Adiponectin Decreased Aquaporin 4 MRNA Expression in Rat Model of Type 1 Diabetic Retinopathy, Can It Prevent Retinal Edema?

Mohamed Hussein Mohamed, Huda Galal Mohamed, Reham Hassan Ebrahim
Department of Physiology, Faculty of Medicine, Zagazig University, Egypt
***Corresponding author** Reham Hassan Ebrahim, **Mobile:** (+20) 01144963609,
Email: phisiology_lover_4@yahoo.com, **Orchid ID:** 0000-0001-8038-4144

ABSTRACT

Background: Diabetic retinopathy is a great ocular diabetic disorder and may cause blindness. It is linked with hyperglycemia, inflammation and oxidative stress. Adiponectin is an adipokine that has antioxidant, anti-inflammatory and anti-angiogenic effects. **Objective:** to detect possible role of adiponectin as a therapy in experimentally induced diabetic retinopathy in male adult rats.

Material and Methods: Thirty male adult rats were divided into 2 groups: group I (control 10 rats) and group II (diabetic type 1 induced by streptozotocin, 20 rats). Diabetic rats were divided four weeks later into 2 subgroups: Subgroup IIA (diabetic retinopathy), Subgroup IIB (adiponectin-treated diabetic retinopathy), in all groups, serum glucose, insulin, lipid profile, superoxide dismutase (SOD), malonaldehyde (MDA), interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) and aquaporin-4 (AQP4) gene expression were estimated. Retinal histopathology and immunohistochemistry of retinal VEGF and tumor necrosis factor (TNF) alpha were also estimated.

Results: Subgroup IIB showed significant decrease in serum levels of glucose, cholesterol, triglyceride, low density lipoprotein (LDL), MDA, VEGF and IL-6, and AQP4 gene expression, with significant increase in insulin, SOD and high-density lipoprotein (HDL) levels. Retinal histopathology showed partial restoration of retinal layers organization and immunohistochemistry showed downregulation of TNF-α and VEGF.

Conclusion: Adiponectin may improve diabetic retinopathy via antioxidant, anti-inflammatory, antidiabetic and anti-angiogenic effect.

Keywords: Adiponectin, Aquaporin 4 MRNA Expression, Diabetic Retinopathy, Retinal Edema.

INTRODUCTION

Diabetes mellitus (DM) is a major disorder that has many complications and high mortality [1]. Many complications occur in diabetic patients, as corneal abnormalities, cataract, glaucoma and diabetic retinopathy (DR). DR is a common vascular disease that threatens all diabetic patients, leading to blindness if left untreated [2].

DR occurs with a higher incidence in T1DM than T2DM patients, and this different incidence is due to longer duration and worse metabolic control of diabetes in patients with T1DM than in T2DM [3]. Diabetic retinopathy development is strongly linked with hyperglycemia, dyslipidemia, oxidative stress, inflammation and pathological angiogenesis [3, 4].

Adiponectin (APN) is an adipokine, it regulates glucose and lipid metabolism with anti-inflammatory, anti- antioxidant, anti-atherogenic and anti-hypertensive effects [5,6]. Adiponectin has two receptors (AdipoR), they are AdipoR1 and AdipoR2 [6].

Both APN receptors are expressed in the retinas of humans and rats, it was stated that diabetic retinopathy patients have low levels of plasma APN [5], in contrast, **Li *et al.*** [7] found high plasma APN level in patients with diabetic retinopathy, so APN may have a role in diabetic retinopathy [8].

Aim of the work was to clarify possible therapeutic effects of adiponectin on rat model of diabetic retinopathy.

MATERIAL AND METHODS

Animals: Thirty adult male albino rats, weighing 180-220 gm and aged 12 weeks old, were obtained from Faculty of Veterinary Medicine. The rats were present in stainless steel cages (5 / cage) in the Physiology Department. They were allowed for acclimatization for two weeks before the experiment. The rats were fed commercial rodent chow.

Drugs and chemicals:
Streptozotocin (STZ), adiponectin globular recombinant protein: MyBioSource, USA.

