RESEARCH ARTICLE

Assessment of Serum Levels of the WNT Pathway Antagonist (Dickkopf-1) in a Sample of Type 2 Diabetic Patients with Retinopathy using Two Groups of Antidiabetics

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ABSTRACT

Objectives: Dickkopf-1 (DKK-1) is WNT/b-catenin pathway antagonist which plays a detrimental role in the development of diabetic retinopathy (DR). This research aimed to assess serum DKK-1 levels in diabetic patients who have and have not developed DR and, compare them with the control subjects finding out whether we can use it as an indicator for DR early diagnosis and to find out which one of the widely used two groups of antidiabetic treatments had the greater effect on this biomarker and hence on the progression of DR.

Methods: The study participants were divided into two subgroups: First, 70 patients (36 male, 34 female) with type 2 diabetes mellitus, among them 35 patients diagnosed with DR and 35 with no evidence of DR, and secondly, non-diabetic controls (11 male, 9 female) were selected from the patients attending Ibn AL-Haitham hospital for ophthalmology and a specialized center for endocrinology and diabetes. Venous blood samples of all participants were drawn after an overnight fast, and serum samples were stored at -20ºC until DKK-1 assay.

Results: Serum DKK-1 showed significantly lower levels in diabetic patients with (6.1 ± 2 ng/mL) or without DR (14 ± 6.2 ng/mL) when compared to those of controls (34 ± 12.25 ng/mL) ($p<0.05$). Furthermore, serum DKK-1 levels were lower in the late stage of DR compared to the early stage 5.6 ± 1.7 and 7 ± 1.9 ng/mL, respectively. Furthermore, DPP-4 inhibitors cause a better increment in DKK-1 levels when compared to SU in the NDR group.

Conclusions: Reduced serum levels of DKK-1 are related to the existence and worsening of DR and have the prospect to serve as an indicator for this condition.

Keywords. Diabetic retinopathy; Dickkopf-1; WNT pathway

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic condition characterized by high blood glucose levels and inadequate insulin production or insulin resistance. Diabetic retinopathy (DR) is a devastating condition that develops slowly and insidiously.[1] In 2030, people affected by this condition are expected to rise to 191.0 million 2030.[2,3] Functional and formal abnormalities in the retina characterize this condition, which is the most prevalent cause of vision loss in working-age adults. Before any macroscopic abnormalities in the retina were seen, several microvascular changes were occurring pathophysiologically in DR.[4] Pericytes loss, increased basal membrane thickness, and endothelial dysfunction will occur in the vascular bed in this disease, resulting in a vascular barrier function abnormality, leading to retinal feeding issues and exudative edema. This causes retinal ischemia and the appearance of new blood vessels, resulting in a proliferative DR process.[5] Non-proliferative diabetic retinopathy (NPDR), on the other hand, is the early stage. The most common symptoms of NPDR are microaneurysms and small elongation of retinal blood vessels.[6] Chronic hyperglycemia is a significant pathogenic marker of diabetic retinopathy, with HbA1c levels and the duration of diabetes being the most relevant determinants in disease start and progression in most (meta-)analyses.[7,8]

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The WNT/b-catenin pathway was found to play a key role in determining the fate of stem cell, it ›s proliferation, differentiation, and cell migration.[9,10] This pathway appears to be activated in the retinas of people and animal models with DR, suggesting that it plays a pathogenic role in this disease.[11] The normal formation of the anterior segment of the eye require the WNT signaling during development. Many developmental processes, such as the retinal pigment epithelium (RPE), the lens, the ciliary edge, dorsoventral patterning in the optic cup, and the retinal vascular system, are regulated by WNT signaling.[12,13]

The Dickkopf-1 protein is an antagonist of Wnt signaling that links to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and prevents the WNT-induced Frizzled (Fz)-LRP5/6 complex from forming. The level of DKK-1 was identified as a diagnostic and prognostic biomarker for a variety of disorders, including carcinoma of the hepatic cells, cancer of the pancreas, lung and esophageal cancer. Reduced serum DKK-1 levels have been involved in diabetic retinopathy presence, in which Wnt pathway activation is involved in it›s pathogenesis, according to a recent study.[14]

