0.4	6.8 ± 0.3*#.	8.1 ± 0.6**^	8.9 ± 0.6***
TC (mg/ dl)	178.6 ± 6.4	228.0 ± 8.4*#.	244.1 ± 10.4**	247.9 ± 12.3***
TG (mg/ dl)	128.8 ± 5.4	174.1 ± 9.7*#.	184.9 ± 9.3**	189.0 ± 4.7***
HDL-c (mg/ dl)	52.8 ± 5.1	46.0 ± 5.9*#.	41.8 ± 5.8**	41.0 ± 4.1***
LDL-c (mg/ dl)	93.1 ± 4.6	151.4 ± 8.9*#.	168.6 ± 7.0**	171.1± 7.7***

* p < 0.05 (Group B versus A)

** p < 0.05 (Group C versus A)

*** p < 0.05 (Group D versus A)

\# p < 0.05 (Group B versus C)

. p < 0.05 (Group B versus D)

^ p < 0.05 (Group C versus D)

Table (3): Comparison of inflammatory markers (TNF-α –IL -6 –CRP) among the studied groups.

	group A (n=25)	group B (n=20)	group C (n=20)	group D (n=20)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TNF-α (pg/ ml)	15.3 ± 1.8	19.5 ± 3.2*#.	22.5 ± 3.7**^	25.7 ± 4.9***
IL6 (pg/ ml)	1.0 ± 0.3	2.1 ± 0.6*#.	3.8 ± 0.7**^	5.2 ± 1.0***
CRP (mg/ dl)	2.1 ± 0.7	3.6 ± 0.9*#.	5.6 ± 1.4**	6.0 ± 1.3***

* p < 0.05 (Group B versus A)

** p < 0.05 (Group C versus A)

*** p < 0.05 (Group D versus A)

\# p < 0.05 (Group B versus C)

. p < 0.05 (Group B versus D)

^ p < 0.05 (Group C versus D)

Table (4): Correlation between serum TNF-α levels and HbA_1c levels in patients groups.

	Groups	TNF-α (pg / ml)	
		r	P value
HbA_1c%	B	+0.755	<0.05
	C	+0.823	<0.05
	D	+0.729	<0.05

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Table (5): Correlation between severity of diabetic retinopathy and serum levels of TNF-α, IL-6 and with duration of diabetes mellitus in patients groups.

	Severity of retinopathy	
	r	P value
IL6 (pg / ml)	**+0.860**	**<0.05**
TNF-α (pg / ml)	**+0.455**	**<0.05**
Duration of diabetes (years)	**+0.817**	**<0.05**

Table (6): Correlation between serum levels of IL-6 and CRP in patients groups.

	groups	IL6 (pg / ml)	
		r	P value
CRP (mg/ dl)	**B**	**+0.710**	**<0.05**
	C	**+0.610**	**<0.05**
	D	**+0.529**	**<0.05**