Grouping of animals:

- **Group I [control group (n=10)]:** healthy adult male albino rats treated with 0.5 ml saline/day intraperitoneally (I.P) 1 week
- **Group II [diabeti**c, **type 1 group (n=20)]:** type 1 diabetic rats by streptozotocin[9] were subdivided to 2 subgroups 4 weeks after induction of diabetes:
  - ***Subgroup II A* [diabetic retinopathy group:** diabetic rats treated with 0.5 ml saline/day I.P for 1 week
  - ***Subgroup II B* [adiponectin-treated diabetic retinopathy group:** diabetic rats treated by globular adiponectin (3.5 ug/day) I.P for 1 week [10].

Experimental design:

Induction of diabetes type 1:

Single I.P injection of STZ 60 mg/kg was given. STZ was freshly diluted in 0.1 M sodium citrate buffer with pH 4.5. After 2 days we measured blood glucose levels, rats used in the study had glucose levels of 250 mg/dl or more [9]. Rats were injected by 0.1- 0.2 units/kg of insulin subcutaneously daily to prevent ketosis and maintain rats alive, without normalizing blood glucose level [11]. 4 weeks after induction of

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diabetes type 1, diabetic retinopathy was confirmed by retinal histopathological changes. These changes are in line with that proved by **Furman [12] and Olivares *et al.* [13]** who stated that retinal changes began 2 weeks after diabetes onset, and progressed to thinning of retinal layers at 4 weeks.

Sampling of blood and tissue preparation:

At the end of the experiment, the rats were anesthetized with 0.4% pentobarbital sodium injection (1 ml / 100 gm) and blood samples were collected from sinus orbitus vein then both eyes were carefully enucleated. Eyes were immediately placed in formalin solution for 24 hours, then rats were sacrificed by decapitation. The blood was allowed to clot. Serum was separated by centrifugation of blood at 3000 rpm for 15 minutes, and was stored frozen at -20ºC. Retinas were removed and either embedded in paraffin for histopathology and immunohistochemical studies, or snap-frozen in liquid nitrogen and stored at –80°C until use in polymerase chain reaction to detect AQP4 in retinal tissue.

Biochemical analysis: Rat kits were used for estimation of the following;

- Serum glucose, insulin and total cholesterol levels (BioSource Europe, 8-B- 1400 Nivelles-Belgium).
- Serum triglyceride (TG) level (BioSource Europe, 8-C- 1150 Nivelles-Belgium).
- Serum HDL-cholesterol level (BioSource Europe, 8-A- 1340 Nivelles-Belgium).
- Serum MDA, SOD, interleukin-6 (Il-6) and VEGF levels (Bioassay technology laboratory, Shanghai, China).
- LDL was calculated: - LDL=TC-HDL-TG/5

Histopathological analysis:

Hematoxylin and eosin (H&E) were used to stain 7 µm sections of retinal tissue.

Immunohistochemical analysis [14]

Antibodies for VEGF, and TNF-alpha immunohistochemical analysis were applied to 4 µm retinal sections for 60 minutes, and treated according to manufacturer instructions.

Reverse transcription polymerase chain reaction to detect AQP4 in retinal tissue [15]:

Total RNA was extracted from the retinas using an RNAeasy Mini Kit, and reverse transcription and quantitative real-time polymerase chain reaction were performed on a LightCycler® 3 according to the manufacturer's instructions, primers for AQP4, and the internal control rat β-actin primers were used.

AQP4 Forward: TCGCCAAGTCCGTCTTCTACAT.
Reverse: AACATCAGTCCGTTTGGAATCAC.
B-Actin Forward: CCTCTATGCCAACACAGTGC.
Reverse: GTACTCCTGCTTGCTGATCC.

Ethical approval

The study was approved by Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC), number of approval is ZU-IACUC/3/F/53/2019. Rats were handled according to National Institutes of Health (NIH) guidelines for animal experimentation.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by one way ANOVA, and LSD as a post-hoc test by using SPSS program (IBM SPSS statistics 26). P value < 0.05 was considered statistically significant.

RESULTS

Table (1) shows mean value of serum glucose (mg/dl) and insulin levels µIU/ml) in all groups:

Serum Glucose:

In subgroup II A [Diabetic retinopathy], there was significant increase in serum glucose by comparing with group I. In subgroup group II B [Adiponectin-treated diabetic retinopathy], serum glucose was increased significantly by comparing with group I, and significantly lower than subgroup II A.

