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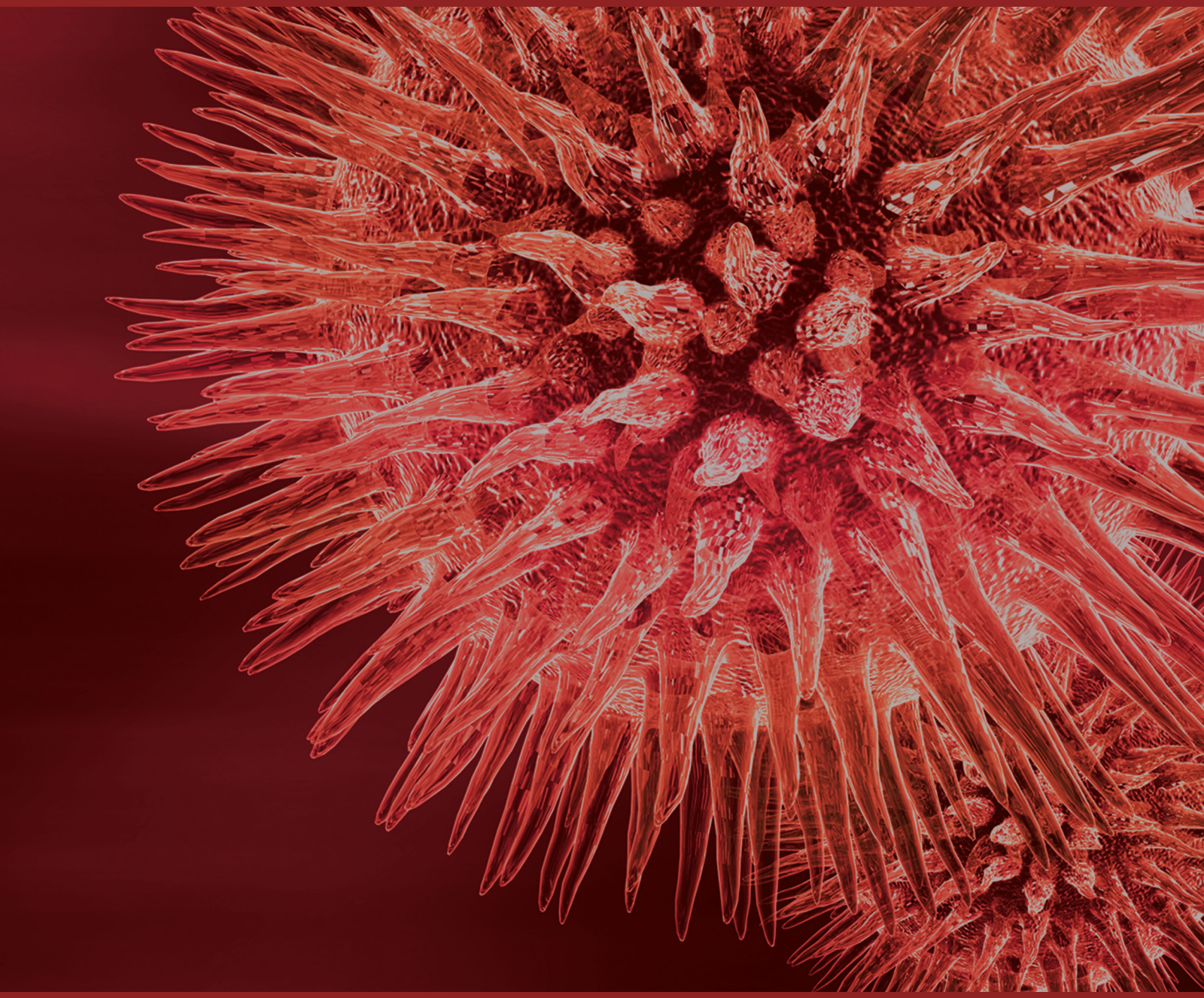
Guest Editors: Mohamed Al-Shabrawey, Wenbo Zhang, and Denise McDonald



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Editorial

Diabetic Retinopathy: Mechanism, Diagnosis, Prevention, and Treatment

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Diabetic retinopathy (DR) is the most common complication of diabetes and remains a major cause of preventable blindness. Anatomical and functional changes occur in various retinal cells including retinal endothelial cells, neurons, and retinal pigment epithelium prior to clinical symptoms of the disease. Early changes include appearance of microaneurysms, leukocyte adhesion, apoptosis of vascular (endothelial cells and pericytes), and neuronal cells. The changes progress to involve breakdown of the inner and outer blood retinal barriers causing diabetic macular edema, the most leading cause of vision loss in DR. Capillary degeneration and development of acellular capillaries lead to impairment of retinal perfusion and subsequent hypoxia and retinal neovascularization, the hallmark of proliferative diabetic retinopathy (PDR). There are several therapeutic strategies to manage the DR including laser photocoagulation, anti-VEGF, and triamcinolone intraocular injection. These therapeutic interventions are still limited by significant side effects. Therefore, there is still an urgent need to find out new therapies to limit the diminution or loss of vision in diabetic patients. The current special issue through a number of investigators and experts in the field of DR presents both research and review articles that highlight novel pathways implicated in the development of DR and review the pathophysiology and management of DR.

Novel Mechanisms of Diabetic Retinopathy. Effective therapeutic approaches to restore sight in diabetic patients with clinically identifiable retinopathy are still lacking and in this issue S. Z. Safi et al. provide a timely review of current thinking in field. Firstly, using several scientific databases (PubMed, Ovid MEDLINE, SPORTDiscus, and EMBASE databases) they have reviewed the literature focusing on the molecular mechanisms involved in the pathogenesis of DR and, secondly on emerging strategies under consideration for development of future pharmacological interventions. Initially, they described the major pathways widely recognized to be involved in disease, namely, the polyol pathway, activation of protein kinase C (KPC) isoforms, increased hexosamine pathway flux and increased advanced glycation end-product (AGE) formation, and oxidative stress along with several other mechanisms such as the potential role of the renin-angiotensin system which are less well researched. Arising from this discussion they summarized established preventive measures including general, primary, and secondary preventive strategies before covering novel and emerging therapeutic targets such as PKC inhibitors, VEGF inhibitors, and ACE inhibitors and drugs such as antioxidants. Finally, the authors draw on current evidence and clinical studies arguing for the use of fenofibrate in halting disease progression. This review, therefore, provides background and context for the research articles included in this special issue.

Reactive oxygen species (ROS) generated from mitochondria, NADPH oxidase, and other oxidases are known

to play an essential role in the pathogenesis of DR ROS modify redox sensitive kinases and transcription factors such as NF-κB, Signal Transducers and Activators of Transcription proteins (STAT), and activator protein 1 (AP-1) and therefore induce inflammatory gene expression in DR. Alternatively, lipid peroxidation and DNA damage induced by ROS cause cell death in DR. In this issue, R. Liu et al. discovered an alternative mechanism on how ROS may play a role in DR. Using human endothelial cells (ECs), they found that ROS treatment induces EC senescence. With gain-and-loss function approaches, they provided the first evidence that ROS-induced EC senescence is at least partially mediated by downregulation of Sirtuin 6 (Sirt6), H3K9, and H3K56 deacetylase recently recognized as a novel antiaging and anti-inflammatory molecule. Further, they found that Sirt6 antagonizes ROS-induced activation of senescence pathways, such as p21 overexpression and activation of retinoblastoma (Rb) protein. As accelerated aging is currently recognized as one of the key mechanisms of many cardiovascular diseases including DR, this study may lead to a hot area to explore Sirtuins as well as other antiaging molecules in DR. Certainly, the role of Sirt6 in diabetic animals and patients remains to be further elucidated. Increased H3K9 acetylation has been found in DR as well as other inflammatory conditions, suggesting that loss of Sirt6 protein and/or function is very likely involved in DR. A challenge to directly address the role of Sirt6 in DR is the lack of transgenic animals. Global Sirt6 knockout mice die within 1 month and EC-specific Sirt6 knockout mice will be important to test whether lack of Sirt6 will accelerate diabetes-induced retinal vascular alterations such as permeability and capillary degeneration if these mice are viable.

There is now much evidence to indicate the involvement of a dysregulated inflammatory response in early disease and defining this role further is likely to offer exciting new avenues for therapeutic intervention. To this end, S. Fulzele et al. have focused on characterizing the regulation of adenosine deaminase 2 (ADA2) by miR-146-3p. They show that the predicted miR-146-3p target site in the UTR of ADA2 is indeed functional. In addition, since the expression of this microRNA is inversely related to ADA2 levels in samples from diabetic patients and similarly treated in vitro modes the authors conclude that miR-146-3p has a regulatory role in diabetes related inflammation.

Most of the research on the pathogenesis of DR has primarily focused on the injury of the neuroretina and the dysfunction of the inner blood retinal barrier (BRB). Contrary, the impact of diabetes on the function of retina pigment epithelium (RPE) has received less attention and also the underlying mechanism for RPE dysfunction during diabetes remains unclear. The paper by S. Beasley et al. demonstrated for the first time the important role of caspase-14 in the development of diabetic macular edema (DME). The expression of caspase-14 in cultured retinal pigment epithelial cells (ARPE-19) is correlated with the disruption of RPE barrier function and hyperpermeability. In addition, knocking down the caspase-14 in RPE abrogated the hyperglycemia-induced RPE hyperpermeability. Interestingly, this paper also demonstrated that caspase-14 might play a role upstream from other caspases to mediate inflammatory and apoptotic responses. In conclusion, this paper suggests caspase-14 as a novel player as well as therapeutic target in DR.

Biomarkers of Diabetic Retinopathy. Diabetic retinopathy is a progressive disease, which is clinically identifiable only at an advanced stage; therefore markers that indicate early disease status would be of major benefit in managing disease progression. Since there are early subclinical changes occurring in retina prior to clinical symptoms, it is necessary to find out specific early biomarkers that predict the pattern and progress of these changes to an advanced stage of DR. The discovery of biomarkers to aid in the identification of patients most likely to develop severe DME and PDR is essential for better treatment of this disease. In support of this concept, B. A. Mysona et al. presented here an interesting clinical study in which they tested whether changes in proNGF/NGF levels observed in vitreous will be matched in serum and thus provide rationale to examine proNGF as a biomarker for DR. This study included analysis of serum and vitreous samples from nondiabetic patients and patients with PDR. Interestingly, the proNGF/NGF imbalance in serum was comparable to the imbalance of proNGF/NGF in vitreous of patients with PDR suggesting that serum proNGF/NGF ratio might act as a novel biomarker that reflects the progress of DR.

Increasing evidence indicates that inflammation is a key player in DR. Increases in vitreous inflammatory cytokines such as IL-6, VEGF, MCP-1, and IP-10 have been found to be positively associated with the progression of DR and the severity of macular edema. However, it is difficult to get vitreous samples and therefore the feasibility of using vitreous cytokines as biomarker is very low. In this issue, N. Dong et al. analyzed an array of inflammatory cytokines in the aqueous humor in relation to macular edema in diabetic patients following uncomplicated phacoemulsification cataract surgery. They found that concentrations of inflammatory cytokines such as IL-1β, IL-6, IL-8, MCP-1, IP-10, and VEGF are positively associated with macular edema whereas levels of anti-inflammatory cytokines such as IL-10 and IL-12 are negatively associated with macular edema. Given that it is easy to obtain the aqueous humor, this study highlights the possibility to use these cytokines as biomarkers for diabetic macular edema. Certainly, it is much easier to obtain tears than aqueous humor; it would be interesting to further explore whether there is an association of tear inflammatory cytokines with diabetic macular edema and neovascularization. This study also brings an interesting question about where these inflammatory cytokines are generated. Are they diffusing into aqueous humor or are they generated from cells in ciliary body or cornea? Further exploration of their source will help to better understand the development of DR.

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Clinical Study

Imbalance of the Nerve Growth Factor and Its Precursor as a Potential Biomarker for Diabetic Retinopathy

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Our previous studies have demonstrated that diabetes-induced oxidative stress alters homeostasis of retinal nerve growth factor (NGF) resulting in accumulation of its precursor, proNGF, at the expense of NGF which plays a critical role in preserving neuronal and retinal function. This imbalance coincided with retinal damage in experimental diabetes. Here we test the hypothesis that alteration of proNGF and NGF levels observed in retina and vitreous will be mirrored in serum of diabetic patients. Blood and vitreous samples were collected from patients (diabetic and nondiabetic) undergoing vitrectomy at Georgia Regents University under approved IRB. Levels of proNGF, NGF, and $p75^{NTR}$ shedding were detected using Western blot analysis. MMP-7 activity was also assayed. Diabetes-induced proNGF expression and impaired NGF expression were observed in vitreous and serum. Vitreous and sera from diabetic patients ($n = 11$) showed significant 40.8-fold and 3.6-fold increases, respectively, compared to nondiabetics ($n = 9$). In contrast, vitreous and sera from diabetic patients showed significant 44% and 64% reductions in NGF levels, respectively, compared to nondiabetics. ProNGF to NGF ratios showed significant correlation between vitreous and serum. Further characterization of diabetes-induced imbalance in the proNGF to NGF ratio will facilitate its utility as an early biomarker for diabetic complications.

1. Introduction

Diabetic retinopathy (DR), a leading cause of blindness in working age adults, is estimated to affect 101 million people worldwide [1]. Although DR is initially asymptomatic, chronic hyperglycemia caused by insufficient insulin damages the retinal microvasculature. Early insults include pericyte loss, microaneurysms, and leukostasis [2–4]. Dysfunction of the blood retinal barrier is evidenced by increased microvascular permeability and diabetic macular edema (DME), which lead to decreased visual acuity [5, 6]. Endothelial cell death and acellular capillary formation further impair the retinal blood supply leading to proliferative diabetic retinopathy (PDR) which is characterized by growth of fragile, leaky blood vessels and loss of vision [7, 8]. Current treatments for DME and PDR such as laser photocoagulation and anti-VEGF injections are invasive with considerable side effects [9–11]. Even though the overall risk of developing DR increases with duration of diabetes, poor glycemic control, and high blood pressure, the rate of development and severity of DR varies greatly from patient to patient [1, 12–14]. Several studies have identified morphological biomarkers associated with the progression of DR including changes in the multifocal electroretinogram, microaneurysm turnover, and subclinical edema [6, 15–22]. In addition, altered expression of cytokines, chemokines, and angiogenic and apoptotic related factors has been identified in the vitreous of patients with DME and PDR [23–27]. Despite these advances, we still

Table 1: (a) Clinical characteristics of control subjects. (b) Clinical characteristics of diabetic patients.

(a)

Patient	Type	Age	Sex	Ethnicity	Sample	Retinal pathology
1	Control	65	Female	White	Both	Macular hole
2	Control	72	Male	White	Both	Macular pucker
3	Control	18	Female	Black	Vitreous	Retinal detachment
4	Control	59	Male	White	Serum	Retinal detachment
5	Control	60	Male	White	Serum	Retinal detachment
6	Control	70	Male	Black	Vitreous	Retinal detachment, globe injury
7	Control	40	Male	White	Serum	Retinal detachment
8	Control	62	Female	Black	Vitreous	Macular hole
9	Control	57	Male	White	Serum	Retinal detachment

(b)

Patient	Type	Age	Sex	Ethnicity	Sample	HbA1c	Retinal pathology
10	Diabetic	46	Female	Black	Both	Unknown	PDR
11	Diabetic	75	Male	White	Both	8.7	PDR
12	Diabetic	57	Female	Black	Both	9.7	PDR
13	Diabetic	38	Female	Black	Both	11.3	PDR
14	Diabetic	51	Female	Black	Both	6.8	PDR
15	Diabetic	58	Female	White	Both	Unknown	PDR
16	Diabetic	31	Female	Black	Both	Unknown	PDR
17	Diabetic	55	Male	Black	Both	Unknown	PDR
18	Diabetic	50	Female	White	Both	6.8	PDR
19	Diabetic	24	Female	Black	Both	6.4	PDR
20	Diabetic	49	Male	Black	Both	Unknown	PDR

do not have a reliable biomarker that is readily detectable in serum and that predicts the likelihood of a patient developing sight threatening complications of DR.

Changes in level of nerve growth factor (NGF) have been previously assessed in diabetic patients in relation to diabetic retinopathy and neuropathy [28–30]. NGF is traditionally released as the proform, proNGF, which is cleaved intracellularly by furins and extracellularly by several proteases including MMP-7 [31, 32]. While NGF binds to the tyrosine kinase receptor A (TrkA) to signal through prosurvival pathways, proNGF binds preferentially to $p75^{NTR}$, which in combination with its coreceptor sortilin generally activates inflammatory and apoptotic pathways (reviewed in [33–35]). Our group has discovered that diabetes causes an imbalance of increased proNGF at the expense of mature NGF due to impaired MMP-7 activity in clinical and experimental diabetes [31]. Specifically, proNGF levels are elevated and NGF levels reduced in the aqueous humor of patients with PDR and in the vitreous of diabetic patients. In experimental STZ-diabetes rat model, decreases in NGF were associated with early retinal neurodegeneration [36–38]. Treatments that enhanced the levels of NGF either endogenously [31, 36] or by exogenous supplement of recombinant NGF protein [37, 39] prevented retinal neurodegeneration in models of diabetes. In the diabetic retina, increased expression of $p75^{NTR}$, the preferred receptor of proNGF, exacerbates the effects of the proNGF/NGF imbalance [36, 38, 40] by favoring the activation of the proNGF/$p75^{NTR}$ signaling pathways that are associated with increases in inflammatory mediators and vascular permeability [36, 38, 40].

In the present work, we performed a small pilot study investigating the feasibility of whether changes in proNGF/NGF levels observed in vitreous will be mirrored in serum and thus provide rationale to examine proNGF as a biomarker for diabetic retinopathy. This study includes an analysis of vitreous and serum samples from patients with PDR and from nondiabetic patients, all undergoing vitrectomy.

2. Subjects and Methods

2.1. Study Participants. Participants were recruited from the Georgia Regents Eye Care Center, Augusta, GA. All participants gave written informed consent and the study was governed under guidelines of the local institutional review board. A total of 9 nondiabetic and 11 diabetic participants were included in this study. Blood was drawn shortly before surgery. Vitreous was collected from participants undergoing pars plana vitrectomy. Characteristics of the study participants are summarized in Table 1.

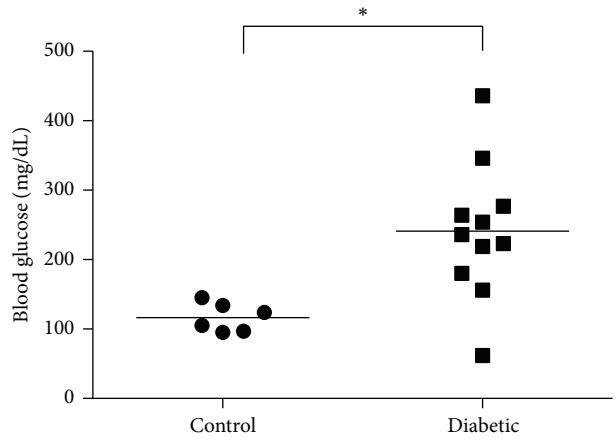

Figure 1: Blood glucose higher in diabetic patients. On average, diabetic participants had significantly higher glucose concentration of 241 mg/dL ± 30 than nondiabetic control participants 117 mg/dL ± 9 in their serum ($N = 6$–11, $^{*}P < 0.05$).

2.2. Serum Sample Preparation. Whole blood was allowed to clot 60–90 minutes at room temperature and then was centrifuged at 3200 g for 15 minutes to separate serum from red blood cells. Serum blood glucose was tested using the ReliOn Ultima blood glucose meter (Abbott Diabetes Care, Inc., Alameda, CA). Serum aliquots were placed into cryovials and stored at −80°C for further analysis.

2.3. Vitreous Sample Preparation. Vitreous wash was collected, filtered, and concentrated. Vitreous was prefiltered through a combination Whatman 1 filter paper circle (GE Healthcare Biosciences, Pittsburgh, PA) fit to a Nalgene Rapid-Flow sterile filter unit (Thermo Fisher Scientific, Waltham, MA). Proteins 10 kilodaltons (kD) and greater were concentrated from the filtrate with a Corning Spin-X UF concentrator (Corning Inc., Corning, NY) and Amicon Ultra centrifugal filters (Merck Millipore, Darmstadt, Germany). Concentrated aliquots were placed into cryovials and stored at −80°C for further analysis.

2.4. Protein Estimation. Protein concentration of serum and vitreous was measured using Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).

2.5. Western Blot Sample Preparation. Serum and vitreous samples were diluted to a concentration of 3 μg/μL in RIPA buffer with protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO), and halt phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Dilution of serum was critical so that samples could be boiled without coagulation. For Western blot, 35–50 μg aliquots with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) plus beta mercaptoethanol (Sigma-Aldrich, St. Louis, MO) were boiled 10 minutes and then loaded on 15% polyacrylamide gels, which results in good separation of proteins in the 10 to 30 kD range. Thus, the larger abundant proteins such as albumin (66 kD) are held back in the upper portion of the gel away from the proteins that we are interested in (proNGF 26–32 kD, NGF 13 kD).

2.6. Western Blot Analysis. Samples of vitreous and serum were separated by SDS-PAGE, transferred to nitrocellulose membrane and stained with Ponceau S (Sigma-Aldrich, St. Louis, MO). After blocking for 15–30 minutes, membranes were probed overnight with primary antibodies per manufacturer's recommendation in 5% milk or 2% BSA followed by secondary antibody (1 : 5000 in 5% milk). Blots were developed using Pierce ECL Western Blot Substrate (Thermo Fisher Scientific, Rockford, IL) or Immoblion Western chemiluminescent HRP substrate (Merck Millipore, Darmstadt, Germany). Films were scanned and the band intensity was quantified using densitometry software (alphEaseFC) and then expressed as relative optical density (ROD) normalized to Ponceau S (due to lack of a specific loading control for serum and vitreous) and expressed as fold change from nondiabetic controls. Representative images chosen for Western blot were non-adjacent bands from the same blot.

2.7. Antibodies. The following antibodies were used for immunoblotting: rabbit polyclonal anti-NGF and anti-proNGF (Alomone Labs, Israel) and rabbit polyclonal $p75^{NTR}$ (1 : 5000), a kind gift from Dr. Bruce Carter, Vanderbilt University, Nashville, TN. Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit (Calbiochem, La Jolla, CA).

2.8. MMP-7 Activity. The activity of matrix metalloproteinase 7 (MMP-7) was determined using the Sensolyte 490 generic MMP-7 fluorimetric assay kit (AnaSpec, EGT Corporation, Fremont, CA) per the manufacturer's instructions and as described by our group previously [31].