Some events in DM may have influenced platelet secretion of DKK-1, resulting in a decrease in DKK-1 levels. This, in turn, may have resulted in the Wnt signaling pathway upregulation, which may lead to retinal inflammation and the formation of new blood vessels through the increased release of inflammatory and proangiogenic factors such as tumor necrosis factor (TNF-a), intracellular adhesion molecule (ICAM-1), and vascular endothelial growth factor (VEGF).[14,15] As a result, it seems acceptable to conclude that lower DKK-1 levels play a key role in the onset and progression of DR, with the ability to be used as an indicator for DR prediction.

The American Diabetes Association recommends metformin and sulfonylurea in conjunction with metformin to achieve tight glycemic control in those with type 2 diabetes and lifestyle changes.[16] Patient adherence is harmed by hypoglycemia and weight gain caused by SU, which may have a deleterious influence on long-term treatment.

According to the "American Association of Clinical Endocrinologists/American College of Endocrinology" guidelines published in 2009, incretin-based medicine is recommended in the management of people with type 2 diabetes[17] Three DPP-4 inhibitors are used to treat type 2 diabetic patients: Sitagliptin, Vildagliptin, and Saxagliptin. They work by inhibiting the enzyme DPP-4 and increasing the activity of incretins like glucose-dependent insulinotropic peptide **(**GIP) and glucagon-like peptide-1 (GLP-1). They also help regulate blood glucose levels and improve 24-hour blood glucose fluctuation. Glycated hemoglobin levels and glucose swings appear to be reduced by Vildagliptin.[18] Linagliptin and alogliptin also lowered HbA1c levels.[19] Furthermore, decreasing postprandial blood glucose rises is likely to alter oxidative stress indicators. Measuring the antagonist of canonical WNT signaling is thought to be important in predicting DR in its early stages since current treatment focuses on late-stage DR when vision has already been compromised and based on the results, we can choose an antihyperglycemic agent that has a better effect on this biomarker and thus on the progression of DR. In this study, we measured serum levels of Dickkopf-1 in diabetic patients that have or have not developed DR and compare the results with healthy controls to correlate them with the severity of DR and to find out whether we can use serum DKK-1 levels as a biomarker for diagnosing diabetic retinopathy at early stages. Furthermore, we aim to find whether SU+ metformin or DPP4is+metformin had a greater effect on this oxidative stress marker, thereby slowing DR's progression.

MATERIALS AND METHODS

Study Participants

From November 2021 to March 2022, a case-control study was conducted at the Ibn Al-Haitham Hospital Of Ophthalmology and a Specialized Center For Endocrinology and Diabetes. The study was approved by the ethical committee of the College of Pharmacy, University of Baghdad before it began, and it followed the Declaration of Helsinki. After the participants were given full information about the study's goal, they gave their informed consent. Plasma samples were drawn from patients with type 2 DM that have developed DR, have not developed DR (NDR), and healthy subjects. All of the patients had diabetes for at least 5 years and were given either DPP4 is or SU to treat it. Healthy controls were age-matched people who didn't have any signs of diabetes, ocular bleeding, exudation, or any abnormal new blood vessels.

Previous intraocular surgery, other neovascular disorders in the eye, such as occlusion of the central retinal vein (CRVO) and age-related macular degeneration, a history of any inflammation in the eye, and glaucoma were all excluded. Other exclusion criteria included significant cardiac, pulmonary, or hepatic insufficiency, autoimmune disorders including type 1 DM, history of newly diagnosed malignant neoplasms, a recent history of thrombotic events, bone disease, pregnancy, or lactation, regular use of non-steroidal anti-inflammatory drugs, antioxidants or antiplatelet agents (since platelets are the major source that releases DKK-1).