Serum Insulin:

In subgroup II A [Diabetic retinopathy]: serum insulin levels were significantly lower than group I. In subgroup II B [Adiponectin-treated Diabetic retinopathy]: serum insulin was significantly lower than group I and significantly higher than subgroup II A.

Table (1): Serum glucose (mg/dl) and insulin levels (µIU/ml) in all groups

Mean ± SD	Group I (control group)	Group II A (diabetic retinopathy group)	Group II B (adiponectin-treated diabetic retinopathy group)
Serum Glucose	84.87±4.94	389.94±45.2	215.56±14.94
Serum Insulin	21.43±1.6	1.59±0.4	13.17±1.2
P value of F-test	P<0.001		
P value of LSD vs group I		P<0.001	P<0.001
P value of LSD vs group IIA			P<0.001

Serum lipid profile

Table (2) shows mean value of serum cholesterol, triglycerides, HDL, LDL levels (mg/dl) in all groups:

In subgroup IIA [diabetic retinopathy]: serum cholesterol, serum triglycerides, serum LDL levels were increased significantly by comparing with group I. In subgroup IIB [Adiponectin-treated diabetic

retinopathy]: serum cholesterol, triglycerides, LDL were increased significantly by comparing with group I, and were significantly lower than subgroup II A.
Serum HDL in subgroup II A [diabetic retinopathy] was significantly lower than group I.
In subgroup II B [Adiponectin-treated diabetic retinopathy]: serum HDL was significantly lower by comparing with group I, and increased significantly by comparing with subgroup II A.

Table (2): Serum lipid profile (mg/dl) in all groups:

Serum lipid profile	Group I (control group)	Group II A (diabetic retinopathy group)	Group II B (adiponectin-treated diabetic retinopathy group)
***Serum cholesterol* Mean ±SD**	110.1±10.4	193.88±9.5	136.11±5.9
***Serum triglycerides* Mean ±SD**	67.4 ± 6.1	124.88±4.91	91.11±8.2
***Serum HDL* Mean±SD**	84.4 ± 6.13	38.63± 4.93	60.44 ± 4.33
Serum LDL Mean ±SD	16.7± 2.87	46.75± 3.01	26.33± 2.35
P value of F-test	P<0.001		
P value of LSD vs group I		P<0.001	P<0.001
P value of LSD vs group II A			P<0.001

Serum oxidative stress and inflammatory markers:
Table (3) shows mean value of serum SOD levels (IU/ml), MDA (nmol/ml), IL-6 (pg/ml) in all groups:

Serum SOD: In subgroup II A [diabetic retinopathy] was significantly lower than group I. In subgroup II B [Adiponectin-treated diabetic retinopathy]: serum SOD was significantly lower than group I, and significantly higher than subgroup II A.

Serum MDA: In subgroup II A [Diabetic retinopathy] was significantly higher than group I. In subgroup II B [Adiponectin-treated diabetic retinopathy]: serum MDA was significantly higher than group I, but significantly lower than subgroup II A.

Serum IL-6: In subgroup II A [Diabetic retinopathy]: serum IL-6 was increased by comparing with group I. In subgroup II B [Adiponectin-treated diabetic retinopathy]: serum IL-6 was significantly higher than group I and significantly lower than subgroup II A.

Table (3): Mean value of serum SOD levels (IU/ml), MDA (nmol/ml), IL-6 (pg/ml) in all groups:

Oxidative stress and inflammatory markers	Group I (control group)	Group II A (diabetic retinopathy group)	Group II B adiponectin treated diabetic retinopathy group)
***Serum MDA* Mean ±SD**	7.37± 0.77	23.39± 2.07	12.78 ± 0.52
***Serum SOD* Mean ±SD**	346.02±3.65	186.14±7.83	291.61 ± 9.62
***Serum IL-6* Mean±SD**	122.21± 5.6	283.09±7.83	188.90± 6.1
P value of F-test	P<0.001		
P value of LSD vs group I		P<0.001	P<0.001
P value of LSD vs group II A			P<0.001