2.9. Data Analysis. Graphs were prepared with the aid of GraphPad Prism 6.04 (GraphPad Software Inc., La Jolla, CA).

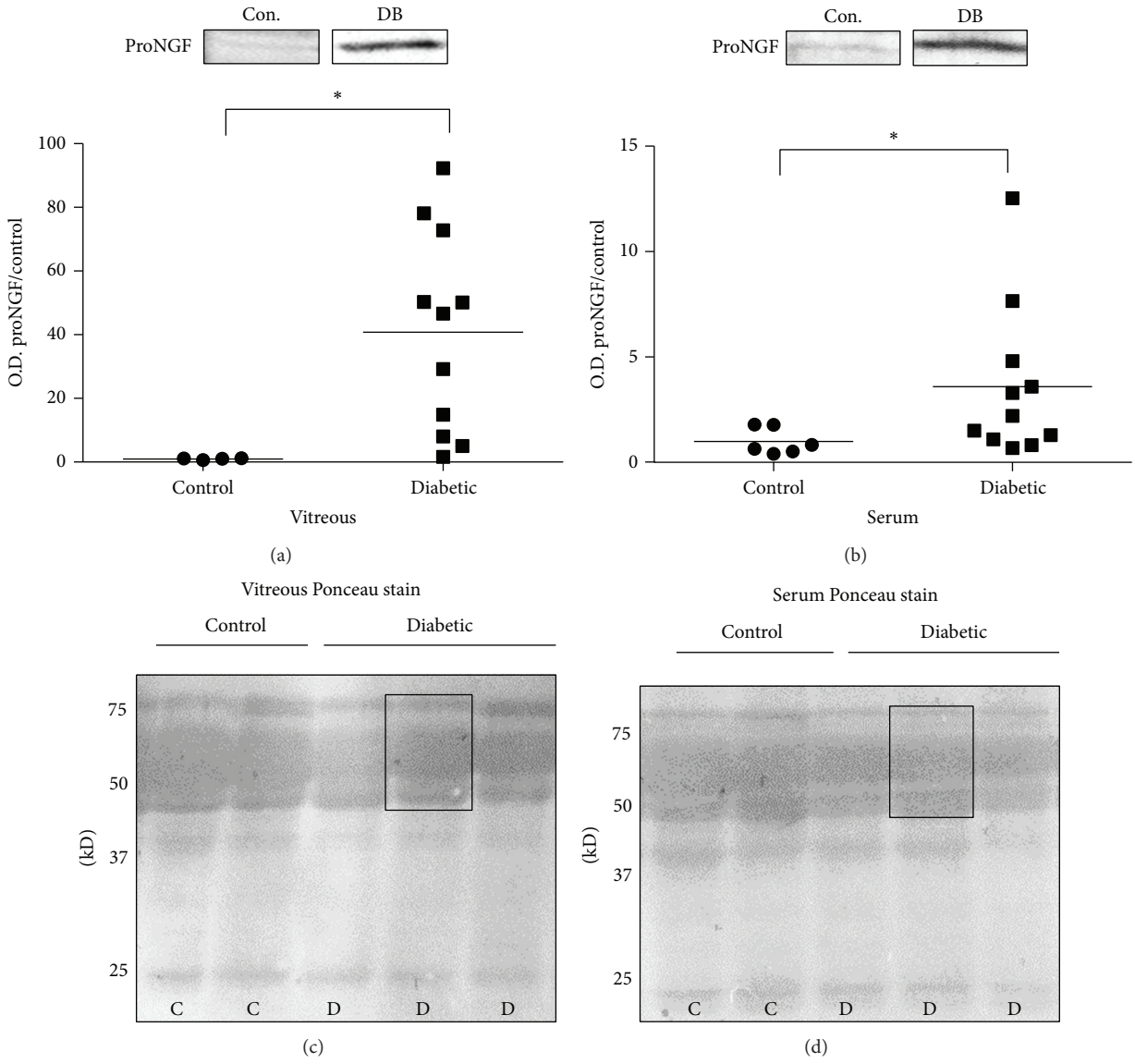

Figure 2: ProNGF increases are consistent in vitreous and serum. Representative blots and statistical analysis of proNGF protein expression are shown. ProNGF band intensities in vitreous and serum of diabetic (DB) and nondiabetic control (Con.) participants were normalized to Ponceau S and respective control group. (a) ProNGF expression is significantly elevated in vitreous of diabetic, 40.8-fold ± 9.5, relative to nondiabetic control group ($N = 4$–11, $^{*}P < 0.05$). (b) In serum, proNGF expression is significantly increased, 3.6-fold ±1.1, compared to nondiabetic controls ($N = 6$–11, $^{*}P < 0.05$). Typical image of Ponceau staining for (c) vitreous blots and (d) serum blots showing the equal loading of control and diabetic samples as well as the area selected for intensity measurements.

Results were expressed as mean ± SEM. Normality of data was verified by the D'Agostino and Pearson omnibus normality test. Normal data sets were compared using unpaired t-test with or without Welch's correction for unequal variances. Nonparametric data sets were evaluated using the Mann-Whitney test. After eliminating outliers, correlation between normalized proNGF/NGF expression ratio in diabetic vitreous and serum was calculated using the Pearson coefficient followed by Deming linear regression between groups with different standard deviations. Outliers were determined to be all proNGF/NGF ratios outside the range of the median ±1.5 × (interquartile range). Data analysis was performed using GraphPad Prism 6.04 and JMP Pro 10.0.1 Release 2 software (SAS, Cary, NC). Significance was defined as $P < 0.05$.

3. Results

3.1. Study Participants. A total of 20 participants, both males and females of various ethnic backgrounds, were recruited from the Georgia Regents Eye Care Center, Augusta, GA. The 9 nondiabetic control patients had an average age of 55.9 ± 5.6 years and were undergoing pars plana vitrectomy for retinal detachment or macular pucker. The 11 diabetic participants were undergoing pars plana vitrectomy for retinal

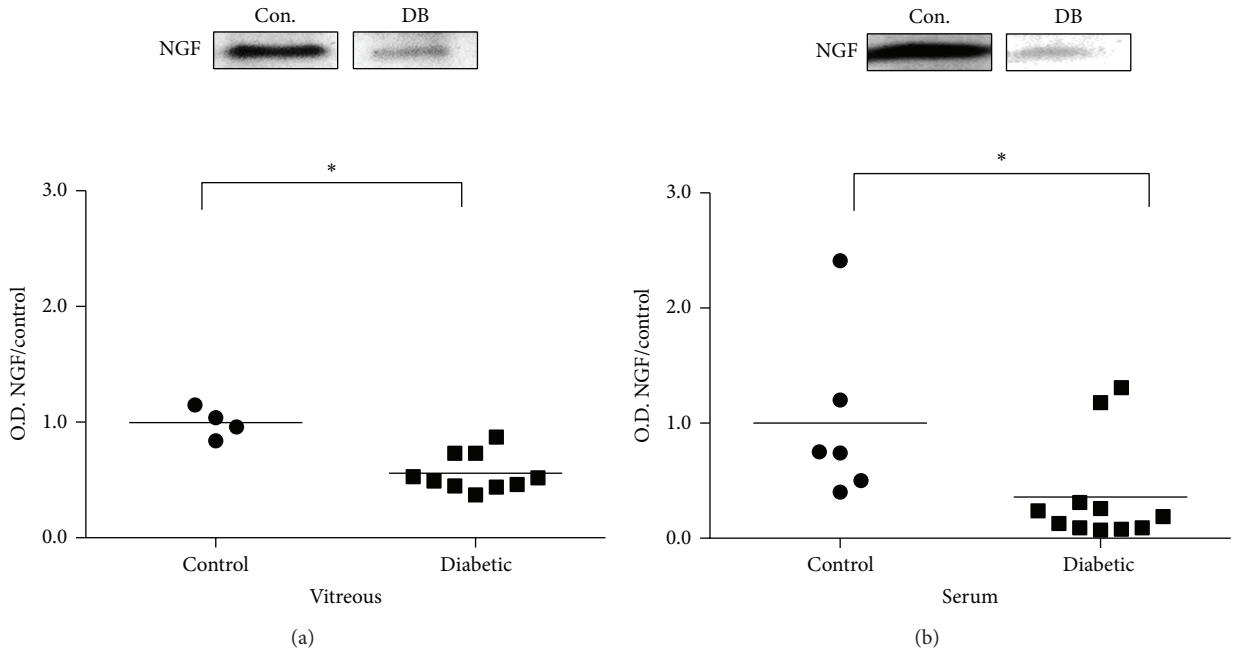

Figure 3: NGF decreases are consistent in vitreous and serum. Representative blots and statistical analysis of NGF protein expression are shown. NGF band intensities in vitreous and serum of diabetic (DB) and nondiabetic control (Con.) participants were normalized to Ponceau S and respective control group. (a) NGF expression is significantly reduced in diabetic vitreous, 0.56 ± 0.05, compared to nondiabetic control group, 1.00 ± 0.07 ($N = 4$–10, $^*P < 0.05$). (b) A significant decrease in NGF expression occurs in diabetic serum, 0.36 ± 0.13, compared to nondiabetic controls 1.00 ± 0.30 ($N = 6$–11, $^*P < 0.05$).

detachment as a complication of PDR and were primarily type 2 diabetic with an average age of 48.5 ± 4.2 years. HbA1c values were available for half of the diabetic participants and averaged 8.3% ± 0.8. Characteristics of the participants appear in Table 1.

3.2. Blood Glucose Levels Are Higher in Diabetic Participants. Screening of participant blood glucose levels showed that all but one of the diabetic patients had significantly higher serum glucose levels than their nondiabetic counterparts (Figure 1). On average, blood glucose in serum from diabetic participants was 241 mg/dL ± 30 while nondiabetic control participants had serum glucose values of 117 mg/dL ± 9.

3.3. ProNGF Increases Are Consistent in Vitreous and Serum. Statistical analysis of Western blot band intensity normalized to Ponceau S staining showed that the observed increases in proNGF in vitreous occurred also in serum, albeit, to a lesser extent. In diabetic vitreous, proNGF was significantly elevated 40.8-fold ±9.5. In serum, the trend towards increased proNGF expression was not as dramatic but still significant with a 3.6-fold ±1.1 elevation in proNGF expression compared to nondiabetic controls (Figures 2(a) and 2(b)). A typical example of the Ponceau S signal demonstrates that this staining can be used as a crude measure of evaluating the equal loading between control and diabetic samples from vitreous and serum (Figures 3(a) and 3(b)). The Ponceau S signal was determined by measuring band intensity of a rectangle width of the band from 50 to 75 kD (Figures 2(c) and 2(d)).

3.4. NGF Decreases Are Consistent in Vitreous and Serum. Statistical analysis of Western blot band intensity normalized to Ponceau S staining showed that the proNGF/NGF imbalance in vitreous occurred also in serum, albeit, to a lesser extent. In diabetic vitreous, NGF expression was significantly decreased 0.56 ± 0.05 compared to the nondiabetic control group (Figure 3(a)). NGF expression was significantly decreased in serum of diabetic participants, 0.36 ± 0.13, relative to nondiabetic controls (Figure 3(b)).

3.5. Imbalance of ProNGF/NGF Ratio Is Consistent in Vitreous and Serum. Next, we evaluated the usefulness of the proNGF/NGF expression ratio as a measure of the proNGF/NGF imbalance in vitreous and serum. In vitreous, the proNGF to NGF expression ratio in individual diabetic participants was significantly 81.0-fold higher than in nondiabetic control participants (Figure 4(a)). In serum the proNGF/NGF expression ratio was significantly increased by a more modest 12.9-fold compared to serum of nondiabetic control participants (Figure 4(b)). After elimination of two diabetic participants because of very high proNGF/NGF ratios determined to be outliers, correlation and linear regression were performed on the remaining proNGF/NGF ratios. Deming linear regression of proNGF/NGF ratios in vitreous as a function of the ratio in serum of diabetic participants resulted in a linear correlation described by the equation

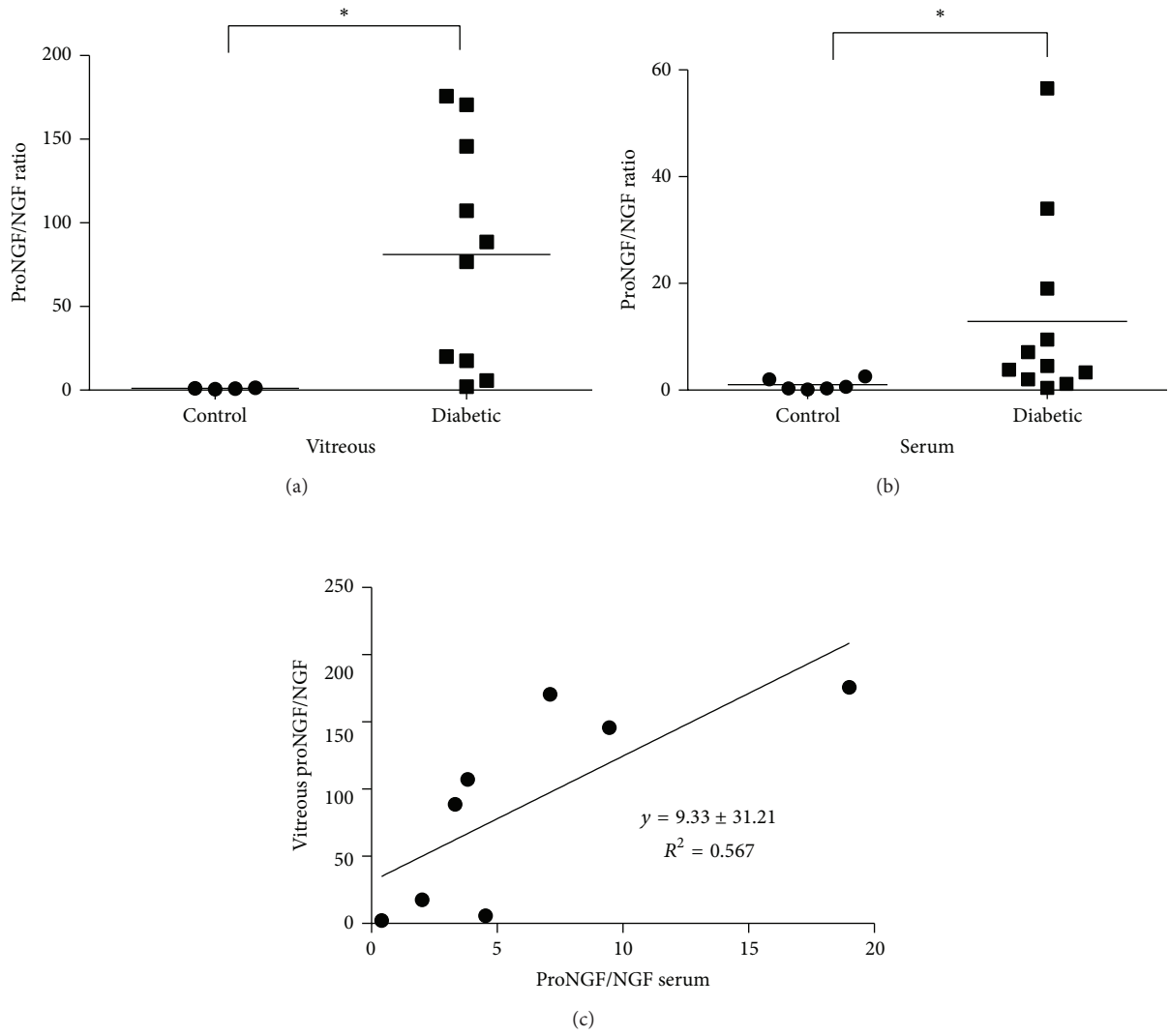

Figure 4: ProNGF/NGF ratio in vitreous correlated to proNGF/NGF ratio in serum. (a) In vitreous, the expression ratio of proNGF to NGF in individual diabetic participants was significantly higher than nondiabetic control participants ($N = 4$–10, $^*P < 0.05$). (b) In serum expression ratio of proNGF to NGF in individual diabetic participants was also significantly higher than nondiabetic control participants ($N = 6$–11, $^*P < 0.05$). (c) Deming linear regression of proNGF/NGF ratios in vitreous as a function of the ratio in serum of diabetic participants had a slope of 9.33 ± 3.33 and an intercept of 31.21 ± 27.65. The linear correlation was significant with a Pearson coefficient of $R^2 = 0.567$ ($N = 8$, $P < 0.05$).

$y = 9.33x + 31.21$ with a Pearson coefficient of $R^2 = 0.567$ (Figure 4(c)).

3.6. MMP-7 Activity Decreased in Vitreous but Not in Serum. The enzyme MMP-7 is known to cleave proNGF to form the mature neurotrophin NGF [32]. MMP-7 proteolytic activity, measured in relative fluorescent units (RFU), was reduced in diabetic vitreous to 66.6 RFU ± 4.4, compared to the nondiabetic control group, 77.5 RFU ± 11.7, but the difference between groups was not significant (Figure 5(a)). Interestingly, in serum, MMP-7 activity detected in the diabetic group, 77.3 RFU ± 2.0, was similar to the activity in nondiabetic controls, 78.7 RFU ± 1.3 (Figure 5(b)).

3.7. Shedding of p75^{NTR} Receptor Is Consistent in Vitreous and Serum. Expression of p75^{NTR} receptor in vitreous and serum samples from diabetic and control participants was analyzed by Western blot. Signaling via p75^{NTR} involves a sequence of proteolytic events including ectodomain shedding and regulated intramembrane proteolysis [41–43]. Accordingly, multiple sized p75^{NTR} variants were detected by WB. Representative blots (Figure 6(a)) of p75^{NTR} showed three main size variants including full length p75^{NTR} (75 kD), the receptor ectodomain (50 kD), a 27 kD possible C-terminal fragment (CTF), and a 22 kD possible intracellular domain (ICD) fragment. At the present time, distinguishing the exact identity of these smaller fragments is not possible. Statistical

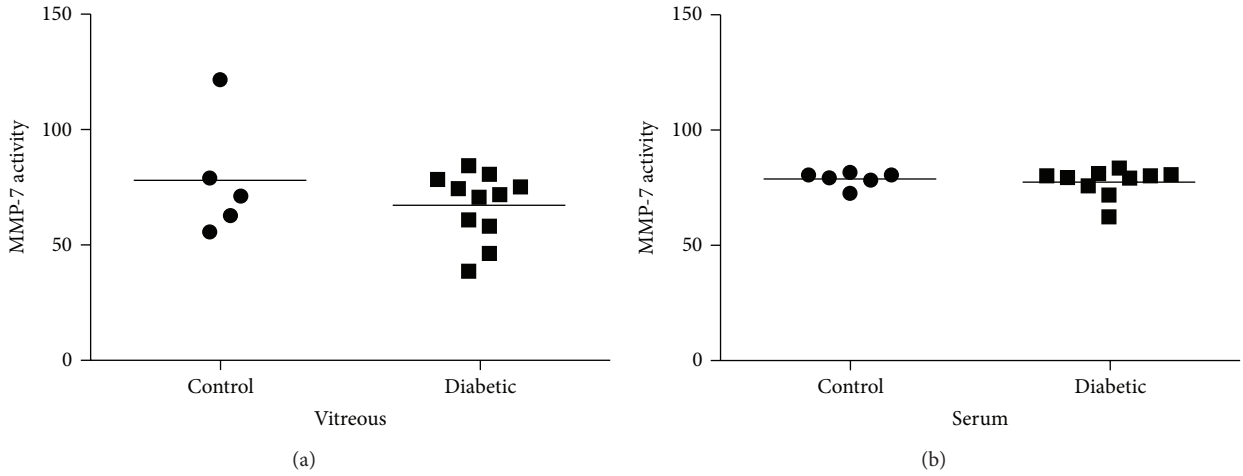

Figure 5: MMP-7 activity is decreased in vitreous but not in serum. (a) In vitreous, MMP-7 activity was decreased in diabetic (66.6 ± 4.4) compared to nondiabetic control group (77.5 ± 11.7). (b) In serum, MMP-7 activity detected in the diabetic groups (77.3 ± 2.0) was similar to the activity in nondiabetic controls (78.7 ± 1.3).

analysis of band intensities showed that, in vitreous, 27 kD $p75^{NTR}$ possible CTF fragment was significantly increased in diabetic (1.65-fold ± 0.23) compared to nondiabetic control group (Figure 6(b)). In serum, a significant increase in 22 kD $p75^{NTR}$ possible ICD fragment occurred in diabetic (1.85 fold ± 0.30) compared to nondiabetic controls (Figure 6(c)).

Statistical analysis of band intensities showed that full length $p75^{NTR}$ receptor (75 kD) was not significantly different in diabetic compared to control groups in either vitreous or serum (Figures 7(a) and 7(b)). The $p75^{NTR}$ ectodomain (50 kD) was also not significantly different in diabetic compared to control groups in vitreous or serum (Figures 7(c) and 7(d)).

4. Discussion

The results of this pilot study shed light on the relationship between the diabetes-induced proNGF/NGF imbalance in vitreous and serum. Three main findings arose from this work. First, on average, the increased proNGF and decreased NGF expression in diabetic vitreous was also observed in diabetic serum. There was significant and positive correlation of the ratio of proNGF/NGF in vitreous to serum in PDR patients. Second, MMP-7 activity in vitreous of participants with PDR was slightly lower than in nondiabetic control subjects; however, no difference between groups was observed in serum. Third, proteolytic shedding of $p75^{NTR}$ receptor fragments was increased in both diabetic vitreous and serum compared to nondiabetics. Together, these results support the feasibility and rationale of investigating the increased serum proNGF/NGF ratio as a biomarker of DR.

While there has been interest in correlating changes in NGF levels with diabetic complications, previous studies have used the enzyme-linked immunosorbent assay (ELISA) that cannot differentiate between NGF and its precursor form, proNGF. These studies showed that diabetes increases serum NGF levels and omitted the impact of diabetes on the homeostasis between proNGF and NGF [28–30]. In the present study, this problem was circumvented by separating serum and vitreous samples by Western blot and by utilizing an anti-proNGF antibody that specifically recognizes the "pro" region of proNGF which can exist as 26, 32, or 40 kD forms depending on the glycosylation state [44–46]. To avoid any confusion between the NGF dimer and 26 kD proNGF, the smaller NGF monomer (13 kD) was evaluated in our studies. The results of our pilot study showed that serum from diabetic participants with PDR had a significant 3.6-fold increase in proNGF expression compared to a more dramatic increase of 40.8-fold in the diabetic vitreous relative to nondiabetic control group. In addition to elevated serum proNGF, low levels of serum NGF in diabetic participants exacerbated the effects of increased proNGF. NGF levels in participants with PDR were decreased significantly to 0.36 ± 0.13 of control levels in serum and 0.56 ± 0.05 of control levels in vitreous compared to the nondiabetic control group. The present study resulted in the novel finding that a vitreous proNGF/NGF ratio is positively correlated with its ratio in serum of PDR patients (Figure 4). Increases in proNGF and decreases in NGF in vitreous from PDR patients lend further support to our previous findings in ocular fluids of diabetic patients [31] and experimental diabetes [36, 38, 40]. One of the caveats in studying vitreous protein changes in PDR is that protein levels will be higher in the diabetic vitreous due to leakage of blood into the vitreous [27]. This effect is partially corrected by the loading of equal amounts of protein for each sample; however, the possibility remains that some of the vitreal protein expression changes may be influenced by leakage of blood into the retina. Overall, the large increase in proNGF expression in vitreous, decrease in NGF expression, and different banding patterns for $p75^{NTR}$ fragment expression in vitreous versus serum lead us to believe that these trends are linked to protein expression changes in the vitreous. Although proNGF is better known

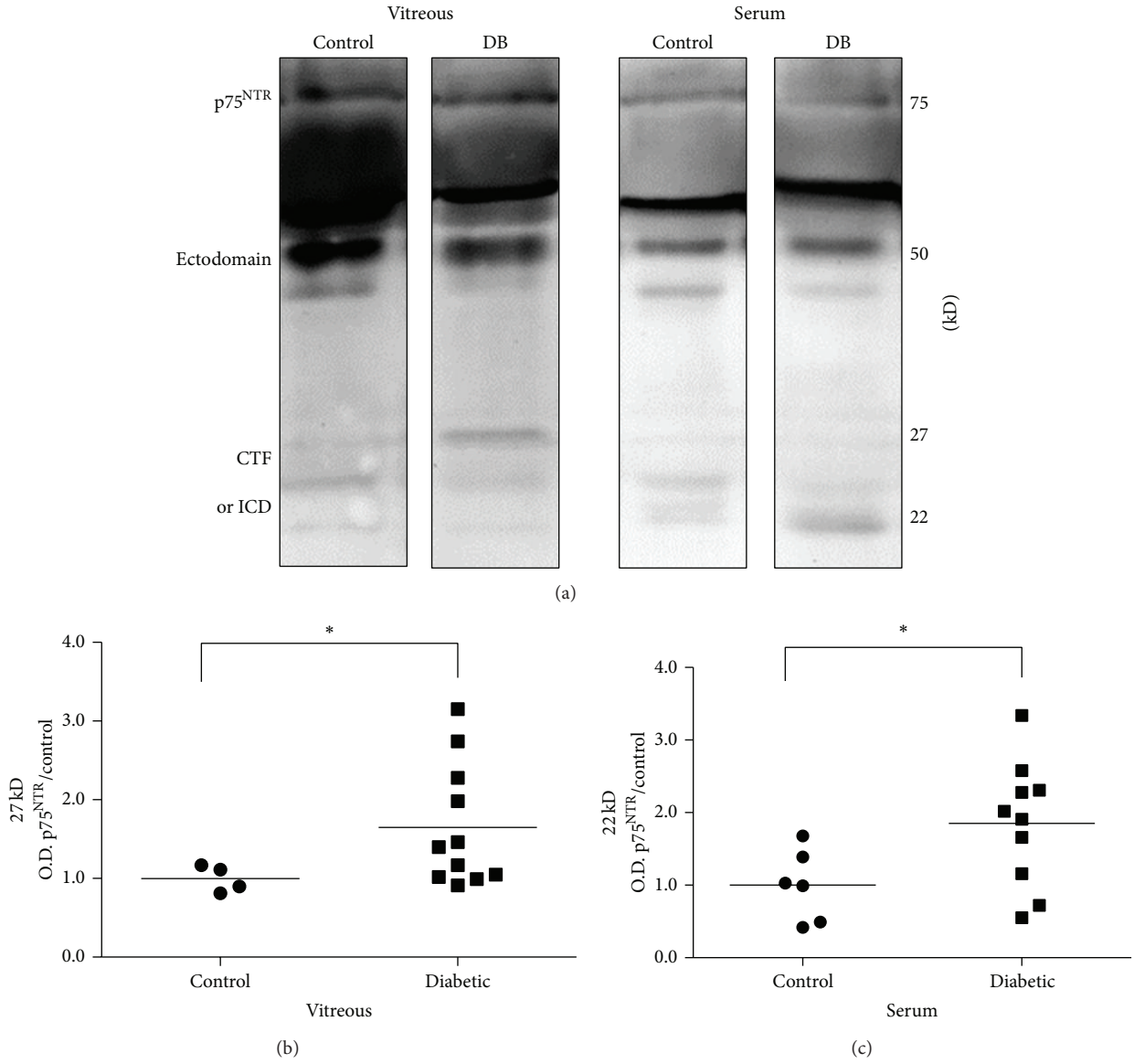

Figure 6: Shedding of $p75^{NTR}$ is consistent in vitreous and serum. (a) Representative bands show $p75^{NTR}$ expression in vitreous and serum for diabetic compared to nondiabetic control groups. The full length $p75^{NTR}$ (75 kD) and receptor ectodomain (50 kD) had similar levels of expression in control and diabetic (DB) groups of both vitreous and serum. The possible proteolytic C terminal fragment (CTF) and intracellular domain (ICD) appeared at 27 kD and 22 kD. Differences in expression patterns between vitreous and serum as well as between diabetic and control groups were evident for both CTF and ICD. (b) In vitreous, 27 kD $p75^{NTR}$ receptor fragment was significantly increased in diabetic (1.65-fold ± 0.23) compared to nondiabetic control group ($N = 4$–11, $^{*}P < 0.05$). (c) In serum, a significant increase in 22 kD $p75^{NTR}$ occurred in diabetic samples (1.85-fold ± 0.30) compared to nondiabetic controls ($N = 6$–10, $^{*}P < 0.05$).

for its roles in neuronal development, CNS injury, and neurodegenerative diseases, proNGF and NGF are increasingly recognized for their important signaling functions in such diverse organs as retina, brain, pancreas, kidney, thyroid, testes, immune system, and vasculature [30, 31, 46–56]. Thus, diabetes-induced alterations in the proNGF/NGF imbalance in serum may well reflect combined contributions from the retina, other organs, the immune system, and the vasculature.