The World Health Organization's criteria were used to make all type 2 diabetes diagnoses[20] and according to the findings in the Early Treatment Diabetic Retinopathy Study (ETDRS), an independent ophthalmologist diagnoses diabetic retinopathy using ophthalmoscopy, fundus photography (FP), or optical coherence tomography (OCT).[21] Diabetic retinopathy is classified into either non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) using' The international clinical DR severity scale'.[22] FBG, HbA1c, lipid profile, urea, and creatinine were all reported by the clinical laboratory. A patient with hypertension means having an arterial blood pressure of greater than 140/90 mm Hg at rest or using antihypertensive medication. Body mass index (BMI) was measured by computing weight and height. Prior histories, personal characteristics, diabetes duration, and antidiabetic therapy were all recorded on a data collection sheet for all participants.

Collection of Blood Samples

Antecubital vein blood samples of 10 mL were obtained. Within one week, two milliliters of blood were transferred to an ethylene diamine tetraacetic acid (EDTA) tube and kept at (+2 to +8°C) for the HbA1c assay. To obtain the serum, the remaining 8 mL of blood was transferred to a gel tube and allowed to coagulate for 30 minutes before being centrifuged for 10 minutes at 3000 rpm. The hospital's laboratory used a portion of the serum to assess fasting blood glucose (FSG), HBA1c, creatinine, urea, and lipid profile on the day of collection. The remaining serum was frozen in Eppendorf tubes at (–20°C) until the Dickkopf-1 levels were measured.

Measurement of DKK-1 Levels

Commercial enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, USA) were used to measure plasma DKK-1 levels. This assay is a "sandwich enzyme immunoassay" that allows the detection of DKK-1 in the range of 0.5 to 150 ng/mL. The procedures were carried out following the manufacturer's recommendations. Variations across and within assays were 8 and 10%, respectively.

Other Biochemical Parameters

Fasting blood glucose was measured using an enzymatic colorimetric method,[23] HBA1C was measured using the D-10™ hemoglobin testing system, which depends on the chromatographic separation of analytes,[23] lipid profile, and urea were measured using an enzymatic hydrolysis method,[24,25] and creatinine was assessed by alkaline hydrolysis method.[25]

Statistical Analysis

Statistical Package for the Social Science (SPSS, IBM, USA version 25) was used to conduct statistical analysis. The Kolmogorov–Smirnov test was used to examine the data distribution. Data were provided as the median, interquartile range, and frequencies, depending on their distribution. When multiple comparisons were assigned, the Kruskal–Wallis H test was employed; the Mann–Whitney U-test was used when just two comparisons were allocated. To compare categorical variables, a Chi-square test was used. The relation between DKK-1 and the study variables was determined using the Spearman correlation test. A receiver-operating characteristic analysis determined the optimal "cutoff score" of serum DKK-1 level for distinguishing non-diabetic controls from patients. A 25% confidence intervals with the area under the curve (AUC) and sensitivity and specificity were used to assess diagnostic accuracy. A two-tailed *p-value* of less than 0.05 was considered statistically significant.

RESULT

The demographic, clinical, and biochemical features of the participants were listed in Table 1. Total 70 diabetic patients, including 35 patients with DR and 35 patients without DR, and 20 non-diabetic controls were included in this study.

Serum Levels of Circulating DKK-1 in Diabetic Retinopathy Patients

Serum DKK-1 levels in the three participating groups showed a statistically significant difference ($p<0.05$, Kruskal–Wallis H test). Significantly lower serum levels of DKK-1 found in patients with DR (median= 6.1 ± 2 ng/mL) in comparison with those in the NDR groups (14 ± 6.2 ng/mL; $p<0.051$, Mann–Whitney U-test) and non-diabetic controls (34 ± 12.25 ng/mL; $p<0.05$, Mann–Whitney U-test) as shown in Figure 1a. These results indicated that increased serum DKK-1 levels have a link to the existence of DR or its development. Furthermore, there is a relationship between DR severity and serum DKK-1 levels. Furthermore, DKK-1 levels in the circulation were less in the PDR group (5.6 ± 1.7 ng/mL) than in NPDR patients (7 ± 1.9; $p<0.05$, Mann–Whitney U-test; Figure 1b). These results find out that decreased DKK-1 levels are associated with the severity of DR or its progression.