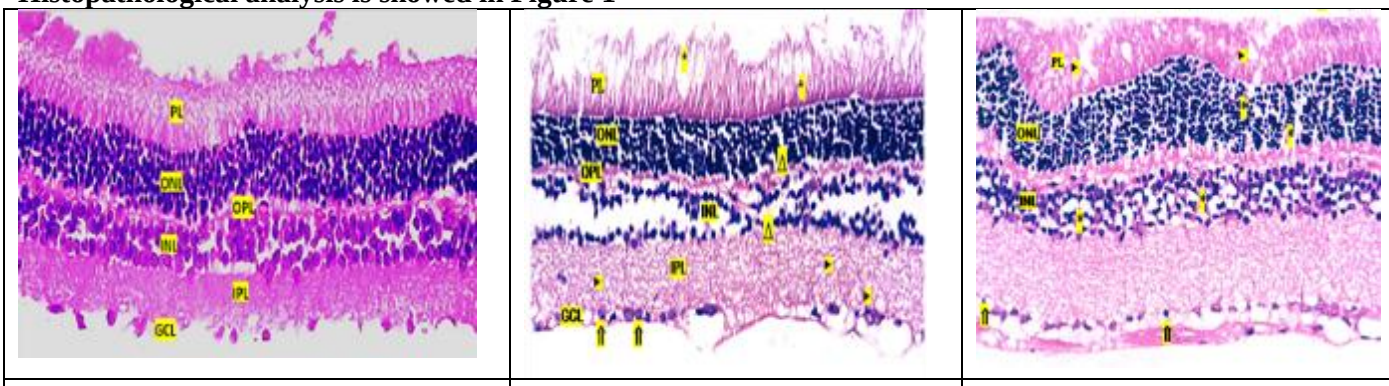
Vascular endothelial growth factor (VEGF):
Table (4) shows mean value of serum VEGF (ng/ml) in all groups.
In subgroup II A [Diabetic retinopathy]: serum VEGF was significantly higher than group I.
In subgroup II B [Adiponectin-treated diabetic retinopathy]: serum VEGF was significantly higher than group I and was significantly lower than subgroup II A.

AQUP 4 gene expression:
Table (4) shows mean value of AQUP 4 gene expression in all groups. In subgroup II A [Diabetic retinopathy]: AQUP 4 gene expression was significantly higher than group I. In Subgroup II B [Adiponectin-treated diabetic retinopathy]: AQUP 4 gene expression was significantly higher than group I and was significantly lower than subgroup II A.

Table (4) serum VEGF (ng/ml) and AQP4 gene expression in all groups

	Group I (control group)	Group II A (diabetic retinopathy group)	Group II B adiponectin-treated diabetic retinopathy group)
VEGF Mean ±SD	42.7 ±4.57	232.49±8.94	128.08± 6.83
AQUP 4 gene expression Mean ±SD	1± 0.02	1.8 ± 0.05	1.3 ± 0.03
P value of F-test	P<0.001		
P value of LSD vs group I		P<0.001	P<0.001
P value of LSD vs group II			P<0.001

Histopathological analysis is showed in Figure 1



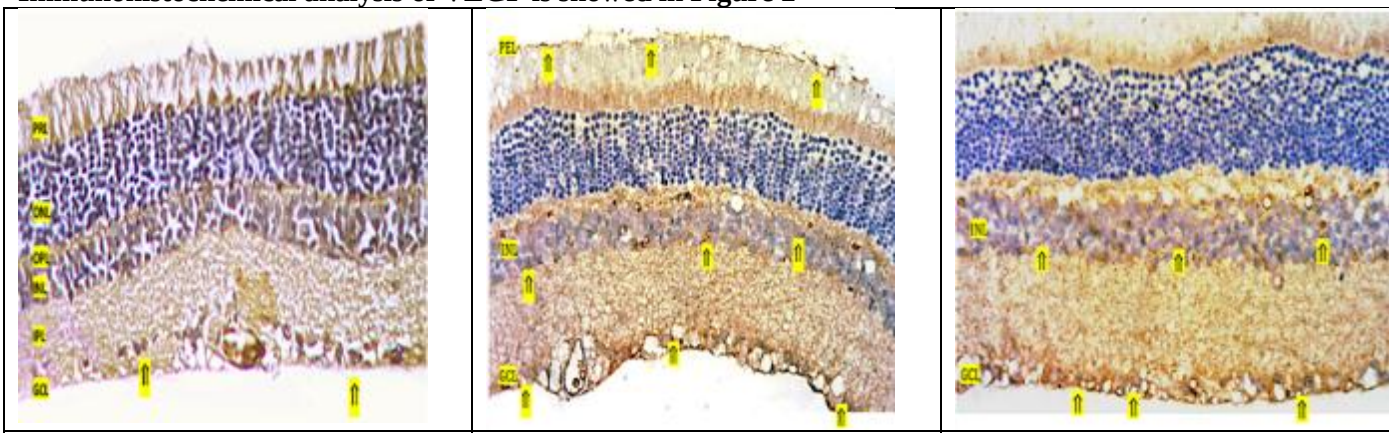
(A)	(B)	(C)