The second finding of this pilot study was that although MMP-7 activity was lower in vitreous of participants with PDR, no apparent activity difference between groups was observed in serum. Although not statistically significant, lower MMP-7 activity in vitreous agrees with previous findings that MMP-7 activity is significantly reduced in aqueous humor of patients with PDR and that MMP-7 protein expression is decreased in diabetic rat retinal lysates [31]. Similar results are reported in kidney with MMP-7 activity and protein expression reduced under conditions of diabetic nephropathy [57]. The correlation, however, between tissue levels of MMP-7 versus the systemic environment of serum is not always straightforward. Two separate studies have documented that serum levels of MMP-7 are higher

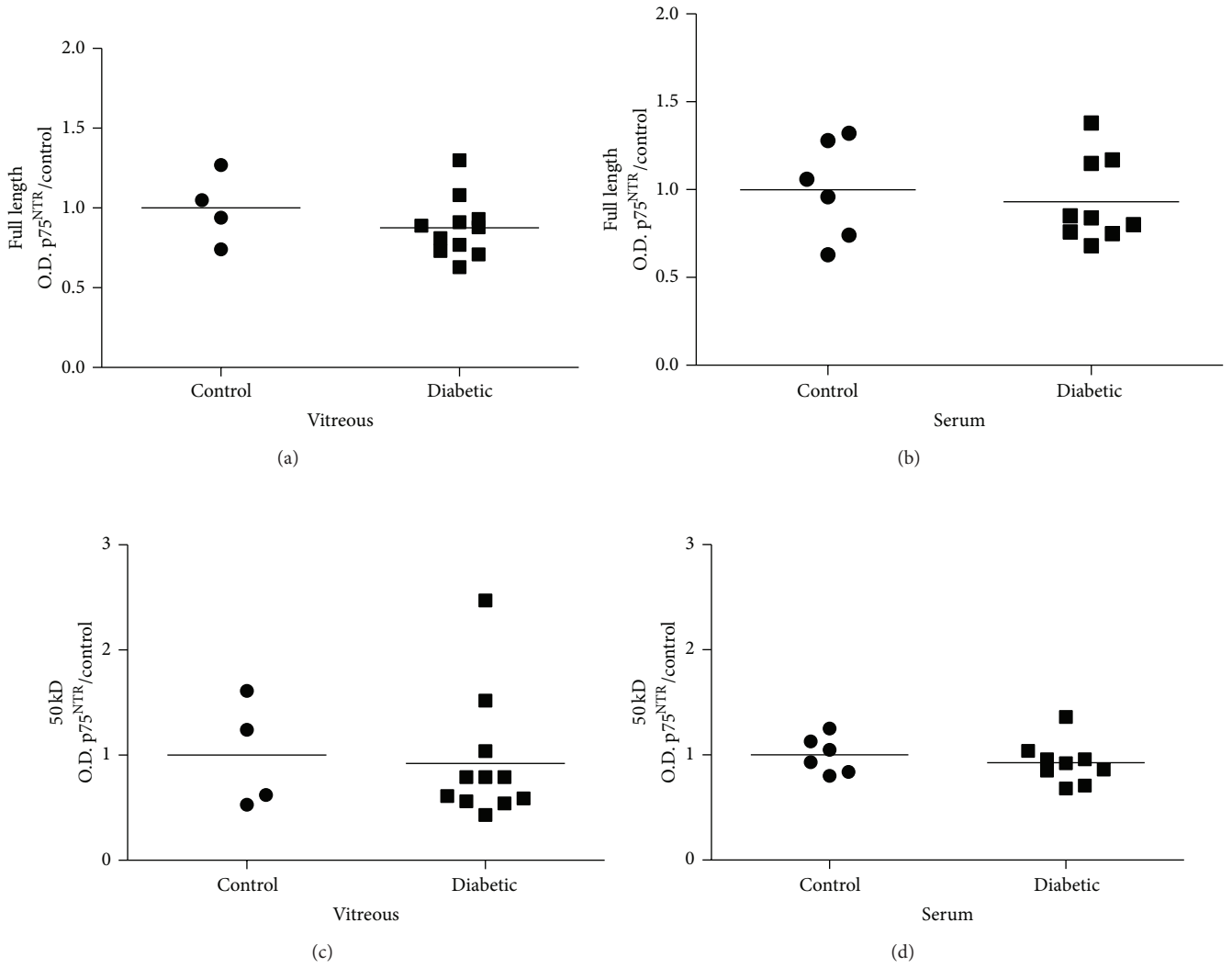

Figure 7: Expression of $p75^{NTR}$ receptor is consistent in vitreous and serum. Results are shown for $p75^{NTR}$ expression in vitreous and serum of diabetic (DB) and control participants normalized to Ponceau S and respective controls. Full length $p75^{NTR}$ receptor (75 kD) was not significantly different in diabetic sample compared to control groups in either (a) vitreous (N = 4–11) or (b) serum (N = 6–9). The $p75^{NTR}$ ectodomain (50 kD) was also not significantly different in diabetic compared to control groups in (c) vitreous (N = 4–11) or (d) serum (N = 6–9).

in diabetic subjects [58, 59]. The dichotomy between MMP-7 action in diabetic serum and retina is further illustrated by findings that the cholesterol lowering drug atorvastatin reduces MMP-7 levels in diabetic serum but increases its expression in diabetic rat retina [31, 59]. Future studies on mechanisms controlling MMP-7 expression in different organ systems would be valuable.

The third finding of this study was that altered expression of $p75^{NTR}$ proteolytic fragments occurred in diabetic vitreous and serum. Since $p75^{NTR}$, the preferred receptor for proNGF, lacks intrinsic kinase activity, the $p75^{NTR}$ receptor signals via ectodomain shedding and regulated intramembrane proteolysis [41–43]. This results in detecting full length $p75^{NTR}$ (75 kD), the receptor ectodomain (50 kD), and smaller fragments, such as the C-terminal fragment (CTF, 27 kD) and p75 intracellular domain (ICD, 22 kD). Western blot analysis of vitreous and serum did not show any significant differences in full length $p75^{NTR}$ or the ectodomain expression between diabetic and control groups (Figure 7). Although expression of full length $p75^{NTR}$ receptor has been reported to be elevated in the plasma of type 2 diabetic rats, our results correspond more closely to a recent study in diabetic patients showing only minor changes in $p75^{NTR}$ expression [60, 61]. We believe that the 50 kD band (Figures 6 and 7) is most likely the ectodomain fragment which should be found in extracellular mostly acellular environment of the vitreous or serum. Previous reports proposed that this fragment might also be a smaller unglycosylated form of $p75^{NTR}$ [41–43]. Our most intriguing observation regarding $p75^{NTR}$ expression was the apparent variations in CTF and ICD

expression in vitreous and serum as well as between diabetic and control groups. The $p75^{NTR}$ CTF and ICD fragments have most commonly been identified in cell culture studies and the reported sizes of these fragments vary somewhat depending on the cell type and experimental conditions used.

In summary, we hypothesized that the ratio of proNGF to NGF more correctly indicates homeostasis in the retina than the level of either neurotrophin alone. High proNGF expression may not necessarily be detrimental if it is accompanied by high levels of NGF. In fact, the linear correlation between either proNGF or NGF levels alone in vitreous with serum did not yield a significant correlation. Using the proNGF/NGF ratio, however, did result in a significant positive Pearson coefficient of $R^2 = 0.567$ showing a correlation between proNGF/NGF ratios in vitreous and serum. Deming linear regression showed that serum proNGF/NGF expression ratios can be used to predict vitreous proNGF/NGF ratios by the equation $y = 9.33x + 31.21$. The current study is limited by a small number of participants and that all the diabetic patients had PDR, the lattermost severe stage of this disease. A study focusing on the association of serum proNGF/NGF imbalance with early microvascular indicators of DR such as microaneurysm formation identified by fundus photography or macular edema visualized by OCT would be informative. The consistent imbalance of proNGF/NGF ratio in both ocular fluids and serum in diabetic patients suggests that treatments to restore NGF levels represent potential therapeutic strategies for diabetic complications. We and others have demonstrated protective effects of restoring NGF in experimental models of diabetes [31, 36, 37, 39]. Clinically, a recent report showed the safety of an exogenous human recombinant NGF in Phase I study, suggesting the feasibility of examining its efficacy as the next step [62]. The discovery of biomarkers to aid in the identification of patients most likely to develop severe DME and PDR is essential for better treatment of this disease. Initial data presented here suggest that on average the proNGF/NGF imbalance in serum was reflective of the proNGF/NGF imbalance in vitreous of patients with PDR. Further investigations to determine if the proNGF/NGF imbalance can be correlated with the progression of DR will be valuable to determine if the proNGF/NGF imbalance can truly be a biomarker of DR.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

MicroRNA-146b-3p Regulates Retinal Inflammation by Suppressing Adenosine Deaminase-2 in Diabetes

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Hyperglycemia- (HG-) Amadori-glycated albumin- (AGA-) induced activation of microglia and monocytes and their adherence to retinal vascular endothelial cells contribute to retinal inflammation leading to diabetic retinopathy (DR). There is a great need for early detection of DR before demonstrable tissue damages become irreversible. Extracellular adenosine, required for endogenous anti-inflammation, is regulated by the interplay of equilibrative nucleoside transporter with adenosine deaminase (ADA) and adenosine kinase. ADA, including ADA1 and ADA2, exists in all organisms. However, because ADA2 gene has not been identified in mouse genome, how diabetes alters adenosine-dependent anti-inflammation remains unclear. Studies of pig retinal microglia and human macrophages revealed a causal role of ADA2 in inflammation. Database search suggested miR-146b-3p recognition sites in the 3′-UTR of ADA2 mRNA. Coexpression of miR-146b-3p, but not miR-146-5p or nontargeting miRNA, with 3′-UTR of the ADA2 gene was necessary to suppress a linked reporter gene. In the vitreous of diabetic patients, decreased miR-146b-3p is associated with increased ADA2 activity. Ectopic expression of miR-146b-3p suppressed ADA2 expression, activity, and TNF-α release in the AGA-treated human macrophages. These results suggest a regulatory role of miR-146b-3p in diabetes related retinal inflammation by suppressing ADA2.

1. Introduction

Diabetic retinopathy (DR) is a leading cause of blindness among working-age adults. Treatment options for DR remain limited and with adverse effects. Major complications in DR include blood-retinal barrier dysfunction and loss of retinal neurons [1–3]. Although these changes may be a major vision-threatening complication in diabetes, by the time they become easily demonstrable, tissue damage has already occurred. Therefore, there is a great need for early detection and intervention of DR during the prediabetic phase.

During early diabetes, retinal immune cell activation causes retinal inflammation leading to major DR complications. These cells are involved in proinflammatory as well as anti-inflammatory processes. Anti-inflammatory process may be induced by extracellular adenosine that activates adenosine receptors (A1AR, A2AAR, A2BAR, and A3AR). A2AAR, a Gs-coupled adenosine receptor, plays a major role in anti-inflammation. Extracellular concentrations of adenosine are regulated by the interplay of the equilibrative nucleoside transporter (ENT) with intra- and extracellular enzymes of adenosine metabolism. Extracellular adenosine and 2′-deoxyadenosine can be internalized through ENT and deaminated to inosine and deoxyinosine by ADA. Two different isoenzymes of ADA, designated as ADA1 and ADA2, were found in mammals, lower vertebrates, and insects [4]. ADA1 is ubiquitous and is critical for the downregulation of adenosine and 2′-deoxyadenosine [5]. Unlike ADA1, the extracellular ADA2 shows a weak affinity

for 2′-deoxyadenosine. During inflammation, an increase in ADA2 has been found in macrophage-rich tissues [6, 7]. ADA2 activity is elevated significantly in pleural fluids of patients with pulmonary tuberculosis [8], sera from HIV-infected individuals [9, 10], and from patients with diabetes [11], making ADA2 activity a convenient marker to improve the diagnosis and follow-up treatment of these disorders. In contrast to ADA1, ADA2 activity for adenosine requires high levels of adenosine and low optimum pH of 6.5, suggesting that ADA2 expresses its activity only at conditions that are associated with hypoxia or inflammation [4]. It was shown that ADA2 is important for monocyte differentiation and stimulation of macrophage proliferation [12]. The search for a rodent ADA2 gene by analysis at the critical region (at or near the human chromosome 22 pericentromere) in humans and the region of conserved synteny in mice has not been successful [13, 14]. The role of ADA2, therefore, has been understudied in mice as the sequencing probes or antibodies to mouse ADA2 are not available [15]. To determine the role of ADA2 in diabetes, the treatment effects of Amadori-glycated albumin (AGA) [2] or HG on the porcine retinal microglia and human monocytes/macrophages (U937) were determined. In the AGA-treated cells, increased ADA2 expression, ADA2 activity, and TNF-α release were induced and these effects were blocked by ADA2-neutralizing antibody or ADA2 siRNA but not by scrambled siRNA [16]. These results suggest that retinal inflammation in DR is mediated by ADA2 and that the anti-inflammatory activity of adenosine receptor signaling is impaired in diabetes due to increased ADA2 activity.

A number of factors regulate gene expression at the transcriptional and translational levels during developmental and diseased conditions. MicroRNAs (miRNAs) are a class of small noncoding RNA molecules, 22 to 25 nucleotides in length that function in the posttranscriptional regulation of gene expression. miRNAs bind partly complementary sequences in mRNAs, targeting them for degradation and/or inhibiting their translation and thereby downregulating the expression of the targeted proteins [17–19]. Dysregulation of miRNAs has been shown to contribute to many types of human diseases, including neuronal disorders [15, 18, 19]. miR-146b-5p, an miRNA regulated by variable globular adiponectin concentrations and acting as an inhibitor of NFκB-mediated inflammation, is decreased in circulating monocytes of obese subjects with type 2 diabetes [15, 20]. miR-146b-5p and miR-146b-3p are two isoforms from the same gene in human chromosome 10 with different sequences and for the regulation of different gene expression. In contrast to the well-studied miR-146b-5p, the targets of miR-146b-3p involved in diabetes are not well studied. In the current study, we demonstrate that ADA2 is one of the targets of miR-146-3p. We identified a role of miR-146b-3p in the regulation of retinal inflammation in diabetes by suppressing ADA2.

2. Materials and Methods

2.1. Postmortem Eye Specimens. Sixteen human eyes, including 8 globes of type 2 diabetes and 8 nondiabetic controls, were obtained from Georgia Eye Bank (Atlanta, GA) according to the following selection criteria: >50 years old, either insulin requiring diabetes or no diabetes, and no life-support measures. The eyes were enucleated an average of 6.71 ± 0.84 h after death. Similarly collected postmortem eye specimens were used in our previous studies [21].

2.2. Immunofluorescence Labeling of Paraffin-Embedded Human Eyes. A microwave oven-based technique for immunofluorescent staining of paraffin-embedded tissues [22] was used to doubly label human eye sections with antibodies for Iba1 (WAKO, code number 016-20001) and ADA2 (Santa Cruz, catalogue number sc-86100).

2.3. ADA2 Activity Assay. The ADA2 assay is based on the enzymatic deamination of adenosine at pH 6 to inosine, which is converted to hypoxanthine by purine nucleoside phosphorylase. Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase. H_2O_2 is further reacted with N-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline and 4aminoantipyrine in the presence of peroxidase to generate quinone dye, which is monitored in a kinetic manner. ADA1 activity is inhibited by ADA1-specific inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Diazyme Laboratories, Poway, CA).

2.4. Drug Treatment Effects on the Cultured Human Macrophages. The human monocyte cell line U937 was grown in ATCC formulated RPMI-1640 medium (catalog number 30-2001) containing 10% fetal bovine serum (Atlanta Biologicals), subcultured in GIBCO RPMI-1640 (Catalog number 11879-020, supplemented with 5 mM D-glucose), and differentiated into macrophages by phorbol 1 2-myristate 13-acetate (PMA, 50 ng/mL) treatment. Cells were then cultured in the same media supplemented with AGA (Sigma, St Louis, MO) which contained undetectable endotoxin (<0.125 units/mL, 10 EU = 1 ng lipopolysaccharide; Lonza, Basel, Switzerland) at a final concentration of 500 mg/mL for 12 hours [2]. Cells were also cultured in HG (GIBCO RPMI-1640 supplemented with 35 mM D-glucose) or in osmotic pressure-control medium (GIBCO RPMI-1640 supplemented with 10 mM D-glucose and 25 mM L-glucose) for 48 hours.

2.5. Human Monocytes Transfection. The human monocyte cell line U937 was cultured in ATCC formulated RPMI-1640 medium (catalog number 30-2001), differentiated into macrophages by PMA treatment at 50 ng/mL. Nonviral transfection of cells was performed using LonzaH NucleofectorH II electroporation system. An aliquot of 10^6 U937 cells were pelleted and resuspended in 100 μL electroporation buffer (VCA-1004) containing negative control (NC), miR-146-5p mimic, or miR-146b-3p mimic at a final concentration of 100 nM. The cells were immediately transferred to a cuvette and electroporated using the program W-001. After transfection, cells were resuspended in medium and grown overnight. The human monocyte cell line was also transfected by lipofectin using Lipofectamine 2000 (Invitrogen) as described by the manufacturer [23].

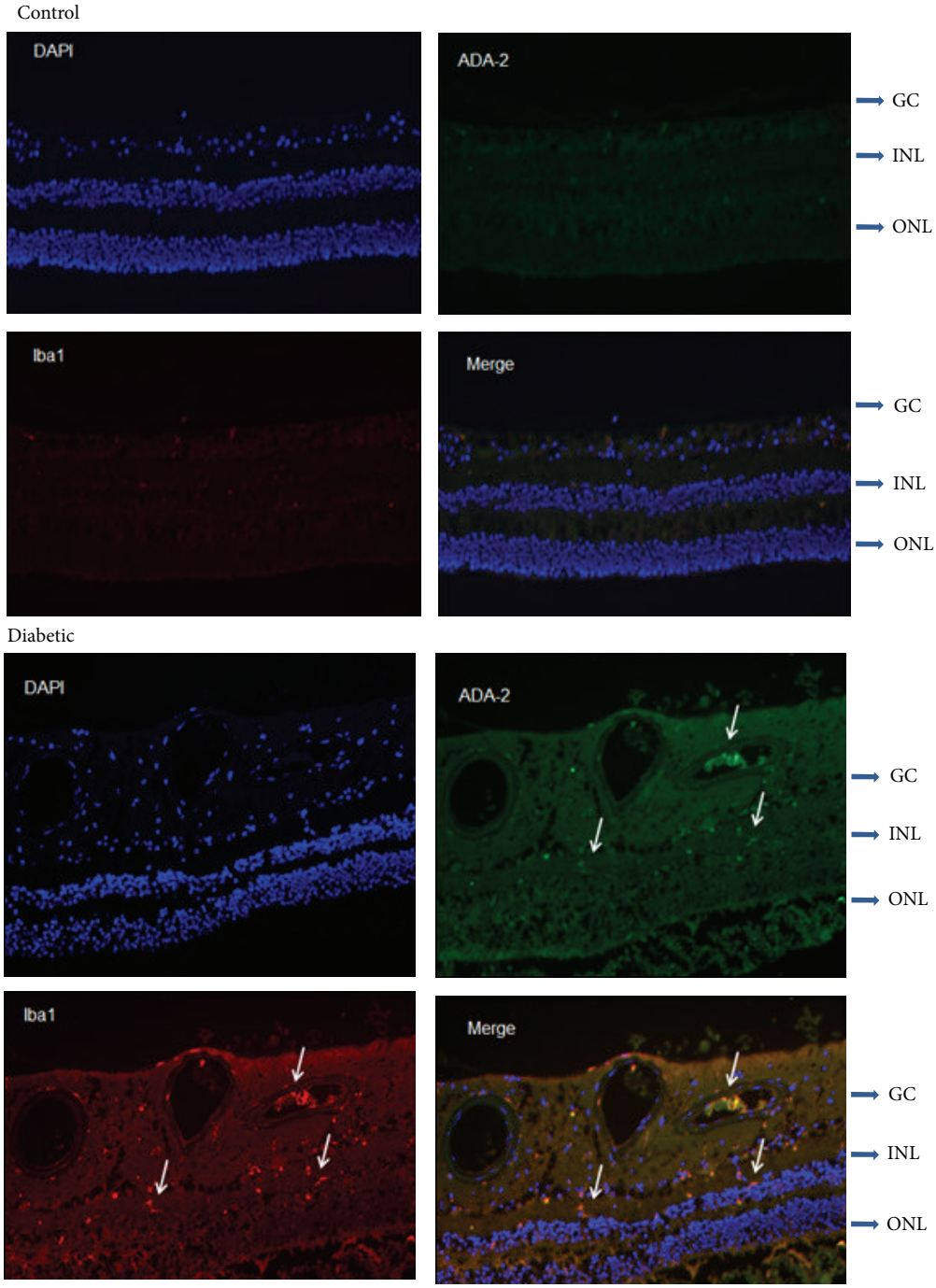

Figure 1: ADA2 is localized in the activated monocytes or macrophages in human retina with diabetes. Immunolabeling of ADA2 (green) and Iba-1 (red), a marker of activated microglia or macrophages, was made in human retinas under normal and diabetic conditions. Colocalization of ADA2 and Iba1, as marked by the orange-stained cells in the inner-retina produced by the merged red and green on the cell surface, is only identified in retinas with diabetes. The figures represent one of three donor eyes in each diabetic and normal group.