There are no statistically significant differences in DKK-1 levels among DR patients taking either SU or DPP4 ($p>0.05$, Mann–Whitney U-test; Figure 2a). in the other hand DKK-1 levels were significantly higher in NDR patients taking DPP4 compared to those taking SU ($p<0.05$, Mann–Whitney U-test; Figure 2b). This means in those without retinopathy DPP-4 inhibitors may delay the incidence or slow the progression of DR.

Table 1: Sociodemographic and clinical characteristics of the participants

Variables	*DR N=35*	*NDR n=35*	*Control n=20*
Age (years)	49 ± 9	488.25	45 ± 14
Gender (m/f)	22/13	14/21	11/9
BMI (kg/m^2)	29.73	293	28.5 ± 4
Smoking	15[a]	8	8
Blood pressure	28	13	……..
Stage NPDR	14	35	……..
PDR	21		
Normal retina	35		
TREATMENT SU/ DPP4	17/18	17/18	
FBS(mg/dl)	200 ± 47[a]	190 ± 90[a]	89.5 ± 9.5
HBA1C %	9 ± 1[a]	8 ± 0.8[a]	5 ± 0.2
TC(mg/dl)	250 ± 90[a]	180 ± 40[a,b]	150 ± 16.75
LDL(mg/dl)	180 ± 20[a]	178 ± 8[a]	149 ± 0.4
HDL(mg/dl)	34 ± 7	33 ± 3	34.5 ± 7
TG(mg/dl)	190 ± 60[a]	178 ± 45[a]	90 ± 11.75
S.CR(mg/dl)	1.5 ± 0.4[a]	1.0 ± 0.2[a]	0.6 ± 0.2
B.U(mg/dl)	49 ± 6[a]	35 ± 11[a]	23 ± 9.5

DR, diabetic retinopathy; NDR, diabetic patients without DR; PDR, proliferative DR; FBG, fasting blood glucose; BMI, body mass index; NPDR, non-proliferative DR; HBA1c, glycated hemoglobin; SU, sulfonylurea; DPP4is, dipeptidyl peptidase 4 inhibitors; TC, total cholesterol; LDL, low-density lipoproteins; HDL, high-density lipoproteins; TG, triglyceride; BU, blood urea; S.Cr, serum creatinine, for categorical variables comparision such as gender, smoking status, and blood pressure, The chi-square test was used, for multiple comparisons of age, BMI, FBS, HBA1c, TC, LDL, HDL, TG, S.CR, BU "Kruskal–Wallis test" was used; when only two comparisons are required while Mann–Whitney U-test was conducted. Data were expressed as numbers, median ± interquartile range, [a] vs control,[b] vs DR group, $p<0.05$.

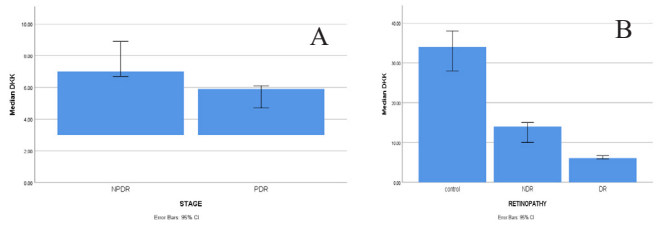

Figure 1: serum DKK-1 levels of the study groups. (a) serum DKK-1 levelsin the healthy individuals, diabetic patients with (DR) and without diabetic retinopathy(NDR). (b) DKK-1 levels among patients in different stages of DR. Data were analyzed with Kruskal–Wallis H test and Mann–Whitney U-test.