Figure 1a: A photomicrograph of retinal section of normal control group showing photoreceptor layer (PL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL).

Figure 1b: A photomicrograph of retinal section of diabetic control subgroup showing disturbance of the normal structure of the retina. Some photoreceptors are lost (*). The outer plexiform layer (OPL) appears thin, inner nuclear layer (INL) is very thin with loss of nuclei, widening of spaces in inner plexiform layer (IPL) (►) with empty spaces (Δ) in both INL and outer nuclear layer (ONL). Ganglion cell layer (GCL) is present with small dark nuclei (arrow) with vacuoles in between.

Figure 1c A photomicrograph of a retinal section of adiponectin treated diabetic subgroup showing partial improvement in the both retinal architecture and retinal layers thickness. There is still some loss of few nuclei in the outer nuclear layer (ONL) and inner nuclear layer (INL) (*) and few photoreceptors are lost (►). Ganglion cell layer contains small dark cells (arrow).

Immunohistochemical analysis of VEGF is showed in Figure 2



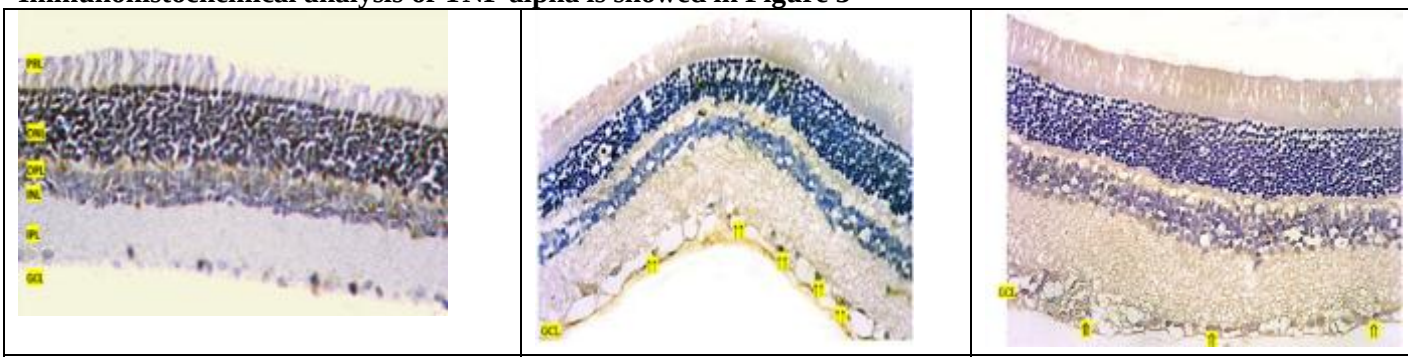
(A)	(B)	(C)

Figure 2a: A photomicrograph of a retinal section of normal control group showing VEGF reaction is very weakly positive and localized to the pigment epithelium layer (PEL) and the ganglion cell layer (GCL).

Figure 2b: A photomicrograph of retinal section of diabetic control subgroup showing strong positive immunoreactions for VEGF (arrows) in many cells of pigmented epithelium layer (PEL), inner nuclear layer (INL) and in ganglion cell layer (GCL).

Figure 2c: A photomicrograph of a retinal section of adiponectin treated diabetic subgroup showing negative reaction for VEGF in most cells in outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL), some weak positive reactions (arrow) are observed in the INL and GCL.

Immunohistochemical analysis of TNF alpha is showed in Figure 3



(A)	(B)	(C)

Figure 3a: A photomicrograph of a retinal section of normal control group showing no reactions for TNF-α.
Figure 3b: A photomicrograph of retinal section of diabetic control subgroup showing strong positive immunoreactions for TNF-α (arrows) in ganglion cell layer (GCL). **Figure 3c:** A photomicrograph of a retinal section of adiponectin treated diabetic subgroup showing some weak positive reactions (arrow) for TNF-α in ganglion cell layer (GCL).