2.6. Vector Construction and Luciferase Reporter Assay. For luciferase reporter analyses, the 3′-UTR of the human ADA2 gene (2184 bp) amplified by PCR from human cDNA was cloned into the pEZX-MT05 (GeneCopoeia, Rockville, MD). U937 cells were seeded into 12-well plates (5×10^5 cells/well), PMA-differentiated, and transfected using nucleofector technology described above. Cells were cotransfected with 100 nM miR-146b-3p mimic, miR-146b-5p mimic, or NC and 1 μg of pEZX-MT05 with or without the 3′-UTR of the human ADA2 gene. After 24 h, culture media were refreshed and after another 48 h, activities of Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) were determined with a luminometer. The relative reporter activity was obtained by normalizing the GLuc activity against SEAP activity.

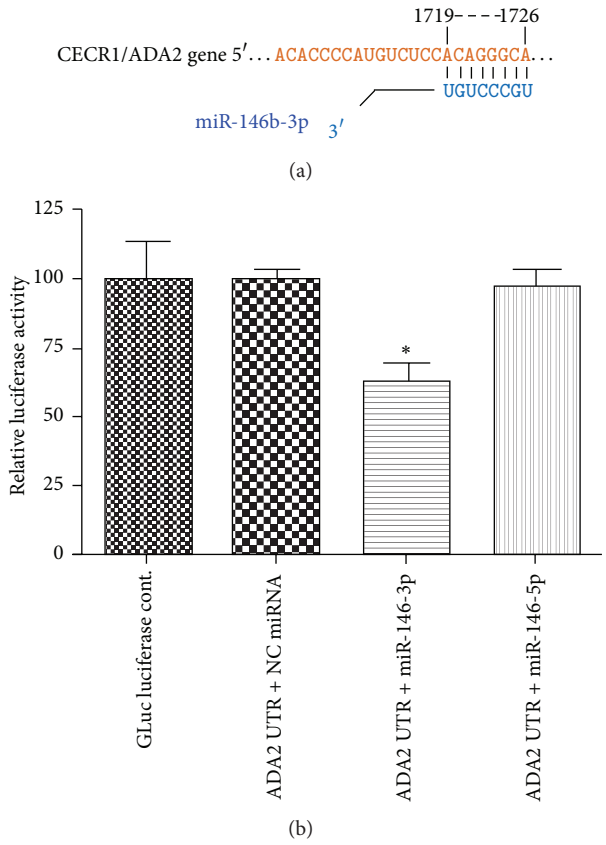

Figure 2: ADA2 is a target of miR-146b-3p. (a) Database searches of the TargetScan sites showed that human ADA2 mRNAs have conserved miR-146b-3p recognition sites. Alignment of the predicted miRNA binding sites in the 3′-UTR of the human ADA2 mRNA was made. Pairing of target region (top) and miRNA (bottom) is shown. (b) Secrete-Pair Dual Luminescence assay system was used to determine if ADA2 expression might be suppressed directly by miR-146b-3p. GLuc luciferase reporter gene linked with or without the 3′-UTR of the ADA2 gene was cotransfected with miR-146b-3p mimic, miR-146b-5p mimic, or negative control (NC, nontargeting miRNA) in differentiated U937 cells. Luciferase activities normalized with SEAP activities were determined with a luminometer. The results are mean ± SD from three independent experiments. $^{*}P < 0.05$.

2.7. Quantitative Real-Time PCR for mRNA and miRNA. For mRNA quantitation, SV Total RNA Isolation kit (Promega) was used for total RNA isolation. RNA was reverse-transcribed into cDNA using iScript reagents (Bio-Rad). Fifty ng of cDNA was amplified in each qRT-PCR using SYBR Green I and appropriate primers. Average of glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA were used as the internal control for normalization. For miR-146b-3p and miR-146b-5p quantitation, total RNA isolated from cells or vitreous were reverse-transcribed into cDNA using miScript reagents (Qiagen). Fifty pg of cDNA was amplified in each qRT-PCR using SYBR Green I and miR-146b-3p or miR-146b-5p primer. HsRNU6 was used as normalization reference gene for miRNA.

2.8. ELISA for TNF-α. TNF-α levels in the supernatants of culture media were estimated with ELISA kits (R & D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

2.9. Statistical Analysis. The results are expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the post hoc test (Fisher's PLSD). Significance was defined as $P < 0.05$.

3. Results and Discussion

3.1. Localization of ADA2 in the Human Retina with Diabetes. During inflammation, increased ADA2 has been found in macrophage-rich tissues [6, 7]. This suggests that ADA2 is involved in inflammation in the macrophages. To investigate the role of ADA2 in the retina during diabetes, we sought to determine cellular localization of ADA2 in the human retina with and without diabetes ($n = 3$ each). Donor eyes with or without type 2 diabetes were paraffin-embedded and sectioned. Immunolabeling was processed for ADA2- and Iba-1-positive cells using antibodies specific for human ADA2 and Iba-1, respectively. Colocalization of ADA2 and Iba1, as indicated by the merged yellow fluorescence on cell surface (*arrows*), is identified in retinas with diabetes but not nondiabetic control (Figure 1). This result suggests that ADA2 upregulation occurs in the activated microglia or macrophages in the diabetic retina.

3.2. ADA2 Is a Direct Target of miR-146b-3p. miRNAs play a major role as negative regulators of protein-coding genes. To determine the role of ADA2 in the retina under the condition of diabetes, we sought to identify miRNA that targets ADA2. TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA [24]. Database searches of the TargetScan sites showed that human ADA2 mRNAs have conserved miR-146b-3p recognition sites (Figure 2(a)). We hypothesize that increased ADA2 production might result from the downregulation of miR-146b-3p. To test this hypothesis, a Secrete-Pair Dual Luminescence assay system (GeneCopoeia) was used in U937 cells. Coexpression of miR-146b-3p mimic, but neither miR-146b-5p mimic nor negative control (NC), significantly suppressed the GLuc luciferase reporter activity of the linked 3′-UTR, indicating that miR-146b-3p suppresses ADA2 expression through miRNA binding [20] sequences in its 3′-UTR (Figure 2(b)). Together, these results suggest that miR-146b-3p suppresses ADA2 expression by binding to the 3′-UTR and that ADA2 is a direct target of miR-146b-3p.

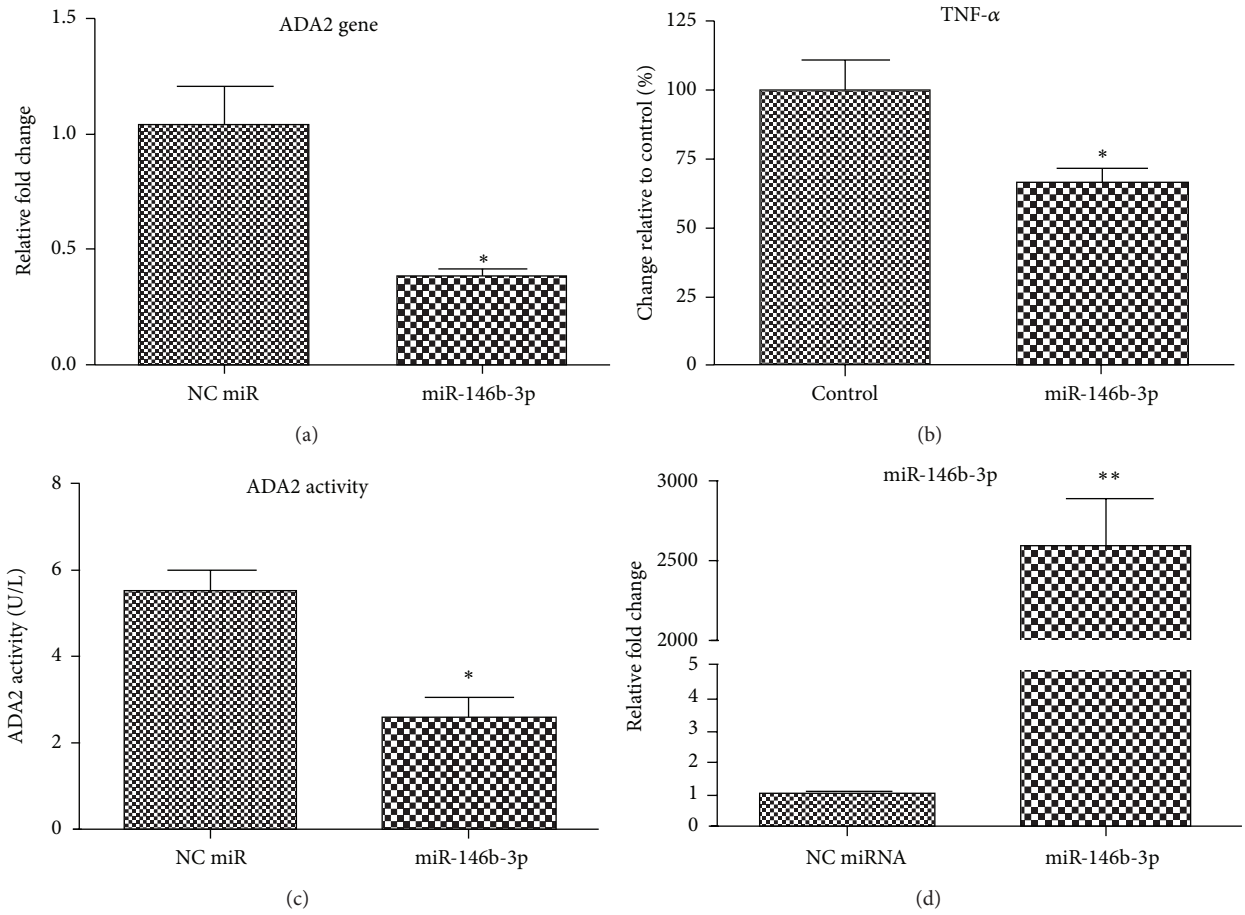

Figure 3: miR-146b-3p is dysregulated in diabetes: ectopic expression of miR-146b-3p inhibits ADA2 and TNF-α expression. PMA-differentiated U937 macrophages were transfected with miR-146b-3p mimic or negative control (NC, nontargeting miRNA). Expression was evaluated by qRT-PCR for (a) ADA2 mRNA and (b) TNF-α quantification (ELISA). (c) ADA2 activity using standard quantification method and (d) miR-146b-3p expression (qRT-PCR). The results are presented as the mean ± SD from three independent experiments. $^{*}P < 0.05$.

3.3. miR-146b-3p Is Dysregulated in Diabetes. Dysregulation of miRNAs has been shown to contribute to many types of human diseases, including diabetes [15] and neuronal disorders [18, 19]. To determine whether miR-146-3p is dysregulated in diabetes, we first determined the effect of miR-146b-3p overexpression in PMA-differentiated U937 monocytes. PMA-treated cells were transfected with nucleofector technology with negative control (NC), miR-146b-3p mimic, and exposed to AGA. ADA2 expression, activity, and TNF-α release were determined by qRT-PCR, standard assay, and by ELISA, respectively. Evaluation of the cell lysates showed that the miR-146b-3p-transfected cells exhibited reduced ADA2 expression, activity, and TNF-α release (Figure 3).

3.4. miR-146b-3p Is Inversely Associated with ADA2 in Diabetes. We hypothesize that as a negative regulator of ADA2, miR-146b-3p expression may be inversely associated with ADA2 expression or activity and the status of inflammation. To test this hypothesis, we measured the miR-146b-3p level, ADA2 expression, and tumor necrosis factor (TNF)-α release after AGA treatment of PMA-differentiated U937 monocytes. AGA, formed as glycated albumin under hyperglycemic conditions and accumulated in the retina in early diabetes, is used to mimic hyperglycemia or diabetic conditions [2]. Proinflammatory cytokines including TNF-α, interleukin- (IL-) 1β, and IL-6 have been implicated in retinal inflammation under diabetic conditions. We have recently provided evidence that TNF-α plays a central role in the pathogenesis of DR [25]. Therefore, TNF-α release was determined to represent the status of inflammation. RNA was extracted from the treated cells, and expression levels of ADA2 and miR-146b-3p were determined by quantitative (q) RT-PCR. ADA2 expression and TNF-α release were both significantly increased after AGA treatment (Figure 4), whereas expression of miR-146b-3p was significantly decreased after AGA treatment (Figure 4).

We then determined ADA2 activity and miR-146b-3p expression in the vitreous of human donor eyes with ($n = 8$) and without diabetes ($n = 4$). The result shows that increased ADA2 activities were present in the vitreous of human donor eyes with diabetes (Figure 5). The result also shows that miR-146b-3p expression levels were downregulated in

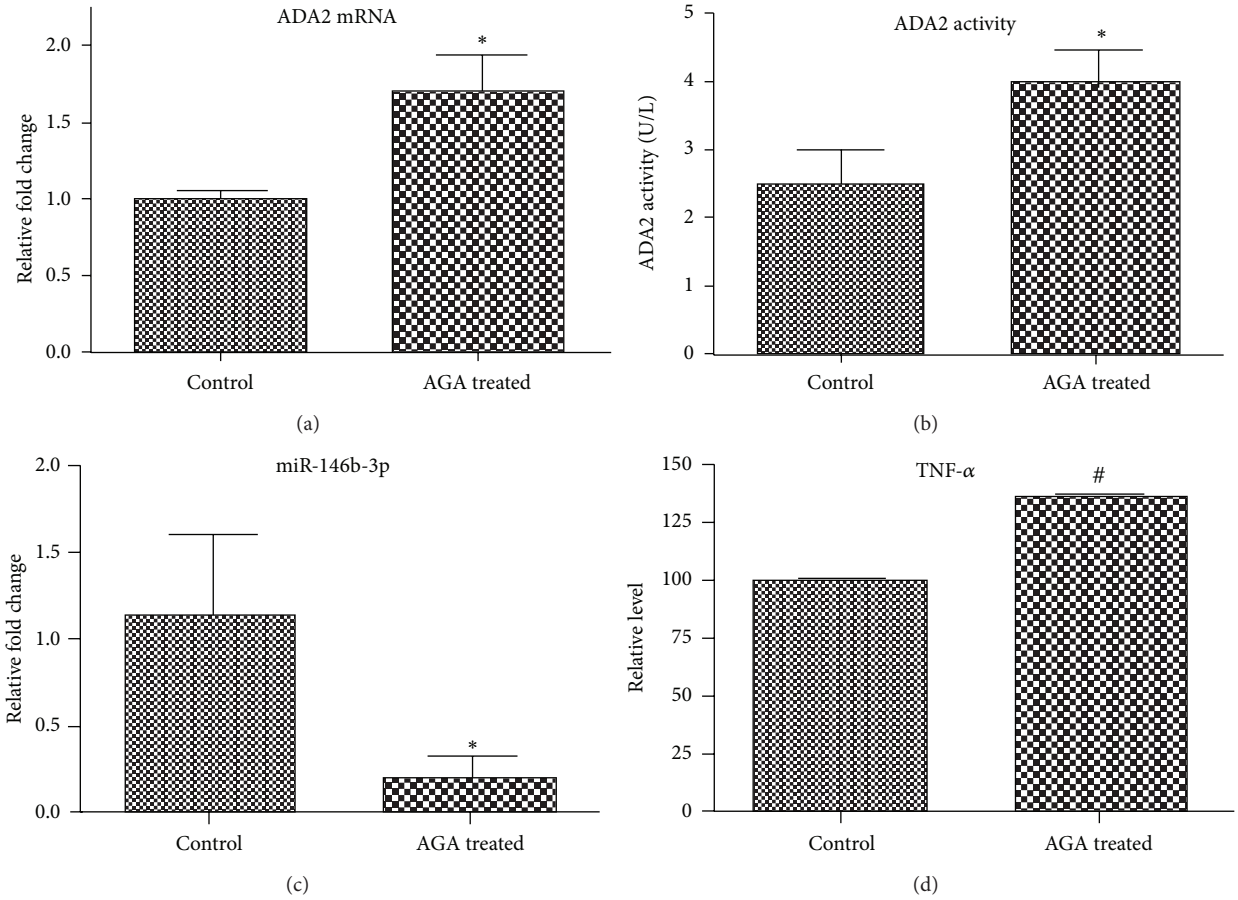

Figure 4: ADA2 is inversely associated with miR-146b-3p: AGA treatment upregulates TNF-α and ADA2 and downregulates miR-146b-3p. PMA-differentiated U937 macrophages were exposed to AGA and media and cell lysates were processed for the following analyses. (a) ADA2 activity was determined by a standard assay (Diazyme Laboratories). (b) ADA2 mRNA expression by qRT-PCR, (c) miR-146b-3p expression by qRT-PCR, and (d) TNF-α release were determined by ELISA. The results are mean ± SD from three independent experiments. $^{*}P < 0.05$.

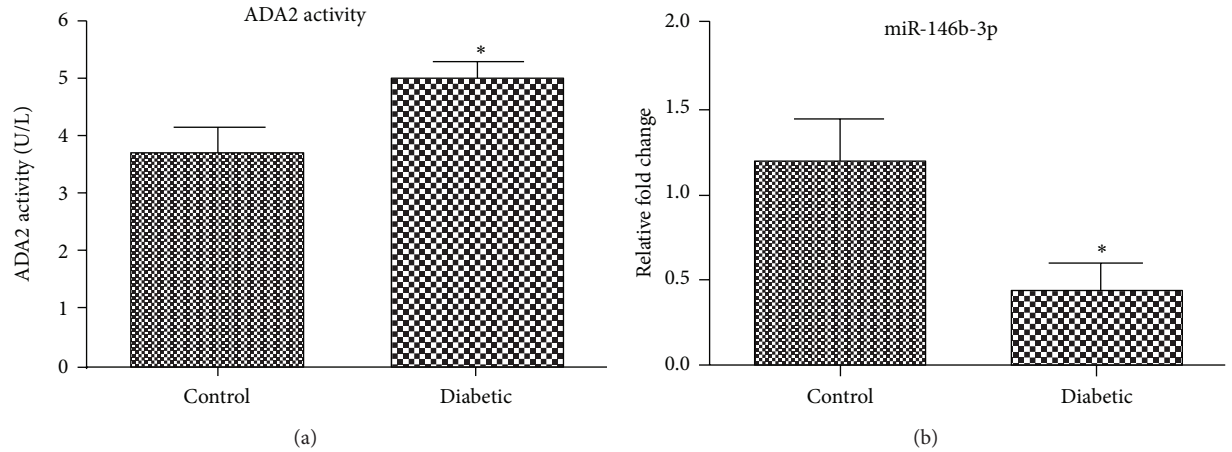

Figure 5: miR-146b-3p is dysregulated in diabetes: 8 donor eyes with type 2 diabetes and 4 nondiabetic donor eyes were evaluated for ADA2 activity and miR-146b-3p expression in the vitreous. $^{*}P < 0.05$.

the diabetic samples after normalizing with housekeeping HsRNU6. Taken together, these results suggest that an inverse correlation between miR-146b-3p and inflammation occurs in diabetes and that reduction or dysregulation of miR-146-3p may contribute to diabetic complications.

As shown in the current study, increased ADA2 activity is accompanied with activation of macrophages and TNF-α release that promote retinal inflammation in diabetes. The positive regulation of ADA2 activity during inflammation is poorly understood. A number of factors such as microRNAs play important roles in regulation of genes. Our bioinformatics analysis showed that ADA2 mRNA has 3′-UTR site which is complimentary to miR-146b-3p. Our results confirm the hypothesis that miR-146b-3p binds to ADA2 3′-UTR and inhibit its expression and activity. Moreover, ectopic expression of miR-146b-3p decreases the ADA2 activity and TNF-α release in PMA-differentiated U937 monocytes. To verify this finding, we constructed luciferases gene containing 3′-UTR of human ADA2 gene. Luciferase reporter analysis shows that luciferase activity decreases with cotransfection of miR-146b-3p but not NC or miR-146b-5p. This study is the first that determined how ADA2 is upregulated in diabetes. The increased ADA2 activity is also associated with decreased expression of miR-146b-3p in diabetes. Dysregulation of miRNA has been shown to contribute to many types of human diseases, including neuronal disorders [18, 19]. In addition, miR-146b-5p, an miRNA regulated by variable globular adiponectin concentrations and acting as an inhibitor of NFκB-, but not ADA2-mediated inflammation, is decreased in circulating monocytes of obese subjects with type 2 diabetes [15]. In contrast to miR-146b-5p, genes that are negatively regulated by miR-146b-3p in diabetes have not been identified. It is likely that, in addition to ADA2, there are other genes that are upregulated by miR-146b-3p silencing in diabetes.

Controlling endogenous adenosine signaling may represent an advantageous way in treating DR. Extracellular concentrations of adenosine are regulated by the interplay of ENT with intra- and extracellular enzymes of adenosine metabolism. We have provided experimental evidence that targeting adenosine kinase can inhibit diabetes-induced retinal abnormalities in the development of DR by potentially amplifying the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine [16, 26]. We have demonstrated that ADA2 has a causal role in microglial activation and retinal inflammation in diabetes. Because increased adenosine production and ADA2 activity occur at specific pathophysiological sites [6], inhibition of ADA2 activity may also represent a mechanism to selectively enhance the actions of adenosine at specific tissue sites. Inhibition of ADA2 is, therefore, potentially with limited side-effects and is of highly translational impact. Delivering an miRNA that reduces the protein levels of target genes linked to a particular disease represents a new therapeutic option. A recent study has shown that the in vivo administration of miR-124 suppresses experimental autoimmune encephalitis by affecting macrophages, suggesting that miRNA delivery could be used to treat some inflammatory diseases associated with microglial activation [19].

4. Conclusions

These results suggest a role of miR-146b-3p in the regulation of retinal inflammation in diabetes by suppressing ADA2. miR-146b-3p may serve as a therapeutic target for early detection and intervention of DR.

Abbreviations

ADA2: Adenosine deaminase-2
AGA: Amadori-glycated albumin
HG: Hyperglycemia
DR: Diabetic retinopathy
ENT: Equilibrative nucleoside transporter.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Sadanand Fulzele and Ahmed El-Sherbini contributed equally to this work.

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Research Article

Aqueous Cytokines as Predictors of Macular Edema in Patients with Diabetes following Uncomplicated Phacoemulsification Cataract Surgery

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This study aims to ascertain whether cytokines in the aqueous humor can predict macular edema (ME) in diabetic patients following uncomplicated phacoemulsification cataract surgery. Undiluted aqueous humor samples were obtained from 136 consecutive type 2 diabetic patients who underwent cataract surgery. The concentrations of 27 cytokines were measured in aqueous humor using the multiplex bead immunoassay. At the final follow-up examination, 116 patients completed 4 weeks of follow-up, and the incidence of macular edema was 29.31% (34 patients) 4 weeks after cataract surgery. Compared to the ME (−) patients, the concentrations of interleukin-1β (IL-1β) ($P < 0.001$), IL-6 ($P < 0.001$), IL-8 ($P < 0.001$), interferon-induced protein-10 (IP-10) ($P = 0.003$), monocyte chemotactic protein-1 (MCP-1) ($P < 0.001$), and vascular endothelial growth factor (VEGF) ($P < 0.001$) in the ME (+) patients were significantly higher. In addition, the aqueous levels of IL-1β ($r = 0.288$), IL-6 ($r = 0.345$), IL-8 ($r = 0.256$), IP-10 ($r = 0.377$), MCP-1 ($r = 0.423$), and VEGF ($r = 0.279$) were positively correlated with the postoperative foveal center point thickness (FCPT). However, the aqueous levels of IL-10 ($P = 0.003$) and IL-12 ($P = 0.017$) were significantly lower in patients with ME. These results suggest IL-1β, IL-6, IL-8, IL-10, IL-12, IP-10, MCP-1, and VEGF may be potential predictors of postoperative macular thickness in patients with diabetes following uncomplicated phacoemulsification cataract surgery.