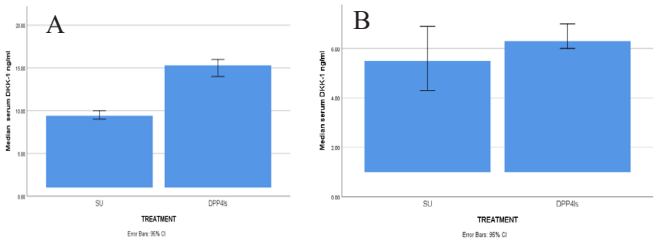

Figure 2: serum levels of DKK-1 levels in diabetic patients taking either SU or DPP-4 inhibitors. (a) DKK-1levels in the DR group. (b) serum DKK-1 levels in the NDR group. Mann–Whitney U-test was used to make the comparison.

Correlation of DKK-1 with the Study Variables

Correlation studies of serum DKK-1 with the studied variables of the pooled data are shown in Table 2. Serum DKK-1 has a negative correlation with FBS, HBA1C, TC, TG, S.CR, and urea. While there was no correlation between serum DKK-1 with HDL and LDL (P>0.05).

Dickkopf-1 Levels as an Indicator for the Diagnosis of Diabetic Retinopathy.

The receiver operating characteristic **(**ROC) analysis was done to determine if serum DKK-1 levels can be considered as an indicator diagnosing of DR. The area under the ROC curve (AUC) was used to determine total accuracy. The optimum diagnostic cutoff for DKK-1 was 9.05 ng/mL, with an AUC of 0.993 (95% CI **0**.982-1.000; p<0.05). This corresponds to a sensitivity of 94%, and specificity of 91% as shown in Table 3 and Figure 3. These results give an idea that the serum DKK-1 level may be used as an indicator for the detection of DR.

DISCUSSION

It has been revealed that Dickkopf-1(DKK-1) is a secretory protein that can inhibit the Wnt signaling transduction pathway. The Wnt signaling pathway has a well-known involvement in embryogenesis, organogenesis, and homeostasis. This signaling pathway is essential for a variety of physiological activities, including cellular proliferation, tissue regeneration, embryonic development, and many other systemic and local consequences, and it can be regulated at multiple levels.[26] Therefore, any disruption in the route may result in complex repercussions.

DKK-1 levels in the circulation were found to be considerably lower in patients with DR compared to NDR

Table 2: Spearman,s correlations of serum DKK-1with the studied variables

Variables	*r-value*	*p-value*
FBS	-0.610	0.000*
HBA1C	-0.734	0.000*
T.C	-0.697	0.000*
LDL	0.04	0.67
HDL	0.08	0.453
TG	-0.722	0.000*
S.Cr	-0.721	0.000*
UREA	-0.775	0.000*

FBG, fasting blood glucose; HbA1c, glycated hemoglobin; TC, total cholesterol; LDL, low-density lipoproteins; HDL, high-density lipoproteins; TG, triglyceride; BU, and S.Cr, serum creatinine;* significant when the p-value of spearman correlation was <0.05.

Table 3: Receiver operating characteristic curve and AUC analysis of DKK-1 for retinopathy

Variable	*AUC*	*95%CI of AUC*	*P-Value*	*Optimal cut-Off*	*SN*	*SP*
DKK-1	0.993	0.982-1.000	0.000*	9.05	0.94	0.91

AUC, the area under the curve; CI, confidence interval; SN, sensitivity; SP, specificity; DKK-1,Dickkopf-1.

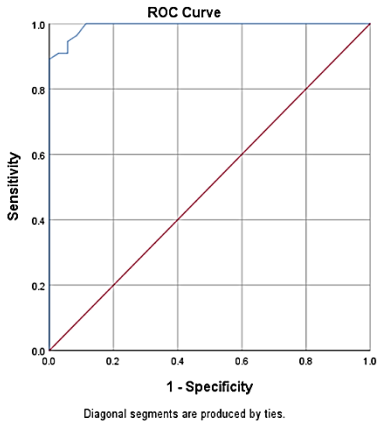

Figure 3: Receiver operating-characteristic curve analysis for PDR prediction using serum DKK-1 levels.