DISCUSSION

Diabetic retinopathy is a serious diabetic complication that causes visual defects and even blindness**,** it is characterized by oxidative stress, inflammation, hypoxia and pathological angiogenesis [3,4].The adipokine adiponectin, has anti-inflammatory, antioxidant and antiangiogenic effects [8,16]**.**

In diabetic retinopathy group, high glucose and low insulin levels can be explained as STZ causes partial destruction of pancreatic β cells with deficiency in insulin secretion and higher blood glucose [17]. Serum glucose level was significantly decreased in adiponectin-treated diabetic retinopathy subgroup, this result was in line with **Yanai and Yoshida** [18] who stated that adiponectin reduced glucose production from the liver and increased skeletal muscles glucose utilization, in addition, adiponectin increases translocation of glucose transporter-4 and glucose uptake by rat skeletal muscle cell**.** Serum insulin level was significantly increased in adiponectin-treated diabetic retinopathy subgroup. It has been reported that adiponectin influences insulin secretion, because its receptor was predominantly expressed in islet β cells, in addition, adiponectin increased insulin exocytosis, accompanied by increase in expression of co-activators of transcription of the insulin gene [6]**.**

The increase in serum lipids level in diabetic retinopathy group was in line with **Warraich *et al.*** [19] who stated that insulin deficiency increased activity of hormone-sensitive lipase, which increases the release of non-esterified fatty acids (NEFA) from triglycerides. Increased NEFA increases triglyceride production from the liver, with increased secretion of apolipoprotein-B (apo-B); the major protein component of LDL. Furthermore, insulin deficiency increases the production of apolipoprotein CIII (apo-CIII), which increases LDL, also, insulin deficiency prevents the actions of lipoprotein lipase (LpL), which is responsible for removal of triglycerides from the circulation [20].

Kurooka *et al.* [20] reported decrease in HDL found in diabetic patients This is a result of increased triglycerides in diabetic patients. Increased level of plasma triglyceride-rich lipoproteins causes transfer of triglycerides from these lipoproteins to HDL through cholesterol esters transfer protein. Catabolism of HDL rich in triglycerides is increased by hepatic lipase, leading to decrease plasma HDL-cholesterol level.

In adiponectin treated-diabetic retinopathy subgroup, adiponectin significantly increased HDL and decreased serum cholesterol, triglycerides and LDL levels. These data are in line with **Christou and Kiortsis** [21] who have shown that adiponectin increases HDL as it increases apolipoprotein A-I production from the liver, which is the major apolipoprotein of HDL. Adiponectin can induce lipoprotein lipase expression and activation, that hydrolyses triglycerides to triglycerides-rich lipoproteins [21]**.**

Serum IL-6 and TNF-α retinal immunoexpression was significantly high in diabetic retinopathy group. This is in line with **Sinclair and Schwartz** [22], who reported that proinflammatory cytokines IL-6, TNF-α, IL-8, IL1β, are increased in serum and retina of diabetic patients.

Sinclair and Schwartz [22] attributed these inflammatory cytokines increase to retinal susceptibility to hypoxia and oxidative stress. Hyperglycemia leads to glial cell dysfunction, activated Müller glial cells contribute to neuronal damage and secreting many proinflammatory cytokine particularly IL-6, and TNF. **Mehrabadi *et al.*** [23] stated that TNF-α is induced by VEGF in diabetic retina, and mediates apoptosis of retinal neurons and vascular endothelial cells in DR.

Decreased serum IL-6 and TNF-α expression within the retinal tissue of adiponectin-treated diabetic retinopathy group goes in line with **Wang *et al.*** [24] who found that adiponectin is effective in inhibiting inflammation, by inhibition of many cytokines (TNF-α, IL-6, IL-1β, and IL-18) through inhibition of the

NF-κB signaling inflammatory pathway. Adiponectin increases activity of anti-inflammatory factors e.g., IL-10. Furthermore, adiponectin interrupts M1 macrophage activation that release pro-inflammatory factors, and support M2 macrophage activation; that prevent inflammatory response [25].