1. Introduction

Diabetes mellitus (DM) has been a leading public health problem in China for the last 10 years and imposes a heavy economic burden on Chinese patients [1]. Diabetic patients have been reported to have a higher prevalence of cataracts and an increased risk of developing cataracts earlier than patients without diabetes [2]. At present, the incidence of postoperative complications is decreasing with the development of phacoemulsification cataract surgery and posterior chamber intraocular lens implantation. However, anterior segment inflammation, progression of diabetic retinopathy, and macular edema (ME) are the most common complications in patients with diabetes following uncomplicated phacoemulsification cataract surgery [3, 4]. ME is one of the main causes of unfavorable visual outcomes following uncomplicated cataract surgery and can result in permanent visual loss [5–7]. The reported incidence of ME ranges from 20% to 50% in patients with diabetes following uncomplicated phacoemulsification cataract surgery [8, 9].

Although the pathogenesis of macular edema is likely multifactorial and remains unknown, it appears to be associated with postoperative inflammation induced by prostaglandins or other inflammatory mediators [10, 11]. Inflammatory mediators break down the blood-retinal barrier (BRB) and the blood-aqueous barrier (BAB), leading to increased vascular permeability [12]. A previous study measured the concentrations of VEGF and IL-6 in aqueous humor in patients with nonproliferative diabetic retinopathy by enzyme linked immunosorbent assay (ELISA) during cataract surgery [13] and demonstrated that high VEGF levels in the aqueous humor predict a significant risk of postoperative exacerbation of macular edema [13]. However, the limitations of the previous study on aqueous humor

cytokines include the examination of a limited number of cytokines. Exploring a greater number of cytokines would provide broader insight into the inflammatory mechanisms involved. Recently, multiplex bead immunoassay has been used to detect cytokines in tears and in the aqueous humor because of the capacity of this assay to simultaneously quantify multiple cytokines in very small sample volumes [14–16].

Our previous study compared the changes in the levels of 27 aqueous humor cytokines between nondiabetic controls and type 2 diabetic patients and showed that the variety of cytokines associated with inflammation and angiogenesis may contribute to the pathogenesis of diabetic retinopathy (DR) [16]. These study participants that consisted of a consecutive cohort of diabetic patients with varying levels of retinopathy, including the absence of retinopathy, were included in this study.

Therefore, in this study, we used the multiplex bead immunoassay to evaluate the concentrations of 27 cytokines in the aqueous humor at the beginning of cataract surgery and correlate their expression levels to the development of macular edema 4 weeks after surgery. In addition, our study explores whether cytokine concentrations in the aqueous humor can predict macular edema in patients with diabetes following uncomplicated phacoemulsification cataract surgery.

2. Materials and Methods

2.1. Subjects. Undiluted aqueous humor samples were obtained from 136 consecutive type 2 diabetic patients (136 eyes; 71 males and 65 females) who were undergoing cataract surgery from January 2010 to April 2012. This study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University, Beijing, China, and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients prior to their participation in the study.

DM was diagnosed according to the 1999 World Health Organization (WHO) criteria. Subjects were considered to have hypertension if their blood pressure was above 140/90 mmHg or they were taking any antihypertensive medications. Hypercholesterolemia was defined as their fasting total plasma cholesterol was above 200 mg/dL. Hypertriglyceridemia was classically defined as fasting plasma triacylglycerols (triglycerides, TG) above 200 mg/dL. Inclusion criteria were the presence of diabetes mellitus and the absence of any retinal or optic nerve disease except diabetic retinopathy in the study group. The exclusion criteria included (1) any other ocular condition (e.g., glaucoma, uveitis), (2) a history of ocular surgery, (3) a history of ocular inflammation, and (4) current presence or history of clinically significant macular edema (CSME).

2.2. Procedure. Patients underwent preoperative ophthalmologic examination and a physical examination that included best-corrected visual acuity (BCVA), slit lamp-assisted biomicroscopy of the anterior segment, a fundus examination, and optical coherence tomography (OCT), which was used to measure the foveal center point thickness (FCPT). The BCVA was measured with a Snellen chart at the preoperative examination, 1 day and 4 weeks postoperatively. The OCT examination (Stratus OCT3; Carl Zeiss Meditec, Dublin, California, USA) was performed by an experienced operator through a dilated pupil. Each study eye underwent OCT testing fewer than 2 weeks before cataract surgery and 4 weeks postoperatively. OCT images were generated with the use of six radial-line scans, 6.00 mm each in length. The maximal foveal center point thickness (in micrometers) was measured at the center point of the fovea by manually placing computerized calipers at the vitreous-retina and retina-retinal pigment epithelium interfaces [5, 17].

2.3. Surgical Technique. All cataract surgeries were performed using the phacoemulsification technique and the insertion of a foldable hydrophilic acrylic intraocular lens (AcrySof IQ IOL, Alcon, Inc.) in the capsular bag. A total of 0.3 mg TobraDex ointment (tobramycin 0.3% and dexamethasone 0.1%, Alcon, Inc.) was used at the end of the surgery in all patients. All patients were instructed to administer TobraDex eye drops (tobramycin 0.3% and dexamethasone 0.1%, Alcon, Inc.) 4 times daily for 2 weeks after surgery and 2 times daily until 4 weeks after cataract surgery. In addition, all patients were instructed to administer 0.1% Diclofenac sodium eye drops 4 times daily for 4 weeks after surgery. All patients were followed for at least 4 weeks after surgery.

2.4. Aqueous Humor Sampling. At the time of cataract surgery, a sterile lid speculum was placed, and a sterile tuberculin syringe was placed in the temporal limbal quadrant. Once inserted, undiluted aqueous humor samples (0.1-0.2 mL) were aspirated into a syringe. The samples were immediately frozen and stored at −80°C until analysis.

2.5. Postoperative Evaluation. Postoperative follow-up visits were scheduled for 1 day and 4 weeks after cataract surgery. The following assessments were performed 1 day after cataract surgery: BCVA, slit lamp-assisted biomicroscopy, fundus examination, and IOP. The following assessments were performed 4 weeks after cataract surgery: BCVA, slit lamp-assisted biomicroscopy, IOP, fundus examination, and OCT.

2.6. Definition of Postoperative Macular Edema. Macular edema was defined as an increase in the center point thickness of more than 30% from preoperative baseline on OCT 4 weeks after cataract surgery [5, 17]. All patients were divided into either the macular edema group [ME (+)] or nonmacular edema group [ME (−)].

2.7. Multiplex Analysis of Cytokines in Aqueous Humors. The Bio-Plex ProTM magnetic color bead-based multiplex assay (Bio-Plex Human Cytokine 27-plex panel; Bio-Rad, Hercules, CA) was used to measure the concentrations of twenty-seven human aqueous humor cytokines: interleukin-1β (IL-1β), IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (b-FGF), EOTAXIN, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating

factor (GMCSF), interferon-gamma (IFN-γ), interferon-induced protein-10 (IP-10 or CXCL10), monocyte chemotactic protein-1 (MCP-1 or CCL2), macrophage inflammatory protein-1α (MIP-1α or CCL3), macrophage inflammatory protein-1β (MIP-1β or CCL4), platelet-derived growth factor-BB (PDGF-BB), regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). The analysis procedure was performed according to the manufacturer's instructions. Standard curves were generated using the Bio-PlexTM 200 System (software version 6.0; Bio-Rad Laboratories) and were used to calculate the cytokine concentrations in the aqueous humor samples.

2.8. Statistical Analysis. Data were recorded as the mean ± SD or as the median and range. The BCVA values were converted to logarithm of the minimum angle of resolution (logMAR). The statistical analyses were performed using SPSS for Windows Version 17.0. The Pearson χ^2 test was used to compare the proportion of qualitative variables. The Student's t-test and Mann-Whitney U test were used to compare means of quantitative variables between two independent groups. The Kruskal-Wallis test was used to compare multiple groups. Pearson correlation coefficients were used to assess the relationship between the concentrations of assayed cytokines and the foveal center point thickness 4 weeks after cataract surgery. A P value less than 0.05 was accepted as statistically significant.

3. Results

3.1. Patient Demographics. A total of 136 consecutive type 2 diabetic patients (136 eyes; 71 males and 65 females) were enrolled, and there were no cases of intraoperative vitreous loss or suprachoroidal hemorrhage. Twelve patients were excluded for currently present macular edema. At the final follow-up examination, 116 patients (116 eyes) completed 4 weeks of follow-up (93.5% completion), and the eight patients who did not complete the protocol were excluded from the study. In the entire study population, 24 eyes (20.7%) were nondiabetic retinopathy, 45 eyes (38.8%) were mild nonproliferative diabetic retinopathy, 31 eyes (26.7%) were moderate nonproliferative diabetic retinopathy, 8 eyes (6.9%) were severe nonproliferative diabetic retinopathy, and 8 eyes (6.9%) were proliferative diabetic retinopathy. At the final follow-up examination, 34 patients (34 eyes; 16 males and 18 females) had an increase in their center point thickness of more than 30% from the preoperative baseline on OCT 4 weeks after cataract surgery. The incidence of macular edema was 29.31%. Table 1 shows demographic and clinical characteristics of patients, including the 34 consecutive ME (+) patients and 82 ME (−) patients (82 eyes; 49 males and 33 females). There were no significant differences in age, hypertension, blood glucose level, cholesterol, triglycerides, type of cataract, and iris color between the ME (+) and ME (−) groups.

3.2. Postoperative Clinical Characteristics. The mean BCVA before surgery was 0.61 ± 0.19 (logMAR) in the ME (−) group and 0.65 ± 0.20 (logMAR) in the ME (+) group. Table 2 shows the BCVA 1 day and 4 weeks after surgery. The postoperative BCVA was not significantly different between the ME (−) and ME (+) groups 1 day after surgery. However, the mean BCVA 4 weeks after surgery was improved compared to the mean BCVA 1 day after surgery in the ME (−) group. Conversely, the mean BCVA 4 weeks after surgery was decreased compared to the mean BCVA 1 day after surgery in the ME (+) group. In addition, the postoperative BCVA was significantly different between the ME (−) and ME (+) groups 4 weeks after surgery ($P < 0.001$).

Table 1: Baseline characteristics of patients with ME (−) and ME (+).

Characteristics	ME (−)	ME (+)	P value
Number	82	34	—
Gender			0.210[a]
Male (%)	49 (59.7)	16 (47.1)	
Female (%)	33 (40.3)	18 (52.9)	
Age (SD)	64.8 (6.33)	67.6 (8.06)	0.310[b]
Hypertension (%)	51 (62.2)	19 (55.9)	0.527[a]
Hypercholesterolemia (%)	20 (24.4)	13 (38.2)	0.132[a]
Hypertriglyceridemia (%)	22 (26.8)	11 (32.4)	0.548[a]
Blood glucose level, mmol/L (SD)	7.8 (2.15)	8.6 (2.56)	0.123[b]
Glycosylated hemoglobin (SD)	7.5 (2.23)	8.03 (1.85)	0.225[c]
Type of cataract			0.205[a]
Cortical (%)	28 (34.2)	6 (17.7)	
Nuclear (%)	38 (46.3)	20 (58.8)	
Posterior subcapsular (%)	16 (19.5)	8 (23.5)	
Iris colour			0.480[a]
Dark (%)	60 (73.2)	27 (79.4)	
Light (%)	22 (26.8)	7 (20.6)	

[a]Pearson χ^2 test; [b]Student's t-test; [c]Mann-Whitney U test.

Table 2: Preoperative, 1-day, and 4-week postcataract surgery BCVA for eyes with ME (−) and ME (+).

	log MAR BCVA		P value[a]
	ME (−), $n = 82$	ME (+), $n = 34$	
Preoperative	0.61 ± 0.19	0.65 ± 0.20	0.275
1-day	0.19 ± 0.13	0.22 ± 0.16	0.341
4-week	0.14 ± 0.12	0.29 ± 0.15	<0.001

log MAR: logarithm of the minimum angle of resolution; BCVA: best-corrected visual acuity.
[a]Student's t-test.

Table 3: Preoperative and 4-week postcataract surgery foveal center point thickness for eyes with ME (−) and ME (+).

	Foveal center point thickness (mean ± SD; μm)		
	ME (−), $n = 82$	ME (+), $n = 34$	P value[a]
Preoperative	159.93 ± 19.84	162.41 ± 21.33	0.091
4-week	185.53 ± 18.35	237.24 ± 24.16	<0.001
P value[a]	0.118	0.003	

[a]Student's t-test.

Table 4: The concentrations of cytokines in aqueous humors of eyes with ME (−) and ME (+) (pg/mL).

Cytokine	ME (−), $n = 82$		ME (+), $n = 34$		P value[a]
	Median	Range	Median	Range	
IL-1β	4.2	0–76	8.6	0–102	<0.001
IL-1rα	13.2	0–325	18.1	0–336	0.445
IL-2	1.5	0–96	1.7	0–106	0.578
IL-4	1.2	0–105	1.5	0–124	0.862
IL-5	1.1	0–133	1.3	0–126	0.653
IL-6	19.8	0–226	28.5	0–362	<0.001
IL-7	4.5	0–82	2.7	0–86	0.203
IL-8	12.6	0–123	17.3	0–186	<0.001
IL-9	3.1	0–102	3.3	0–169	0.580
IL-10	8.2	0–23	5.6	0–21	0.003
IL-12	7.2	0–42	4.6	0–36	0.017
IL-13	2.1	0–26	1.9	0–36	0.453
IL-15	1.6	0–56	1.8	0–38	0.686
IL-17	—	—	—	—	—
b-FGF	12.4	0–165	11.3	0–156	0.560
Eotaxin	5.9	0–86	6.2	0–95	0.753
G-CSF	—	—	—	—	—
GM-CSF	9.2	0–86	9.8	0–79	0.876
IFN-γ	—	—	—	—	—
IP-10	3.3	0–56	5.1	0–72	0.003
MCP-1	189.5	58–1623	325.6	124–2388	<0.001
MIP-1α	—	—	—	—	—
MIP-1β	27.8	0–156	26.5	0–178	0.539
PDGF-BB	3.3	0–45	3.1	0–42	0.756
RANTES	4.6	0–75	4.9	0–76	0.577
TNF-α	—	—	—	—	—
VEGF	535	26–1298	856	123–1756	<0.001

[a] Mann-Whitney U test.

The mean foveal center point thickness before surgery was 159.93 ± 19.84 μm in the ME (−) group and 162.41 ± 21.33 μm in the ME (+) group. Table 3 shows the FCPT 4 weeks after surgery. At 4 weeks, there was an increase of 25.6 μm and 74.83 μm in the FCPT of the ME (−) and ME (+) groups, respectively. The postoperative FCPT was significantly different between the ME (−) and ME (+) groups ($P < 0.001$).

Tables 2 and 3 show that the average increase in center point thickness at 4 weeks for eyes with ME was 74.83 μm, which resulted in a nearly 1-line loss of vision (0.07 logMAR units) compared to eyes without ME, which improved approximately 1 line of vision (0.05 logMAR units).

3.3. Cytokines Concentrations in the Aqueous Humor. Table 4 shows the concentrations of the assayed cytokines. The positive detection rates were more than 80% for 22 cytokines. The positive detection rates for the other 5 cytokines were as follows: TNF-α (60%), IL-17 (40%), G-CSF (32%), IFN-γ (22%), and MIP-1α (20%). These 5 cytokines were not included in the statistical analysis because of the low detection rates.

Compared to the ME (−) group, the concentrations of IL-1β ($P < 0.001$), IL-6 ($P < 0.001$), IL-8 ($P < 0.001$), IP-10 ($P = 0.003$), MCP-1 ($P < 0.001$), and VEGF ($P < 0.001$) from the ME (+) patients were significantly higher. However, the concentrations of IL-10 ($P = 0.003$) and IL-12 ($P = 0.017$) in the samples from the ME (+) patients were significantly lower than the concentrations in the ME (−) patients. There were no significant differences in other cytokine concentrations between the ME (−) and ME (+) patients.

3.4. Association between Cytokines Concentrations and the Severity of DR. In the 34 patients (34 eyes) with ME (+), 3 eyes (8.8%) were nondiabetic retinopathy, 9 eyes (26.5%) were mild nonproliferative retinopathy, 13 eyes (38.2%) were moderate nonproliferative retinopathy, 4 eyes (11.8%) were severe nonproliferative retinopathy, and 5 eyes (14.7%) were proliferative retinopathy.

Tables 5 and 6 show the relationship between the concentrations of the assayed cytokines and the severity of DR. The aqueous humor levels of IL-1β, IL-6, IL-8, MCP-1, IP-10, and VEGF increased with increasing severity of DR, and this correlation was significant. In addition, the aqueous humor levels of IL-10 and IL-12 decreased with increasing severity of DR, and this negative correlation was significant.

3.5. Association between Cytokines Concentrations and Foveal Center Point Thickness. Table 7 shows the relationship between the concentrations of assayed cytokines and the postoperative FCPT. The aqueous levels of IL-1β ($r = 0.288$), IL-6 ($r = 0.345$), IL-8 ($r = 0.256$), IP-10 ($r = 0.377$), MCP-1 ($r = 0.423$), and VEGF ($r = 0.279$) were found to positively correlate with postoperative FCPT. In addition, the aqueous level of IL-10 ($r = -0.327$) and IL-12 ($r = -0.264$) was negatively correlated with postoperative FCPT.

4. Discussion

The incidence of ME peaks at approximately 4 to 6 weeks after uneventful cataract surgery [18, 19]. The incidence of ME has been reported to range from 4% [10] to 11% [17] in nondiabetic patients following uncomplicated phacoemulsification; however, the prevalence of ME ranges from 20% to 50% in patients with diabetes following uncomplicated phacoemulsification [8, 9]. The different rates may be caused by the inflammation which is an important factor that induces DR-related changes. Recent clinical and laboratory investigations have shown that diabetic subjects have an overall increased level of inflammatory activity relative to nondiabetic subjects [20–25]. Our previous study demonstrated that the levels of multiple cytokines associated with inflammation and angiogenesis in the aqueous humor from diabetic patients were increased compared to nondiabetic controls [16]. Therefore, the analysis of the aqueous humor provides useful tool in understanding the pathophysiology and treatment responses of macular edema in patients with diabetes following uncomplicated phacoemulsification cataract surgery.

Table 5: Relationship between the concentrations of the assayed cytokines and the severity of DR (pg/mL).

Level[a]	N	IL-1β (SD)	IL-6 (SD)	IL-8 (SD)	IP-10 (SD)	MCP-1 (SD)	VEGF (SD)
1	3	5.1 (8.4)	28.4 (23.1)	17.3 (13.3)	1.8 (1.5)	236.7 (212.8)	468.6 (304.3)
2	9	9.8 (7.6)	34.8 (25.3)	21.5 (15.5)	3.2 (2.1)	486.7 (163.1)	632.1 (368.1)
3	13	13.3 (11.1)	42.3 (31.8)	25.3 (13.1)	7.3 (3.6)	656.1 (256.7)	787.3 (357.3)
4	4	16.2 (13.3)	76.2 (47.3)	35.8 (29.7)	12.2 (9.1)	837.6 (367.9)	867.4 (423.8)
5	5	21.7 (14.2)	121.7 (69.3)	65.8 (52.1)	18.4 (17.9)	1306.5 (683.4)	976.2 (476.4)
P value[b]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]Level: 1 = nondiabetic retinopathy; 2 = mild nonproliferative retinopathy; 3 = moderate nonproliferative retinopathy; 4 = severe nonproliferative retinopathy; 5 = proliferative retinopathy.
[b]Kruskal-Wallis test.

Table 6: Relationship between the concentrations of the assayed cytokines and the severity of DR (pg/mL).

Level[a]	N	IL-10 (SD)	IL-12 (SD)
1	3	9.8 (4.3)	17.1 (9.3)
2	9	7.2 (3.5)	14.6 (7.6)
3	13	5.6 (4.6)	12.3 (8.5)
4	4	4.9 (3.8)	9.2 (7.7)
5	5	4.6 (3.2)	7.1 (6.2)
P value[b]		<0.001	<0.001

[a]Level: 1 = nondiabetic retinopathy; 2 = mild nonproliferative retinopathy; 3 = moderate nonproliferative retinopathy; 4 = severe nonproliferative retinopathy; 5 = proliferative retinopathy.
[b]Kruskal-Wallis test.

Table 7: Correlations between concentrations cytokines in aqueous humors and 4-week postcataract surgery foveal center point thickness.

Cytokine	Correlation coefficients	P value[a]
IL-1β	0.288	0.005
IL-6	0.345	0.008
IL-8	0.256	0.016
IP-10	0.377	0.007
MCP-1	0.423	0.001
VEGF	0.279	0.012
IL-10	−0.327	0.013
IL-12	−0.264	0.036

[a]Pearson correlation coefficient.

Consistent with previous studies [8, 9], our study showed that 34 patients (34 eyes; 16 males and 18 females) had an increase in their center point thickness of more than 30% from the preoperative baseline on OCT 4 weeks after cataract surgery. The incidence of macular edema was 29.31%. The average increase in center point thickness at 4 weeks for eyes with ME was 74.83 μm, which resulted in a nearly 1-line loss of vision (0.07 logMAR units) compared to eyes without ME, which improved approximately 1 line of vision (0.05 logMAR units). All these show that ME is a main cause of poor postoperative visual gain following uncomplicated cataract surgery.

The pathophysiology of ME involves the accumulation of transudate in the outer plexiform and inner nuclear layers of the retina; the microcysts coalesce into cysts [26]. The pathogenesis of ME is associated with the destruction of BRB and BAB induced by prostaglandins or other inflammatory mediators [10–12]. Elevated levels of angiogenic factors, inflammatory cytokines, chemokines, and growth factors in the aqueous humor may play a role in the breakdown of the vascular barrier [22, 23, 27, 28].

Aqueous analysis provides useful tools in understanding the pathophysiology and treatment response to many ocular conditions. However, aqueous samples consist of very small volumes, limiting the usefulness of the analysis with traditional ELISA techniques. In the current study, multiplex bead immunoassay was used to analyze the aqueous humor levels of cytokines and chemokines in diabetic patients following uncomplicated phacoemulsification cataract surgery. To our knowledge, this is a comparatively large number of samples, and it is the first investigation of these 27 aqueous cytokines as predictors of macular edema in diabetic patients following uncomplicated phacoemulsification cataract surgery.

In our study, positive detection rates were more than 80% for 22 cytokines. Compared to the ME (−) group, the concentrations of IL-1β ($P < 0.001$), IL-6 ($P < 0.001$), IL-8 ($P < 0.001$), IP-10 ($P = 0.003$), MCP-1 ($P < 0.001$), and VEGF ($P < 0.001$) from the ME (+) patients were significantly higher. However, the concentrations of IL-10 ($P = 0.003$) and IL-12 ($P = 0.017$) in the samples from the ME (+) patients were significantly lower than the concentrations in the ME (−) patients.

IL-1β is a proinflammatory cytokine and an angiogenic mediator in several systems in diabetic patients, including the aqueous humor, vitreous, and tears [29, 30]. IL-1β upregulates several inflammatory mediators, including IL-1β itself, TNF-α, cyclooxygenase 2 (COX-2), prostaglandins, inducible nitric oxide synthase (iNOS), and chemokines [31]. The intravitreal injection of IL-1β accelerates the apoptosis of retinal capillary cells via the activation of NF-κB, and this process is exacerbated under high-glucose conditions [32]. A previous study demonstrated that animals were protected from diabetes-induced retinal pathology by IL-1β receptor knock-out [33]. In the current study, the IL-1β concentrations in the ME (+) patients were significantly higher than those of the ME (−) group. Our study suggests a possible role of IL-1β in the development of ME after cataract surgery.