patients and non-diabetic controls in this investigation. Furthermore, when compared to people with NPDR, they were much lower in the advanced stage of DR (PDR). DR's presence, severity, or progression is associated with reduced DKK-1 levels. The present study's findings agreed with previous studies; In a Chinese clinical study, Qiu *et al.* wanted to determine whether plasma and vitreous DKK-1 levels are linked with diabetic retinopathy in type 2 diabetes mellitus. They discovered that blood DKK-1 levels were considerably lower in the DR group than in the non-diabetic control group. In addition, they examined the association between DKK-1 levels and the acuity of DR. PDR patients have lower serum DKK-1 levels than NPDR patients.[27]

Chen *et al.* did another investigation using the eyes of human donors and diabetic animal models to evaluate the

probable impact of the Wnt signaling pathway in DR. The findings suggest that the Wnt pathway may play a pathogenic role in DR by inducing oxidative stress and, as a result, inflammation in the retina. They discovered that blocking Wnt signaling pathway by intravitreal injection of different doses of purified DKK1 into streptozotocin-diabetic rats inhibits the ROS generation induced by high glucose levels, alleviated retinal inflammation, vascular leakage, and NV in those DR.[14]

This may support our findings in Figure 2 in which DKK-1 levels were higher in those who taking DPP4is compared to those taking SU due to the ability of DPP-4 inhibitors to prevent nitrosative (reactive nitrogen and oxygen species) stress, inflammation, and cell death in retinal cells.[28]

DPP4 inhibitors have been designed to prevent incretins such as GIP and GLP-1 from breaking down, extending their effect. These incretins could limit WNT pathway activation by inhibiting the formation of reactive oxygen species (ROS) and plasminogen activator inhibitor-1 (PAI-1) via the cAMP pathway.[29] DPP-4 inhibitors slowed the advancement of diabetic retinopathy, according to Chung *et al*., who reviewed the medical records DR patients and examined the effects of DPP-4 inhibitors on the DR progression based on the" diabetic retinopathy severity scale". Vildagliptin was compared to Sulfonylurea for the treatment of diabetic retinopathy in a large retrospective cohort study conducted by Kolaczynski *et al.* using data collected from the German electronic medical record database. In this clinical scenario, the incidence of retinopathy was considerably reduced in the vildagliptin group compared to the sulfonylurea group.[31]

However, there is a lack of clinical data on the effectiveness of using DPP-4 inhibitors in treating diabetic retinopathy at this time. Existing research suggests that this class can improve vascular homeostasis and even reverse the hemodynamic abnormalities associated with early diabetic retinopathy in diabetic patients.[32]

Depending on the ideal "cut-off" score of serum DKK-1 obtained from the ROC curve, we want to find if circulating DKK-1 levels can be considered as an indicator for DR. The findings revealed that DKK-1 had a 99.3% probability of distinguishing DR samples from normal people, with a sensitivity of 94% and a specificity of 91%.

The following are some of the limitations of our research. First, further longitudinal clinical investigations are needed to evaluate whether decreased serum levels of DKK-1 make people more subjected to DR, and whether detecting DKK-1 levels can help with early diagnosis and prognosis. second, we should measure whether DKK-1 levels in local tissues like the vitreous are connected with circulating levels. Third, lifestyle factors like nutrition and exercise were not taken into account. Fourth, 26 weeks was the duration of the majority of clinical trials of DPP-4 inhibitors as monotherapy or in combination with metformin. As a result, it's unclear whether a full therapeutic response has been obtained. Finally, due to the small number of study participants, we were unable to find whether clinical characteristics such as gender, age have a relationship with DKK-1 levels which should be confirmed in future research.

Our findings revealed that the existence and progression of DR are related to lower DKK-1 levels. Reduced circulating DKK-1 levels may result in activation of Wnt pathway in the retina in DR, and could be used as a biomarker to forecast the disease. We believe that drugs that promote DKK-1 expression or exogenous DKK-1 supplements could be useful in preventing and treating DR. However, more research into DKK-1's therapeutic potential in the treatment of DR in humans is needed.

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