In diabetic retinopathy group, serum and retinal expression of VEGF was increased. **Duan *et al.*[26]** reported upregulation of serum VEGF and its expression in all retinal layers, 4 weeks after STZ induction of diabetes. They found that diabetic hyperglycemia induces hypoxia in retinal tissues, followed by increased production of hypoxia-inducible factor 1 which binds to VEGF gene and initiates its transcription. Decreased serum and retinal expression of VEGF in adiponectin-treated diabetic retinopathy group is consistent with **Gao *et al.* [27]** who reported adiponectin to be an inhibitor of angiogenesis and suppresses VEGF-mediated proliferation in human dermal vascular endothelium. This antiangiogenic activity can be explained by the ability of adiponectin to reduce hyperglycemia-induced oxidative stress leading to decreased vascular permeability and attenuated retinal edema [27].

In diabetic retinopathy group, decrease in SOD accompanied by increase in MDA levels, are in consistent with what reported by **Darenskaya *et al.*[28]** who stated that overproduction of reactive oxygen species in diabetic patients causes inhibition of SOD activity and level with increased of lipid peroxidation and MDA level. In diabetes, increased hydrogen peroxide formation resulted from autoxidation of glucose and non-enzymatic glycation inactivates SOD enzyme.

The increase in serum SOD, and decreased serum MDA in adiponectin-treated diabetic retinopathy group are in line with **Bushra *et al.*[29]** who revealed that adiponectin treatment reduces reactive oxygen species production in human retinal vascular endothelium under hyperglycemic condition, and reported that treatment with adiponectin attenuates the oxidative status in experimentally induced diabetic rats.

In diabetic retinopathy group, aquaporin 4 (AQP4) retinal gene expression was increased. This result agrees with what reported by **Qin *et al.* [30].** AQP4 is the most available osmotic membranous water channel, which is important in regulation of retinal fluid distribution [31-34]. AQP4 is present on astrocytes, which envelope brain and retinal capillaries, and Müller cells [30], during the early stages of DR, AQP4 expression and distribution were changed [31], which lead to abnormalities in movement of water through the glial vascular surface resulting in osmotic swelling of Müller cells and retinal edema, in addition, expression of AQP4 is increased by oxidative stress [32].

One of the most important stimulants of AQP4 expression in DR is angiotensin II, Angiotensin II receptors are present on retinal neurons, glia and blood vessels [33], in addition, there is increased levels of angiotensin II in vitreous of diabetic retinopathy patients. Moreover, angiotensin II receptor blockers decreased AQP4 expression, and prevent retinal edema [30,34]. In adiponectin diabetic retinopathy group, there was significant decrease in AQP4 in comparison to diabetic retinopathy group, so adiponectin may have a role in improvement of retinal edema that accompanies DR. Adiponectin may decrease AQP4 through antagonism of angiotensin II as in angiotensin II induced cardiac fibrosis rat model, adiponectin rescued the angiotensin II–induced reduction of AMP-activated protein kinase (AMPK) phosphorylation and partially suppressed extracellular signal–regulated kinase 1/2 (ERK1/2) activity, which was increased by angiotensin II[35].

Sakaue *et al.*[16] stated that decrease in adiponectin resulted in increased retinal vascular permeability with increase in vascular cellular adhesion molecule-1 and decrease in endothelial claudin-5, which leads to leukocyte activation and blood retinal barrier (BRB) leakage.

Retinal histopathological examination showed apparent reduction of retinal thickness and disorganized retinal layers (Figure 1b). Immunohistochemical analysis showed strong positive expression of VEGF (Figure 2 b) and TNF-α within the retinal tissue (Figure 3 b). Adiponectin showed partial restoration of retinal thickness and organization of its different layers (Figure 1c). Immunohistochemical analysis showed weak positive retinal expression of VEGF (Figure 2 c), and TNF-α (Figure 3 c). The histopathological improvement noticed with adiponectin administration was in accordance with **Deng *et al.*[8]** who demonstrated that adiponectin reduces oxidative stress, limits retinal inflammation, and prevents vascular remodeling and accelerated apoptosis of retinal capillary cells in diabetic rats.

CONCLUSION

Adiponectin may improve diabetic retinopathy via anti-oxidant, anti-inflammatory, antidiabetic and anti-angiogenic effect.

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