It is well known that IL-6 is a multifunctional cytokine that has proinflammatory and angiogenic functions through

the induction of VEGF [22]. In addition, it has been reported that IL-6 is involved in the breakdown of the blood-retinal barrier [34]. In the patients with DR, the level of inflammation gradually increases as the proliferative pathogenic process and neovascularization progress. In our study, the concentrations of IL-6 from the ME (+) patients were significantly higher than those of the ME (−) patients. There is evidence that inflammation is an important molecular mechanism in the development and progression of ME after uncomplicated phacoemulsification cataract surgery.

Chemokines, including four subfamilies, C, CC, CXC, and CX3C, are small heparin-binding proteins that bind to their cognate G-protein coupled receptors (GPCRs) to elicit cellular responses [35, 36]. IL-8 and IP-10 are categorized as CXC chemokines, and MCP-1 is categorized as a CC chemokine. IL-8 is the major attractant and activator of neutrophils and T lymphocytes but not monocytes, and increased levels of IL-8 in PDR are associated with a higher extent of large-vessel gliotic obliteration [35]. IP-10 selectively attracts activated T lymphocytes, which are the only inflammatory cells that express the chemokine receptor CXCR3 [36]. MCP-1 regulates the migration and infiltration of monocytes/macrophages via the chemokine receptor CCR2 but has no effect on neutrophils [37]. In our study, the concentrations of IL-8, IP-10 and MCP-1 from the ME (+) patients were significantly higher than those of the ME (−) patients. All of the evidence indicates that inflammation is an important molecular mechanism in the development and progression of ME after uncomplicated phacoemulsification cataract surgery.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that induces increases in vascular permeability and angiogenesis, enhances collateral vessel formation, and increases the permeability of the microvasculature [38]. The aqueous humor levels of VEGF have been found to be markedly increased in patients with DR, and the VEGF level has been reported to be significantly correlated with the severity of diabetic retinopathy [39]. In accordance with previous results, levels of VEGF in the aqueous have been found to be markedly increased in postoperative exacerbation of macular edema patients [13]; therefore, aqueous cytokine may be a predictor of macular edema in diabetic patients after cataract surgery.

IL-10, which is produced by monocytes and macrophages, is one of the main anti-inflammatory cytokines. IL-10 limits inflammation by reducing the synthesis of proinflammatory cytokines such as IL-1 and TNF-α, by suppressing cytokine receptor expression and by inhibiting receptor activation [40]. In addition, IL-10 prevents angiogenesis by downregulating VEGF expression [41]. IL-12 possesses antiangiogenic activity that is mediated by the stimulation of T-helper lymphocytes and the induction of IP-10 expression [42]. In our study, the IL-10 and IL-12 concentrations of samples from the ME (+) patients were significantly lower. Our results suggest that low levels of circulating IL-10 (anti-inflammatory and antiangiogenic activity) and IL-12 (antiangiogenic) are involved in the pathogenesis of ME after cataract surgery.

The limitations of our study should be noted. First, the number of patients with severe PDR enrolled in the study was relatively low. Aqueous cytokines as predictors of macular edema in patients with severe PDR following uncomplicated cataract surgery needs to be studied further. Second, the concentrations of the cytokines in vitreous samples and serum were not determined. The cytokine levels in the vitreous are usually higher, and the analysis of vitreous would more accurately reflect the intraocular levels of cytokines and the status of the retina. However, in contrast to vitreous samples, obtaining aqueous fluid samples from the anterior chamber is easier, faster, and less risky. In addition, the ME was assessed at 4 weeks of cataract surgery and consequently the ME formation may also be the result of surgery related anterior segment inflammation during this period. Hence, in order to reduce the influence of operation on the result of ME, all phacoemulsification cataract extractions were performed by the same surgeon. Finally, multiplex bead immunoassay has the limitation if the cytokine levels are very low, so the positive detection rates for the 5 cytokines were not more than 80% and these cytokines were not included in the statistical analysis because of the low detection rates in the current study.

5. Conclusions

The present study showed that aqueous levels of IL-1β, IL-6, IL-8, IP-10, MCP-1, and VEGF were increased in patients with postcataract surgery macular edema and were positively correlated with FCPT 4 weeks following cataract surgery in diabetic patients. In addition, aqueous levels of IL-10 and IL-12 were significantly lower in patients with postcataract surgery ME and were negatively correlated with postoperative FCPT. These results indicate that IL-1β, IL-6, IL-8, IL-10, IL-12, IP-10, MCP-1, and VEGF may be potential predictors to determine postoperative macular thickness in diabetic patients following uncomplicated phacoemulsification cataract surgery.

Disclosure

This work neither has been published nor is it being considered for publication elsewhere in any form. All authors have read and approved the paper.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Oxidative Stress Induces Endothelial Cell Senescence via Downregulation of Sirt6

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Accumulating evidence has shown that diabetes accelerates aging and endothelial cell senescence is involved in the pathogenesis of diabetic vascular complications, including diabetic retinopathy. Oxidative stress is recognized as a key factor in the induction of endothelial senescence and diabetic retinopathy. However, specific mechanisms involved in oxidative stress-induced endothelial senescence have not been elucidated. We hypothesized that Sirt6, which is a nuclear, chromatin-bound protein critically involved in many pathophysiologic processes such as aging and inflammation, may have a role in oxidative stress-induced vascular cell senescence. Measurement of Sirt6 expression in human endothelial cells revealed that H_2O_2 treatment significantly reduced Sirt6 protein. The loss of Sirt6 was associated with an induction of a senescence phenotype in endothelial cells, including decreased cell growth, proliferation and angiogenic ability, and increased expression of senescence-associated β-galactosidase activity. Additionally, H_2O_2 treatment reduced eNOS expression, enhanced p21 expression, and dephosphorylated (activated) retinoblastoma (Rb) protein. All of these alternations were attenuated by overexpression of Sirt6, while partial knockdown of Sirt6 expression by siRNA mimicked the effect of H_2O_2. In conclusion, these results suggest that Sirt6 is a critical regulator of endothelial senescence and oxidative stress-induced downregulation of Sirt6 is likely involved in the pathogenesis of diabetic retinopathy.

1. Introduction

Increasing evidence indicates that diabetes accelerates the process of aging, especially in patients who are at high risk of developing complications [1]. Diabetic retinopathy (DR) is one of the most common complications of diabetes. It is a leading cause of blindness in people of working age in industrialized countries [2]. The mechanisms by which elevated blood glucose causes tissue injury and disease progression in the retina are not yet clear. However, studies in animal models and patients suggest that diabetic retinopathy is associated with vascular dysfunction, similar to changes seen during aging [3, 4]. Increased cellular senescence was also observed in retinal blood vessels from diabetic animals [4].

Aging is a normal process of any living mammal. The proliferative lifespan of primary mammalian cells is limited. After a given number of replication cycles, they enter permanent cell-cycle arrest, referred to as replicative senescence and linked to a reduction in telomerase activity and telomere shortening [5, 6]. In contrast to replicative senescence, stress-induced premature senescence is elicited by stressful stimuli and does not require telomere shortening and extensive cell division [5, 7]. Although caused by different mechanisms,

replicative senescence and stress-induced premature senescence share many similarities, including a specific set of alternations in cell function, morphology, gene expression, and positive staining for senescence-associated β-galactosidase activity (SA β-gal) [7].

Premature senescence of endothelial cells is recognized to play a key role in the pathogenesis of diabetic vascular complications including diabetic retinopathy [4, 8, 9]. When endothelial cells acquire a senescent phenotype, their homeostatic functions become impaired as indicated by a decrease in production of nitric oxide and an increase in expression of adhesion molecules such as ICAM-1 [10]. These alternations lead to endothelial dysfunction, vascular inflammation, and impaired angiogenesis and vascular repair [9]. Endothelial senescence can be induced by a variety of factors; among them, oxidative stress has a major role [11].

Oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms endogenous antioxidant systems and/or when the endogenous antioxidant systems are impaired. ROS are a group of oxygen-based molecules characterized by their high chemical reactivity [12, 13]. ROS include free radicals such as superoxide (O_2^{-}) and hydroxyl radicals ($OH^{\bullet}$), and nonradical species such as hydrogen peroxide (H_2O_2) [12, 13]. Excessive production of ROS critically contributes to development of many signs of DR ranging from vascular dysfunction and vascular leakage to pathological angiogenesis [2, 14–19]. Although oxidative stress is one of the major factors causing the onset of senescence [20], the specific mechanisms underlying ROS-induced endothelial senescence are not completely clear.

Mammalian sirtuins (Sirts) are the homologue of the yeast silent information regulator (Sir) 2, a nicotinamide adenine dinucleotide- (NAD-) dependent histone deacetylase regulating life span of yeast [21]. Among the seven mammalian Sirts, Sirt6 most closely resembles the yeast Sir2 regarding its intracellular location and function and the animal phenotype caused by loss of Sirt6 [21, 22]. It is a nuclear, chromatin-bound protein that functions as a NAD-dependent histone H3 lysine 9 (H3 K9) deacetylase, repressing the activities of several transcription factors involved in aging and inflammation, including NF-κB, c-JUN, and hypoxia-inducible factor- (HIF-) 1α [23–26]. Mice deficient in Sirt6 exhibit severe metabolic defects and premature aging phenotype and 60% of them die at about 4 weeks of age [21]. In this study, we determined the role of Sirt6 in ROS-induced endothelial cell senescence.

2. Materials and Methods

2.1. Cell Culture and Transfection. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Lonza Walkersville Inc., Walkersville, MD) and grown in EGM Endothelial Cell Medium (Lonza). Cells between passages 4 and 7 were used for all experiments. To overexpress Sirt6, cells were infected with adenovirus carrying empty vector (control, Ad-Con) or Sirt6 (Ad-Sirt6) as described previously [27]. These adenoviruses were purchased from Vector BioLabs (Philadelphia, PA). One day after infection, HUVECs were treated with 75 μM H_2O_2 (EMD Millipore, Billerica, MA) for 2–4 days, with medium being changed daily. To moderately knock down Sirt6, cells were transfected using HiPerFect (Qiagen, Valencia, CA) following the manufacturer's instructions. Human Sirt6 siGENOME SMARTpool consisting of a mixture of four sequences and a control nontargeting siRNA were purchased from Thermo Fisher Scientific Inc. (Waltham, MA) and used at a concentration of 34 nM. The transfection complexes were removed 24 hours later. Four and eight days after initiating transfection, cells were subjected to two additional transfections.

2.2. Immunocytochemistry. HUVECs were seeded on fibronectin-coated slides and infected with adenovirus carrying Sirt6 (Ad-Sirt6). At 24 hours after infection, cells were washed with Hank's Balanced Salt Solution (HBSS) and fixed in 2% paraformaldehyde-Phosphate Buffered Saline (PBS) for 15 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton-PBS for 5 minutes, blocked with PBS containing 2% bovine serum albumin (BSA) for 30 minutes, followed by incubation with primary antibody to Sirt6 (1 : 100, Cell Signaling Technology, Danvers, MA) in PBS containing 1% BSA for 2 hours at 4°C. After washing three times with PBS containing 0.1% BSA, cells were incubated with secondary antibody Alexa Fluor 488 donkey anti-rabbit (1 : 250, Invitrogen, Carlsbad, CA) for 1 hour at 4°C and washed and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Slides were imaged using an Olympus 1X71 fluorescence microscope (Olympus, Center Valley, PA) at a magnification of 100x.

2.3. Senescence-Associated β-Galactosidase (SA-β-gal) Staining. Senescence-associated β-galactosidase (SA-β-gal) staining was performed using a Senescence Detection kit (BioVision, Milpitas, CA) according to the manufacturer's instructions. Briefly, cells were washed with HBSS, fixed using a fixative solution for 10–15 minutes, again washed with PBS, and then incubated with Staining Solution Mix overnight at 37°C. Cells were imaged under a bright-field microscope at a magnification of 100x. The number of positive cells with blue color was counted and normalized to the number of total cells in the same field.

2.4. Western Blot. Western blots were performed as described [27]. Briefly, cells were lysed in SDS sample buffer without dithiothreitol and bromophenol blue and then cleared of debris by centrifugation. Protein concentration in the lysates was determined by a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Dithiothreitol was added to a final concentration of 125 mM and samples were boiled for 10 minutes. Equal amounts of protein were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes, and the membranes were incubated overnight with one of the following primary antibodies: anti-Sirt6 (1 : 1000), anti-eNOS (1 : 1000, BD Biosciences, San Jose, CA), anti-tubulin (1 : 10000, Sigma-Aldrich, St. Louis, MO), anti-p21

(1 : 200, Santa Cruz Biotechnology, Dallas, TX), and anti-phosphorylated Rb (1 : 1000, Cell signaling) followed by appropriate second antibodies for 1 hour. Immunoreactive proteins were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.5. BrdU Cell Proliferation Assay. HUVECs were allowed to reach 50–70% confluency and then incubated with BrdU (25 μM) (Sigma-Aldrich) at 37°C for 2 hours. Cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with PBS containing 0.3% Triton X-100 for 15 minutes at room temperature, and incubated with 2 M HCl for 30 minutes at 37°C. After DNA denaturation, the remaining HCl was neutralized by incubating cells with 0.1 M borate buffer. Cells were then washed three times with PBS containing 0.05% Tween 20 (PBST), blocked with 2% goat serum in PBST for 30 minutes at 37°C, followed by incubation with a mouse anti-BrdU antibody (1 : 1000, Sigma-Aldrich) for 30 minutes at 37°C. After washing three times with PBST, cells were incubated with Alexa Fluor 488 goat anti-mouse (1 : 400, Invitrogen) for 30 minutes at 37°C and counterstained with DAPI. Cells were imaged using a fluorescence microscope at a magnification of 100x. BrdU positive cells were counted and normalized to the total cells in the same field.

2.6. Tube Formation Assay. HUVECs were seeded on Matrigel matrix- (BD Biosciences)- coated 96-well plates at a density of 1.5×10^4 cells/well in the EGM Endothelial Cell Medium without serum and incubated in humidified 5% CO_2 at 37°C for 2 hours. Cells were washed twice with HBSS and labeled with 8 μg/mL Calcein AM (Invitrogen) in 50 μL HBSS. Plates were incubated for 30 minutes at 37°C. After washing twice with HBSS, cells were imaged using a fluorescence microscope at a magnification of 100x. Tube length was measured using ImageJ software.

2.7. RT-PCR Assay. RNAqueous-4PCR Kit (Invitrogen) was used to isolate total RNA, and cDNA was produced by reverse transcription with M-MLV reverse transcriptase (Invitrogen). Quantitative PCR was performed with StepOne PCR system (Invitrogen) using Power SYBR Green. The fold difference in various transcripts was calculated by the ΔΔCT method using Hprt as an internal control [27]. Subsequently, a melting curve, constructed in the range of 60 to 95°C, was used to evaluate the specificity of the amplification products. Primer sequences for human transcripts were as follows: Hprt For-5′-CCT TGG TCA GGC AGT ATA ATC CA-3′; Hprt Rev-5′-GGT CCT TTT CAC CAG CAA GCT-3′; Sirt1 For-5′-TGC GGG AAT CCA AAG GAT AA-3′; Sirt1 Rev-5′-CAG GCA AGA TGC TGT TGC A-3′; Sirt2 For-5′-TCC CAG CGC GTT TCT TCT-3′; Sirt2 Rev-5′-ACC AGG AGG AGG TCC ACC TT-3′; Sirt3 For-5′-ACA TCG ATG GGC TTG AGA GAG T-3′; Sirt3 Rev-5′-CAT GAG CTT CAA CCA GCT TTG A-3′; Sirt4 For-5′-CCT CTT GGT GGT GGG ATC AT-3′; Sirt4 Rev-5′-CAG GCA GTG AGG ATA AAC CTG TAA-3′; Sirt5 For-5′-CTC GCC CAC TGT GAT TTA TGT C-3′; Sirt5 Rev-5′-GCT GCT GGG TAC ACC ACA GA-3′; Sirt7 For-5′-CGT CCG GAA CGC CAA ATA-3′; Sirt7 Rev-5′-ACG CTG CCG TGC TGA TTC-3′.

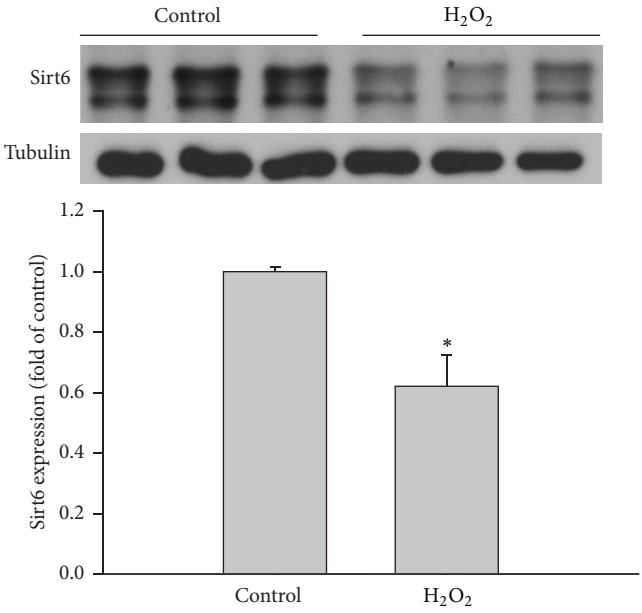

Figure 1: H_2O_2 decreases Sirt6 expression in HUVECs. After human umbilical endothelial cells (HUVECs) were incubated in the absence or presence of 75 μM H_2O_2 for 4 days, Sirt6 protein was measured by Western blot and normalized to control ($n = 3$). Tubulin was used as loading control. $^*P < 0.05$ compared to control.

2.8. Statistical Analysis. Data are presented as mean ± standard deviation. Group differences were evaluated with one way ANOVA followed by post hoc Student's t-test using the Student-Newman-Keuls Method. Results were considered significant if $P < 0.05$. Data shown are representative of at least three independent experiments.

3. Results

3.1. Oxidative Stress Reduces Sirt6 Protein in Endothelial Cells. H_2O_2 is a major reactive oxygen species generated during oxidative stress and has been widely used as an oxidative stress inducer in oxidative stress-related research [11, 28–30]. In order to investigate whether Sirt6 has a potential role in endothelial cell dysfunction induced by oxidative stress, we determined amounts of Sirt6 protein in H_2O_2-treated human endothelial cells (ECs). Our results showed that Sirt6 protein was significantly reduced (by 38%) in H_2O_2-treated ECs compared to vehicle-treated cells (Figure 1).

3.2. Sirt6 Overexpression Attenuates Endothelial Cell Senescence Induced by Oxidative Stress. Since Sirt6 expression was downregulated by oxidative stress, we next determined if Sirt6 has a role in oxidative stress-induced endothelial cell senescence. Adenovirus-mediated gene delivery was utilized to introduce Sirt6 into ECs. We observed that when ECs were infected with adenovirus at multiplicity of infection (MOI)

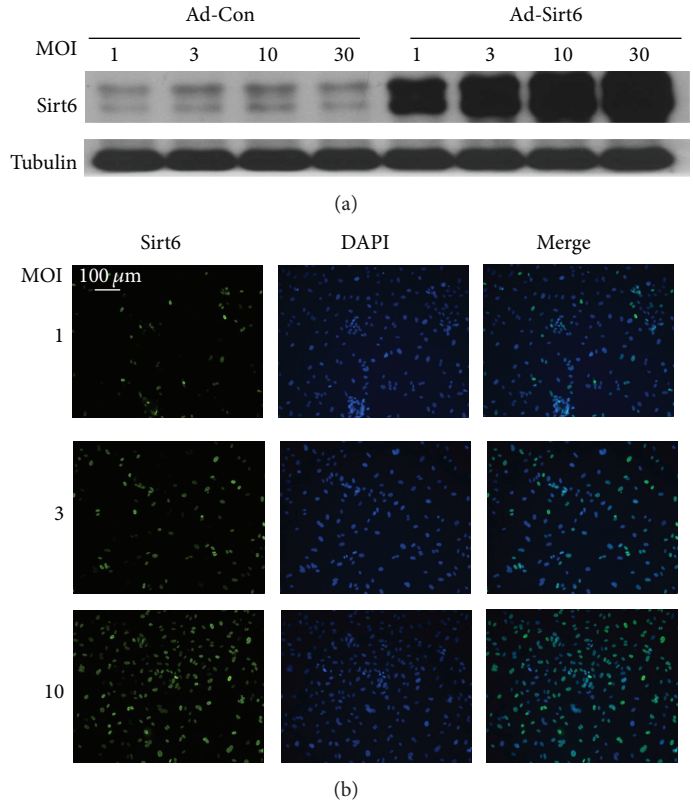

Figure 2: Establishment of overexpression of Sirt6 in endothelial cells. (a) HUVECs were infected with adenovirus carrying Sirt6 gene (Ad-Sirt6) or empty vector (control, Ad-Con) at different multiplicity of infection (MOI). (a) At 24 hrs after infection, cells were lysed and Sirt6 protein level was determined by Western blot. (b) Cells infected with Ad-Sirt6 were stained with DAPI (blue) for nuclei and anti-Sirt6 antibody (green). Images were taken at 100x magnification.

from 1 to 30, Sirt6 protein increased in a dose-dependent manner (Figure 2(a)). A MOI of 10 achieved near 100% infection efficiency (Figure 2(b)) and was used in subsequent experiments to overexpress Sirt6 in ECs.

Senescence-associated β-galactosidase (SA-β-gal) activity is a characteristic feature of cell senescence. To investigate whether Sirt6 is involved in oxidative stress-induced EC senescence, ECs were infected with adenovirus carrying empty vector (Ad-con) or Sirt6 (Ad-Sirt6) and analyzed for SA-β-gal activity after H_2O_2 treatment. Compared with vehicle-treated ECs, H_2O_2 treatment increased the percentage of SA-β-gal positive ECs by 4.1-fold in Ad-con cells. In contrast, in cells infected with Ad-Sirt6, there was only 1.2-fold increase in SA-β-gal positive ECs after H_2O_2 treatment (Figure 3(a)). As the senescence phenotype suggests a change in growth, we analyzed endothelial cell proliferation by BrdU incorporation. We observed that H_2O_2 treatment reduced the percentage of proliferative cells from 27.4% to 3.4%, and overexpression of Sirt6 significantly reversed this effect. The percentage of proliferative cells was increased to 10.5% in cells overexpressing Sirt6 (Figures 3(b) and 3(c)). Similar results were observed by staining cells with the proliferation marker proliferating cell nuclear antigen (PCNA) (data not shown). Consistent with the above markers of EC senescence, EC growth was inhibited by H_2O_2 treatment and overexpression of Sirt6 significantly blocked the effect (Figure 3(d)). These results indicate that Sirt6 negatively regulates oxidative stress-induced endothelial cell senescence.

3.3. Sirt6 Overexpression Blocks Endothelial Cell Dysfunction Induced by Oxidative Stress. EC dysfunction, manifested by reduced production of nitric oxide and impaired angiogenic activity, is a hallmark of vascular diseases. Since EC senescence has been causally linked to EC dysfunction, we analyzed angiogenic activity by tube formation assay and eNOS expression by Western blot in ECs exposed to H_2O_2. The total length of tube formation in H_2O_2-treated groups was decreased 53% compared with control group. Overexpression of Sirt6 partially restored the angiogenic ability of ECs, with the tube length increased by 46% versus cells treated with H_2O_2 (Figure 4(a)). NO is generated by endothelial nitric oxide synthase (eNOS) in ECs. Compared with vehicle-treated control cells, there was a 73% reduction of eNOS protein after H_2O_2 treatment (Figure 4(b)). In contrast, H_2O_2 treatment did not reduce eNOS protein in Ad-Sirt6 cells. These results suggest that Sirt6 overexpression blocks H_2O_2-induced EC dysfunction.

3.4. Effects of Sirt6 Are Not Mediated by Other Sirtuin Family Members. Since other members of the sirtuin family have

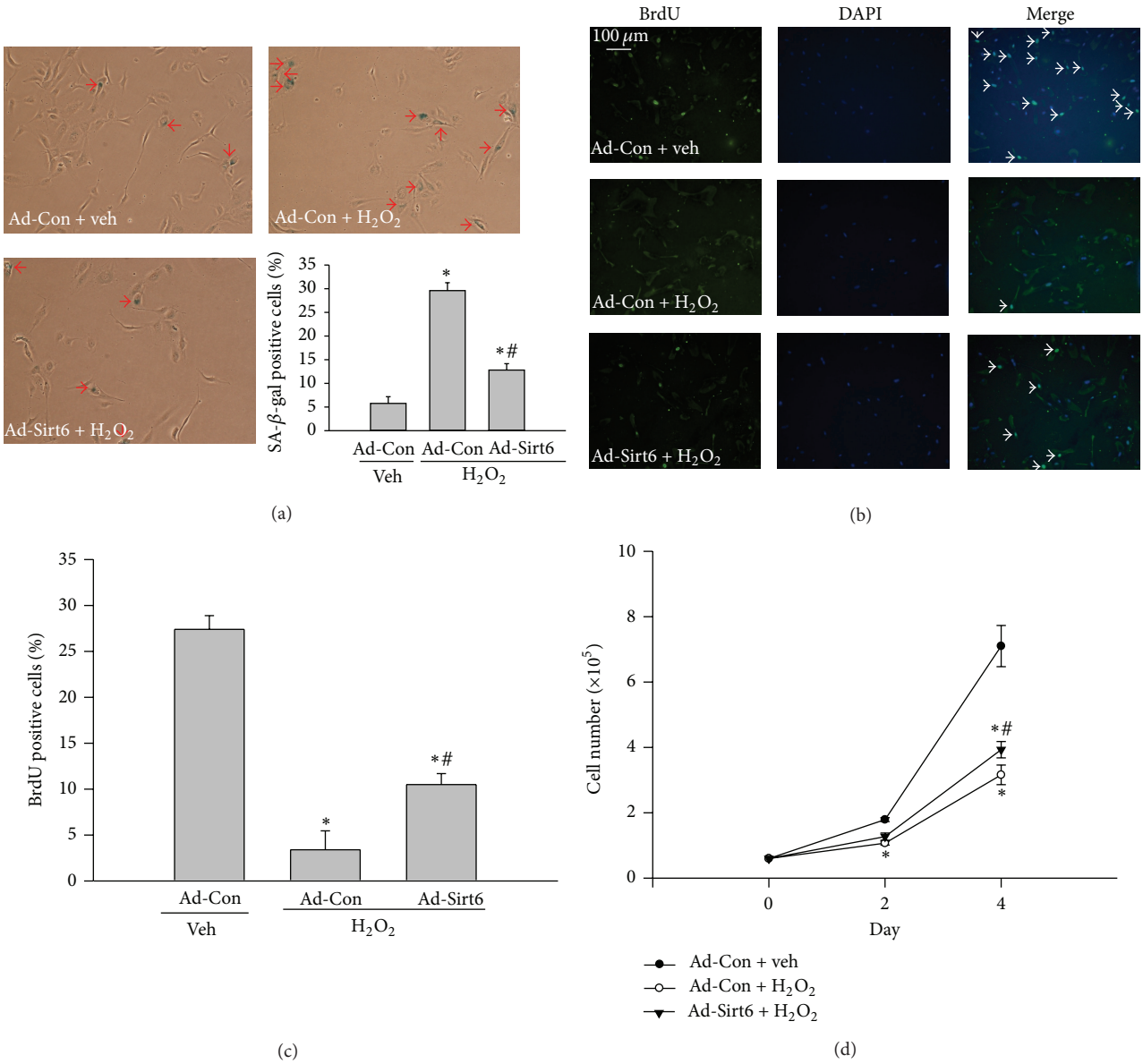

FIGURE 3: Sirt6 overexpression attenuates endothelial cell senescence induced by H_2O_2. ECs were infected with adenovirus carrying Sirt6 gene (Ad-Sirt6) or empty vector (control, Ad-Con) and treated with 75 μM H_2O_2 or vehicle (H_2O) for 4 days. (a) SA-β-gal staining was performed using a Senescence Detection kit. Images were taken under a bright-field microscope at a magnification of 100x. Arrows show SA-β-gal positive cells (blue cells). The percentage of SA-β-gal positive cells versus total cells was determined and quantitative data were shown in the bar graph. (b) Cells were labeled with BrdU followed by staining with DAPI (blue) for nuclei and anti-BrdU antibody (Green). Images were taken at 100x magnification. Arrows show BrdU positive nuclei (green). (c) BrdU positive nuclei (green) and total nuclei (blue) were counted. The percentage of BrdU positive nuclei versus total cell nuclei was calculated and quantitative data were shown in the bar graph. (d) The cell number in each treatment was counted at different times. $^*P < 0.05$ compared with cells infected with Ad-Con and treated with vehicle. $^\#P < 0.05$ compared with cells infected with Ad-Con and treated with H_2O_2.

been shown to prevent EC senescence [31], the effects of Sirt6 may be indirect, caused by alterations of other Sirts induced by Sirt6 overexpression. To exclude this possibility, we analyzed mRNA levels of other Sirts in ECs with Sirt6 overexpressed. We observed no significant change in the expression of other Sirts despite elevated Sirt6 by RT-PCR assay (Figure 5). These results indicate that the endothelial cell protective effects of Sirt6 are directly mediated by Sirt6 itself and not indirectly by other members of the sirtuin family.

3.5. Moderate Loss of Sirt6 Mimics the Effect of Oxidative Stress. Since overexpression of Sirt6 can attenuate oxidative stress-induced EC dysfunction and senescence, we tested if moderate knockdown of Sirt6 would mimic effects of oxidative stress. We optimized experimental conditions to reduce endogenous Sirt6 protein level with Sirt6 siRNA (Sirt6-si) by ~60% (Figure 6). At early time points, such as 48 hrs and 72 hrs, we did not observe significant changes in SA-β-gal activity and EC growth. However, at 10 days after

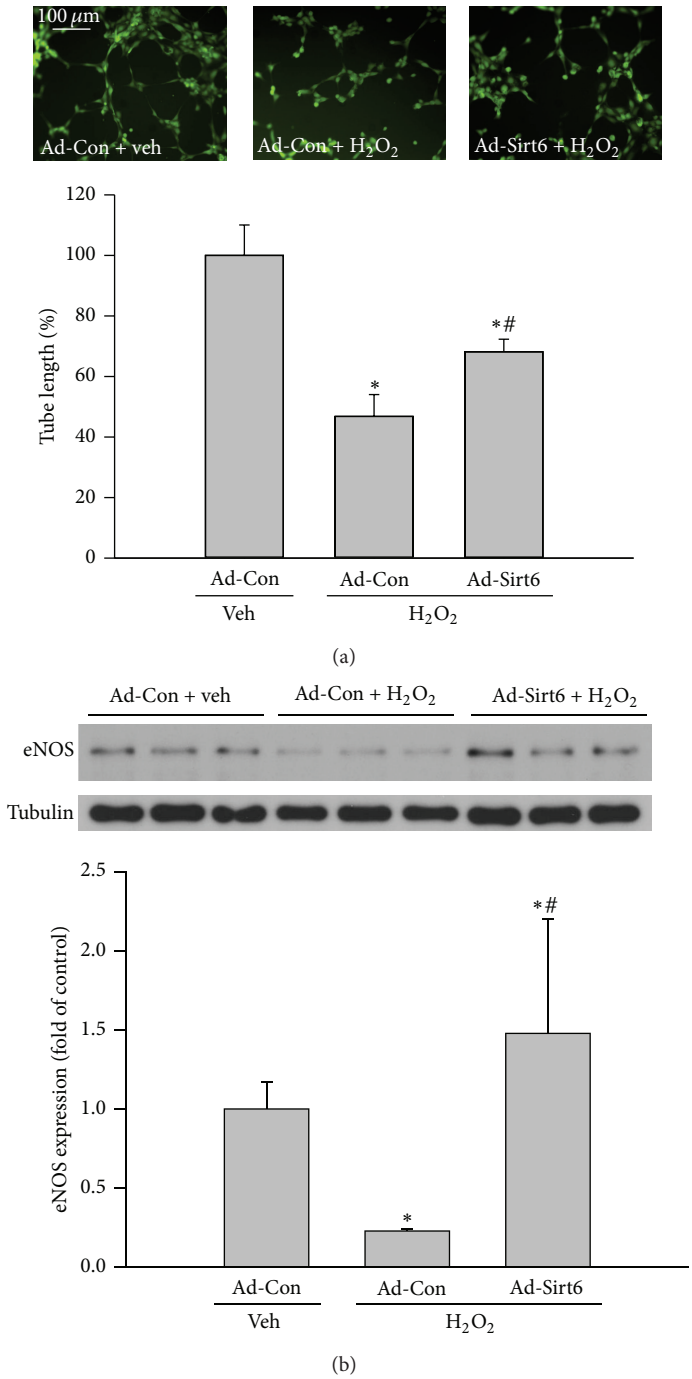

Figure 4: Sirt6 overexpression reduces endothelial cell dysfunction induced by H_2O_2. ECs were infected with adenovirus carrying Sirt6 gene (Ad-Sirt6) or empty vector (control, Ad-Con) and treated with 75 μM H_2O_2 or vehicle (H_2O) for 4 days. (a) Cells were subjected to Matrigel-based tube formation assay to determine the angiogenic ability. Images were taken by fluorescence microscopy at 100x magnification and tube length was measured by ImageJ software. (b) eNOS expression in cells was determined by Western blot. $^*P < 0.05$ compared with cells infected with Ad-Con and treated with vehicle. $^\#P < 0.05$ compared with cells infected with Ad-Con and treated with H_2O_2.

Sirt6-si RNA transfection, there is a 1.8-fold increase in SA-β-gal positive cells, 47% decrease in BrdU positive cells, and 52% decrease in EC growth (Figure 7), suggesting that a moderate knockdown of Sirt6 accelerated EC senescence. Sirt6 siRNA-transfected cells also exhibited signs of EC dysfunction associated with EC senescence, including reduced angiogenic ability and eNOS expression (Figure 8). In summary, these data indicate that a moderate loss of Sirt6 mimics effects of oxidative stress, albeit at a slower pace.

3.6. Sirt6 Suppresses Activation of Senescence Pathways Induced by Oxidative Stress. The retinoblastoma (Rb) protein

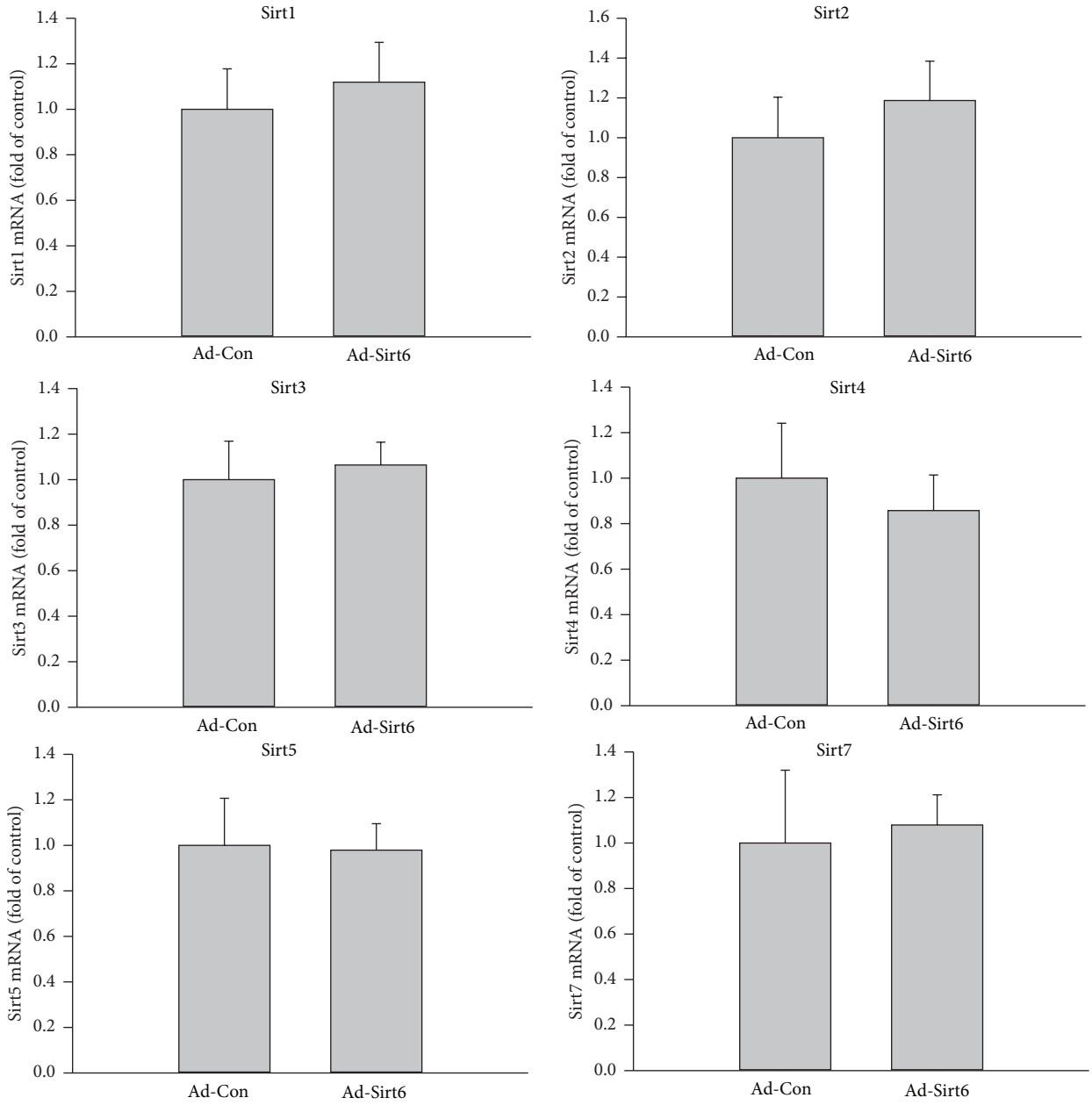

Figure 5: Sirt6 overexpression does not affect the expression of other Sirts. ECs were infected with adenovirus carrying Sirt6 gene (Ad-Sirt6) or empty vector (control, Ad-Con) for 2 days. The levels of other Sirts were determined by quantitative PCR and normalized to control.

plays a critical role in the induction of cell senescence. Rb protein is phosphorylated by cyclin-dependent kinases (CDKs), resulting in loss of its ability to bind E2F/DP transcription factor complexes and allowing cells to enter S-phase [32]. However, when the activity of CDKs is blocked by CDK inhibitors, p21Cip1/Waf1/Sdi1, and p16INK4a, Rb protein is hypophosphorylated and activated and inhibits the transcriptional activity of the E2F protein family members [32]. Consequently, cells are arrested in the G1 cell cycle and develop a senescent phenotype [32]. To further elucidate the downstream signaling mechanisms by which Sirt6 inhibits oxidative stress-induced EC senescence, we determined phosphorylation of Rb in cells treated with H_2O_2. We found the level of phosphorylated Rb protein was dramatically decreased after H_2O_2 treatment (Figure 9), suggesting that activation of Rb protein was involved in H_2O_2-induced EC senescence. Consistent with Rb activation, analysis of p21, its upstream activator, also revealed a significant increase in the level of p21 protein. These changes were significantly attenuated by Sirt6 overexpression. p16 protein was undetectable in our experimental conditions (data not shown). These suggest that Sirt6 inhibits oxidative stress-induced EC senescence at least partly through the p21-Rb pathway.

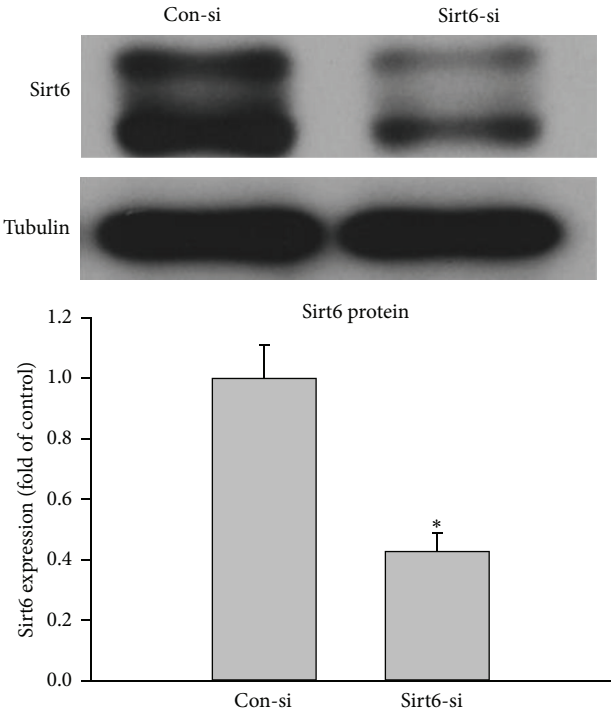

Figure 6: Knockdown of Sirt6 expression in ECs. ECs were transfected with Sirt6 siRNA (Sirt6-si) or Control siRNA (Con-si). 48 hours after transfection, the protein level of Sirt6 was determined by Western blot and normalized to control ($n = 3$). Tubulin was used as loading control. $^{*}P < 0.05$ compared to control.

4. Discussion

Oxidative stress is a key player in the pathogenesis of diabetic retinopathy (DR) [2, 14–19]. It contributes to endothelial senescence by decreasing NO production, promoting inflammation, and perturbing normal endothelial cell functions. However, molecular mechanisms underlying oxidative stress-induced endothelial senescence remain to be defined. Here, we provide novel evidence that Sirt6, a molecule with antiaging and anti-inflammatory properties, is a target of oxidative stress and is involved in oxidative stress-induced EC senescence. We report that Sirt6 protein was markedly reduced in endothelial cells following treatment with H_2O_2 and that overexpressing Sirt6 partially reversed H_2O_2-induced EC dysfunction and senescence. This included decreases in EC growth, proliferation and angiogenic ability, loss of eNOS protein, and increases in senescence markers. A partial knockdown of Sirt6 mimicked effects of oxidative stress induced with hydrogen peroxide. Our data, together with a previous observation that lipopolysaccharide-induced reduction of Sirt6 expression is linked to lipopolysaccharide-induced endothelial cell inflammatory reactions [33], highlight the importance of Sirt6 in preventing endothelial dysfunction caused by cellular stress mechanisms.

Similar to a previous publication [34], we observed that knockdown of Sirt6 accelerates endothelial senescence. However, in our study, a significant reduction of EC growth occurred at later time point (10 days) after cells were transfected with Sirt6 siRNA compared to that in Cardus's study (3 days) [34]. This difference between the two studies can be explained by the observation that Sirt6 protein level was reduced by ~60% in our study, mimicking effects of H_2O_2, whereas it was decreased by 75% in Cardus et al.'s study [34]. These observations suggest that the phenotype caused by loss of Sirt6 is dose-dependent and a threshold must be reached to induce pathophysiological alternations. Consistent with this suggestion is the observation that Sirt6 heterozygous mice display a normal phenotype regardless of a reduced Sirt6 level [21].

A variety of mechanisms may be involved in the antiendothelial senescence effect of Sirt6. It has been shown that Sirt6 inhibits cell senescence by promoting resistance to oxidative stress-induced DNA damage, suppressing genomic instability, preventing telomere dysfunction, and inhibiting NF-κB mediated expression of age-related genes [21, 23, 35, 36]. In our study, we identified a novel mechanism that involves crosstalk between Sirt6 and Rb protein. Rb, in its hypophosphorylated form, is active and inhibits expression of proteins required for cell-cycle progression by binding to the E2F protein family members and repressing their transcriptional activity [37]. Our results indicate that H_2O_2-induced EC senescence was associated with Rb hypophosphorylation and an increase in the expression of p21, an upstream activator of Rb. However, such changes were reversed by Sirt6 overexpression, suggesting Sirt6 may exert antisenescence effect by preventing oxidative stress-induced Rb activation. As p21 is a downstream target of p53 [37], our observations also suggest that Sirt6 is involved in regulation of p53 activity despite the observation that total levels of p53 protein remain unchanged. Previous studies have shown that Sirt1 negatively regulates p53 activity and protects ECs from H_2O_2-induced senescence [31]. However, we did not observe changes of other members in the sirtuin family after modulating Sirt6 expression, indicating an independent role of Sirt6 in regulation of EC senescence.

Previous studies have suggested that Sirt6 is involved in protecting cells from oxidative stress by mono-ADP-ribosylating poly(ADP-ribose) polymerase-1 (PARP-1), stimulating PARP1 poly-ADP-ribosylase activity and enhancing repair of double-strand breaks that occur during oxidative stress [38]. Our results suggest that Sirt6 expression could be directly compromised during oxidative stress, and enhanced Sirt6 expression can attenuate oxidative stress-induced endothelial cell senescence. Therefore, agents that can prevent Sirt6 downregulation or enhance Sirt6 activity will be useful in protecting Sirt6 from oxidative stress-induced loss of the normal Sirt6 function during diseases.

Currently, our understanding of mechanisms regulating Sirt6 function is still limited. Sirt6 protein can be ubiquitinated and degraded through the proteasome, and degradation of Sirt6 is substantially prevented when Sirt6 is noncanonically ubiquitinated at K170 by CHIP [39]. Since CHIP protein is downregulated by H_2O_2 [40], it is possible that oxidative stress reduces CHIP protein level, resulting in a reduction of Sirt6 protein by accelerating its proteasome-dependent degradation. Alternatively, oxidative

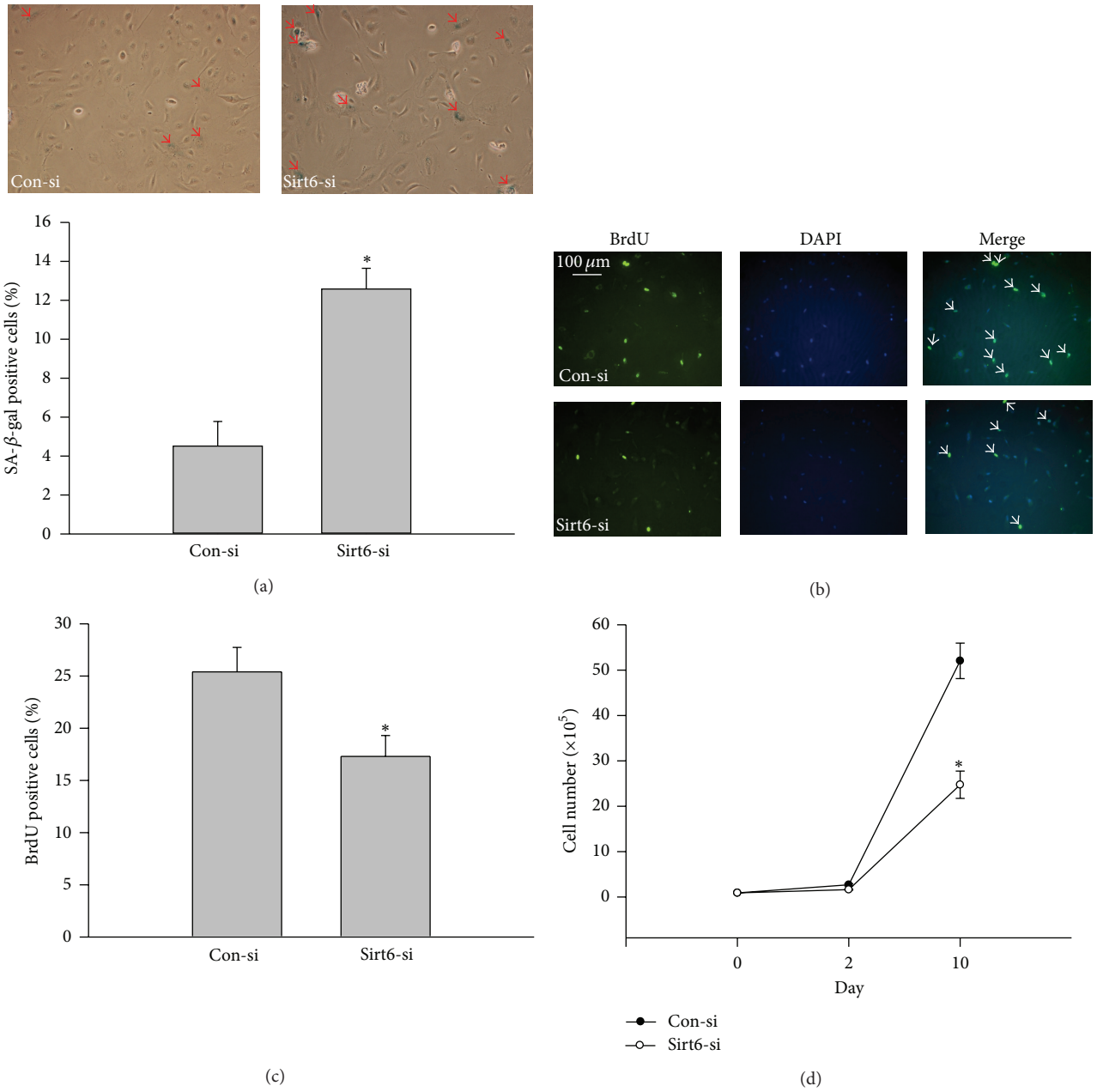

FIGURE 7: Sirt6 deletion induces endothelial cell senescence. ECs were serially transfected with Sirt6 siRNA (Sirt6-si) or Control siRNA (Con-si) for 10 days. (a) Cells were stained for SA-β-gal activity using a Senescence Detection kit. Images were taken under a bright-field microscope at a magnification of 100x. Arrows show SA-β-gal positive cells (blue cells). The percentage of SA-β-gal positive cells versus total cells was determined and quantitative data were shown in the bar graph. (b) Cells were labeled with BrdU followed by staining with DAPI (blue) for nuclei and anti-BrdU antibody (green). Images were taken under fluorescence microscopy at 100x magnification. Arrows show BrdU positive nuclei (green). (c) BrdU positive nuclei (green) and total nuclei (blue) were counted. The percentage of BrdU positive nuclei versus total cell nuclei was calculated and quantitative data were shown in the bar graph. (d) The cell number after siRNA transfection was counted at different times. $^*P < 0.05$ compared with cells transfected with Con-si.

stress may regulate Sirt6 expression and function via other posttranslational modifications of the protein, reduction of the level of NAD+, or inhibition of Sirt6 transcription and translation similar to previously described mechanisms regulating Sirt1 function [41]. Future studies are needed to address these possibilities and investigate whether oxidative stress is involved in loss of Sirt6 expression and function in cancer, cardiomyocyte hypotrophy, and inflammation [25, 33, 42].

In summary, our data demonstrate that an oxidative stress-induced decrease in Sirt6 expression has an essential role in oxidative stress-induced endothelial senescence.

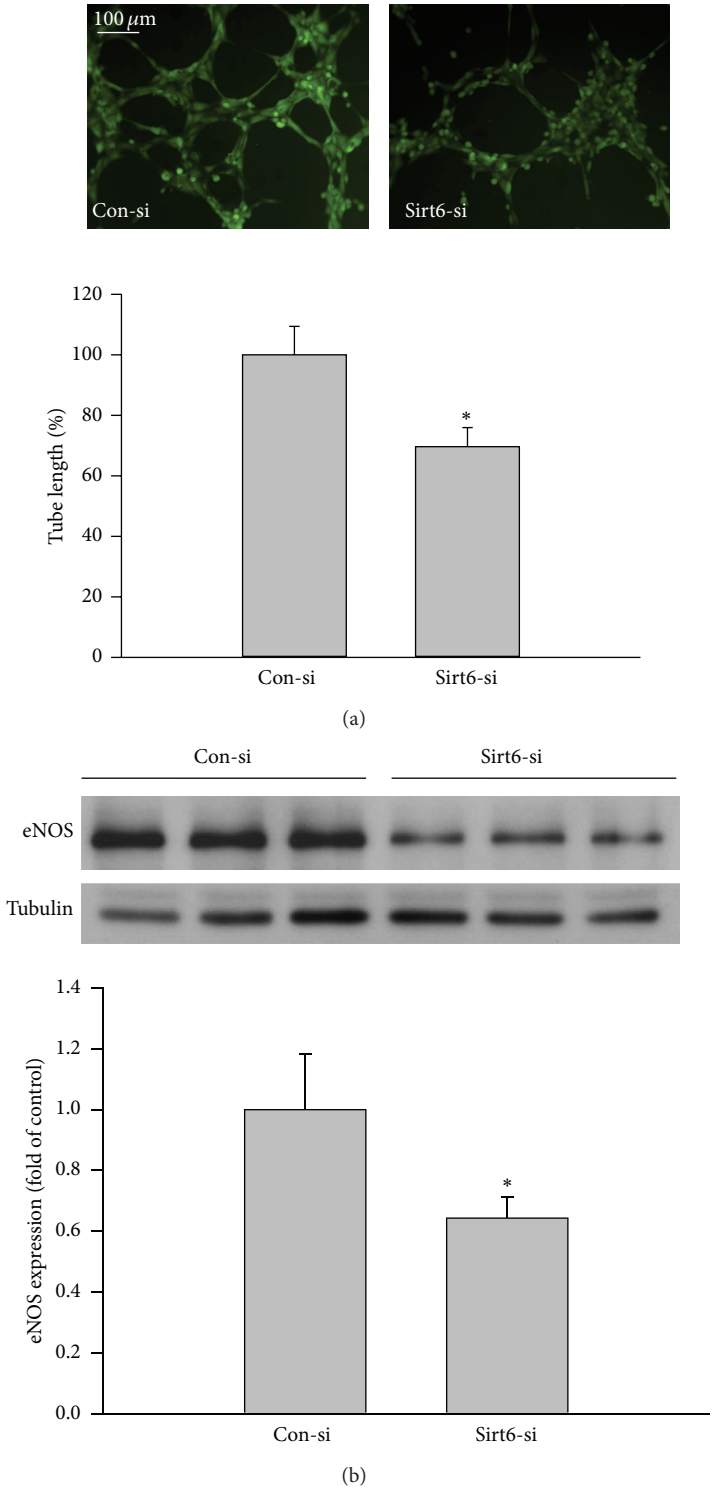

Figure 8: Sirt6 deletion impairs endothelial cell function. ECs were transfected with Sirt6 siRNA (Sirt6-si) or Control siRNA (Con-si). (a) 10 days after transfection, the angiogenic ability of cells was determined by tube formation assay. Images were taken by fluorescence microscopy at 100x magnification and tube length was measured by ImageJ software. (b) eNOS expression in cells was determined by Western blot. $^{*}P < 0.05$ compared with cells transfected with Con-si.

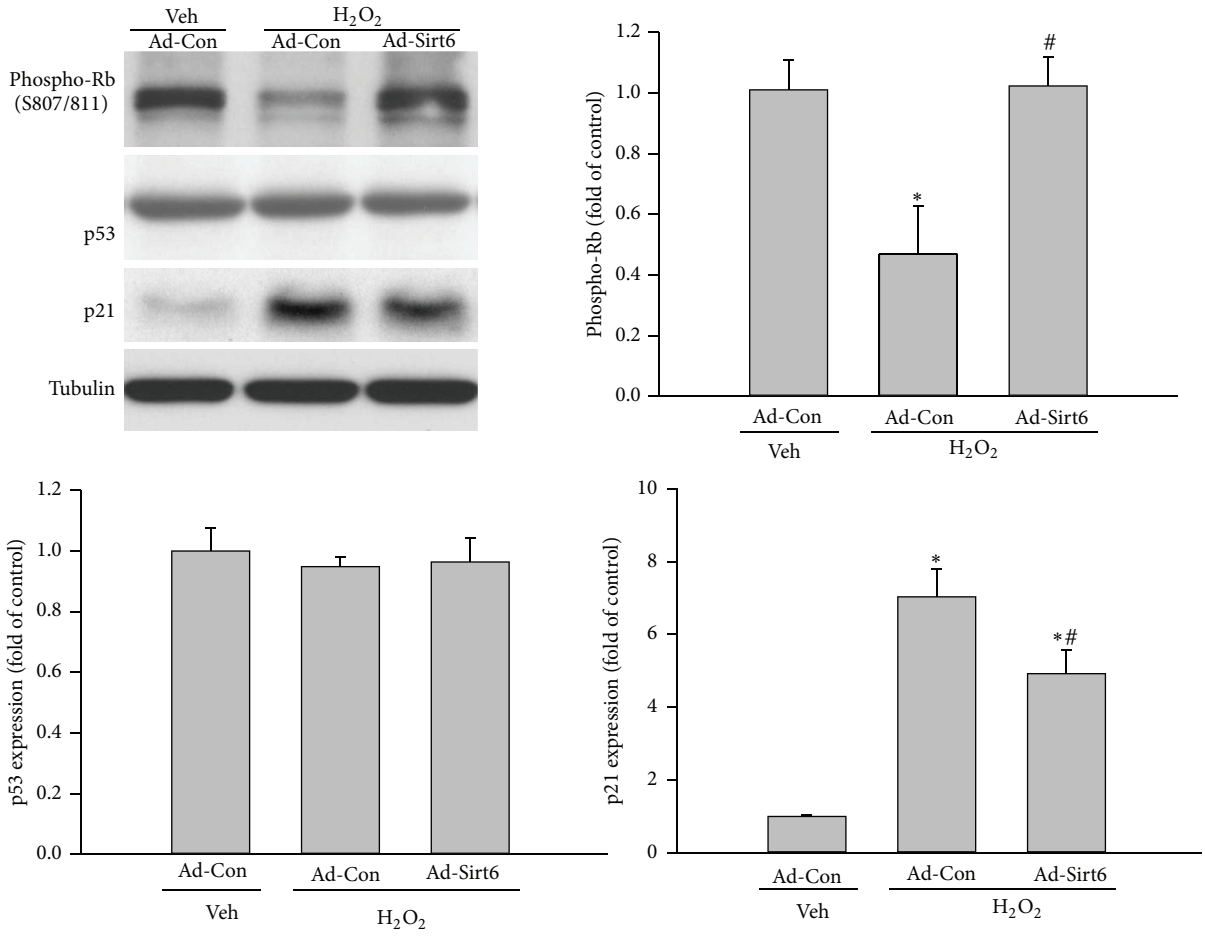

FIGURE 9: Sirt6 overexpression inhibits the activation of senescence pathways induced by H_2O_2. ECs were infected with adenovirus carrying Sirt6 gene (Ad-Sirt6) or empty vector (control, Ad-Con). At 1 day after transduction, cells were exposed to 75 μM H_2O_2 or vehicle (H_2O). At 1 day after H_2O_2 treatment, cells were lysed and phosphorylated-Rb, p53, and p21 were determined by Western blot. Tubulin was used as loading control. $^*P < 0.05$ compared with cells infected with Ad-Con and treated with vehicle. $^\#P < 0.05$ compared with cells infected with Ad-Con and treated with H_2O_2.

Given that endothelial senescence is implicated in the development of diabetic vascular complications [8, 9], our results warrant further investigations of the role of Sirt6 in various vascular alternations in DR, such as vascular dysfunction, inflammation, leakage, and pathological angiogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Caspase-14 Expression Impairs Retinal Pigment Epithelium Barrier Function: Potential Role in Diabetic Macular Edema

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We recently showed that caspase-14 is a novel molecule in retina with potential role in accelerated vascular cell death during diabetic retinopathy (DR). Here, we evaluated whether caspase-14 is implicated in retinal pigment epithelial cells (RPE) dysfunction under hyperglycemia. The impact of high glucose (HG, 30 mM D-glucose) on caspase-14 expression in human RPE (ARPE-19) cells was tested, which showed significant increase in caspase-14 expression compared with normal glucose (5 mM D-glucose + 25 mM L-glucose). We also evaluated the impact of modulating caspase-14 expression on RPE cells barrier function, phagocytosis, and activation of other caspases using ARPE-19 cells transfected with caspase-14 plasmid or caspase-14 siRNA. We used FITC-dextran flux assay and electric cell substrate impedance sensing (ECIS) to test the changes in RPE cell barrier function. Similar to HG, caspase-14 expression in ARPE-19 cells increased FITC-dextran leakage through the confluent monolayer and decreased the transcellular electrical resistance (TER). These effects of HG were prevented by caspase-14 knockdown. Furthermore, caspase-14 knockdown prevented the HG-induced activation of caspase-1 and caspase-9, the only activated caspases by HG. Phagocytic activity was unaffected by caspase-14 expression. Our results suggest that caspase-14 contributes to RPE cell barrier disruption under hyperglycemic conditions and thus plays a role in the development of diabetic macular edema.

1. Introduction

Diabetic retinopathy (DR) is the most common complication of diabetes and remains a major cause of preventable blindness worldwide [1, 2]. Anatomical and functional changes occur in the retina and retinal pigment epithelium prior to clinical symptoms of the disease and RPE plays a key role in the pathogenesis of DR [3–5]. Most of the research on the physiopathology of DR has been focused on the impairment of the neuroretina and the breakdown of the inner blood retinal barrier (BRB). By contrast, the effects of diabetes on the RPE have received less attention and also the molecular mechanisms responsible for these early changes in the RPE remain unclear [6].

The retinal pigment epithelium is densely pigmented hexagonal monolayer of cells located between the neural retina and choroid blood vessels [7]. RPE cells are joined together by junction adhesion (JA) molecules and tight junction (TJ) proteins such as occludin, claudins, and zonula occludens [8, 9] which is linked to the actin cytoskeleton. Integrity of TJ and JA is important to keep the subretinal space dry and preserve the outer retinal barrier [10]. RPE is also responsible for light absorption and phagocytosis of shed photoreceptor of outer segments [11, 12]. Furthermore, RPE

secretes several factors, which are involved in maintaining normal retinal vascular homeostasis such as platelet-derived growth factor (PDGF) [13], pigment epithelium-derived factor (PEDF) [14], and vascular endothelial growth factor (VEGF) [15–17]. RPE cell malfunction is involved in many eye diseases including age related macular degeneration (AMD) [18, 19] and DR through production of inflammatory cytokines and caspase-mediated inflammatory and apoptotic pathways [20]. High glucose treatment of RPE cells leads to disruption in the levels of gap junction protein connexin and the TJ protein claudin-1, causing epithelial barrier dysfunction [21, 22].

Caspases exist as inactive proenzymes, activation of which requires proteolytic processing at conserved internal aspartic residues to generate a heterotetrameric enzyme consisting of two large and two small subunits [23, 24]. Caspase-14 is expressed and activated mainly in the epidermis [25] and in several tissues related to barrier function such as choroid plexus, hair follicles, epidermis, RPE, thymic Hassall's bodies, and keratinized oral epithelium [26, 27]. Caspase-14 is thought to be associated with epidermal barrier formation that protects against dehydration and ultraviolet radiation-induced apoptosis [25]. Recently we demonstrated that caspase-14 is expressed in the retina and different retinal cells under normal conditions, and increased expression of caspase-14 occurs in the retinas of diabetic human subjects and experimental diabetic mice, as well as in retinal microvascular cells cultured under high glucose conditions [27]. We also showed that caspase-14 overexpression induced apoptosis of retinal endothelial cells and pericytes suggesting that caspase-14 plays a role in the pathogenesis of DR via accelerating retinal microvascular cell death [27] which contributes to the breakdown of the inner retinal barrier [28].

In this study, we investigated the functional involvement of caspase-14 in RPE cells. Our data showed that caspase-14 is involved in barrier function of RPE cells but not in phagocytic function. We evaluated the effect of high glucose (HG) on caspase-14 expression in RPE cells and we examined the effect of caspase-14 expression or knockdown on the RPE cell barrier function, activation of other caspases, and apoptosis under normal and hyperglycemic conditions. This study revealed upregulation of caspase-14 in RPE by HG treatment and disruption of RPE barrier by caspase-14 expression and HG treatment and this was associated with a significant increase in the activity of caspase-1 and caspase-9. Furthermore, the effects of HG treatment on RPE cells barrier function and caspase-1 and caspase-9 activities were prevented by caspase-14 knockdown.

2. Material and Methods

2.1. Cell Culture. The ARPE-19 cells are available from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). They are cultured under standard conditions (37°C in a humidified chamber of 5% CO_2 in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12) (Thermo Scientific, Wyman, Massachusetts) supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin, 10 mg/mL streptomycin sulfate, and 25 μg/mL amphotericin (Cellgro, Manassas, Virginia) which was changed every 3 days. Cells were passaged weekly at a ratio of 1 : 10.

2.2. High Glucose Treatment of RPE Cells. RPE cells were grown until 70–80% confluent, and then the serum-free DMEM/F-12 was added to the cells for 24 h before switching to high or normal glucose treatment (30 mM or 5 mM D-glucose + 25 L-glucose, resp.). The cells were then incubated in 37°C in a humidified chamber of 5% CO_2 for 5 days. After the incubation, the medium was removed and the cell lysates were used for Western blot analysis of caspase-14.

2.3. Caspase-14 Transfection. The preparation of caspase-14 expression vector and empty vector was previously described [27], and the cDNA sequences of the vectors were confirmed by DNA sequencing. ARPE-19 cells were transfected with the pCMV plasmid containing human caspase-14 cDNA, the empty vector, scrambled siRNA (Integrated DNA Technologies, Coralville, Iowa), and caspase-14 siRNA (Santa Cruz, Dallas, Texas), by electroporation using ECM 830 electroporation system (Harvard, Holliston, Massachusetts). ARPE-19 cells were grown in 75 cm^2 flasks. When cells reached 90%–95% confluence, the cells were passaged and resuspended in 4 mL PBS (Life Technologies, Grand Island, New York) and placed in an electroporation cuvette and 1 μg of DNA or RNA was added to the cuvette. The cuvette was placed in the ECM 830 and the electroporation was done at 450 V for 75 μs, repeated twice with 100 ms intervals. The cell mixture was mixed and the electroporation was repeated. The cells were then reseeded in 75 cm^2 flasks.

2.4. Assessment of Retinal Pigment Epithelial Cell Barrier Function. Integrity of RPE barrier is essential for normal retinal function and it is disrupted by hyperglycemia contributing to the pathogenesis of diabetic macular edema. Therefore, we assessed whether caspase-14 is implicated in RPE barrier function by studying the effect of modulating its expression on RPE permeability, transcellular electrical resistance, and cytoskeleton.

2.5. FITC-Dextran Flux Assay. ARPE-19 cells transfected with caspase-14 or empty vector were seeded on noncoated membranes with 0.4 μm pores (Transwell; Corning Costar), in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12). After becoming completely confluent, FITC-dextran (1 mg/mL) was then added to the upper chambers and samples from the lower and upper chambers were obtained at different time points (1, 3, and 6 h), and fluorescence intensity measurements were performed using a plate reader. The FITC-dextran that passed across the ARPE-19 cell monolayer was normalized, and P_o was calculated. The equation for P_o is $[(F_l/\Delta t)V_A]/(F_A A)$ whereas P_o is in cm/s; F_l is basolateral fluorescence, F_A is apical fluorescence, Δt is the change in time, A is the surface area of the Transwell, and V_A is the volume of the basolateral chamber [29]. Similar experiments were performed with ARPE-19 cells treated with HG or NG for 5 days. In addition, one group was transfected with caspase-14 siRNA or the scramble siRNA before HG

treatment. The knockdown of the caspase-14 was confirmed by Western blotting prior to initiating the experiment.

2.6. Measurement of the Transcellular Electrical Resistance (TER) by Electric Cell Substrate Impedance Sensing (ECIS). Since RPE barrier dysfunction includes changes in the TER across the confluent monolayer, we evaluated the effect of modulating caspase-14 expression on RPE TER under NG or HG condition. ARPE-19 cells were transfected with caspase-14 siRNA or scrambled siRNA and placed into DMEM/F-12 medium with 1% FBS for 24 hours in 25 cm^2 flasks. Once the cells were 90–95% confluent, ARPE-19 cells were seeded onto 8-well 10w3 + arrays (20,000 cells per well) and placed onto the ECIS machine (Applied Biophysics, Grand Island, New York). After 4 hours, the medium was changed to the NG or HG treatment in DMEM/F-12 medium and the measurements of TER were taken over 24 hours. Additional experiments were performed on ARPE-19 cells expressing caspase-14 following transfection with the caspase-14 or control plasmid.

2.7. Assessment of ARPE-19 Cell Cytoskeleton. ARPE-19 cells, which were previously transfected with the caspase-14 vector or the empty vector, were seeded in 8-well chamber slides (Lab Tek II) (about 2000 cells/well) for 24–48 h. Cells were then fixed with 4% paraformaldehyde, permeabilized for 5 min with 0.1% Triton X-100, and blocked with a 5% bovine serum albumin (BSA) (ACROS Chemical, Wyman, Massachusetts) for 30 min at room temperature. Diluted antivinculin 1 : 100 (Millipore, Billerica, Massachusetts) containing 5% BSA was added to the cells and incubated for 1 h at room temperature. Diluted goat anti-mouse FITC conjugated secondary (1 : 200) and TRITC conjugated phalloidin (1 : 200) (Millipore, Billerica, Massachusetts) were added to each well and incubated for 1 h at room temperature. Cells were incubated with DAPI solution (1 : 1000) (Millipore, Billerica, Massachusetts) for 5 minutes at room temperature. The slides were mounted using mounting media (Vector Laboratories, Burlingame, California) and images were obtained using immunofluorescence microscopy (LSM 510; Carl Zeiss, Thornwood, NY).

2.8. Measurement of the Activity of Caspase-1, -3, -4, -5, -8, and -9. ARPE-19 cells were transfected as previously described (with caspase-14 siRNA, caspase-14 plasmid, and control plasmid) and once the cells were 70% confluent the medium was changed to serum-free DMEM/F-12 medium for 24 h. The medium was then changed to HG or NG medium and the cells were grown in this medium for 5 days, and afterwards the cells were lysed. The cell lysates were collected and used to measure caspase activity by caspase family activity colorimetric II kit (Abcam, Cambridge, Massachusetts). This included the activity of caspase-1, -3, -4, -5, -8, and -9. The kit protocol was followed and the activity was measured by microplate reader at 400 nm.

2.9. Western Blotting. We evaluated the expression of caspase-14 and cleaved caspase-4 in RPE cells lysate. Confluent monolayers of ARPE-19 cultures were lysed using lysis buffer (5 mL of 1X RIPPA; Millipore, Billerica, Massachusetts) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then scraped with a sterile scraper (Fisher Scientific, Wyman, Massachusetts). Cell lysate was incubated for 30 minutes on ice and then centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected for protein assay and the pellet was discarded. ARPE-19 cell lysates (50 μg protein) were separated by sodium dodecyl sulfate-PAGE using a 10% ready precast gel (Bio-Rad, Hercules, CA), transferred to polyvinylidene fluoride membrane, and reacted with rabbit polyclonal caspase-14 antibody (Sc-5628; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with horseradish peroxidase-conjugated secondary antibody and examination by enhanced chemiluminescence (Amersham Pharmacia, San Francisco, CA). The membranes were then stripped and reprobed with β-actin to demonstrate equal loading, and the results were analyzed using Image-J program.

2.10. Assessment of Phagocytic Activity of RPE Cells. ARPE-19 cells were transfected with Lipofectamine 2000 according to the previously described protocol [27] with caspase-14 plasmid and control plasmid. Triplicates were seeded in 96-well plates at 100,000 cells/well in 1X DMEM medium supplemented with 1% FBS and 1% PSN. The medium (100 μL) was placed into three wells for a no cell background. Once the cells adhered to the plate, culture medium was removed and replaced with pHrodo red phagocytosis particle (Invitrogen, Grand island, NY) (1 mg/mL) suspension. Immediately, the plate was transferred to 37°C incubator for 2 h. Fluorescence intensity was measured using a plate reader at an excitation wavelength 560 nm and an emission wavelength 585 nm.

2.11. TUNEL Assay. ARPE-19 cells that were previously transfected were grown in four-well chamber cells (Lab Tek 11) for five days in treatment medium. TUNEL assay (Promega, Madison, WI) was done according to the kit protocol. The nuclear stain propidium iodide was used to stain the nuclei of the cells on the chamber slides. The samples were analyzed under a fluorescence microscope and ten pictures per group were taken. The number of apoptotic cells relative to the number of normal cells in each microscopic field were counted using image J.

2.12. Statistical Analysis. Statistical analysis was done in GraphPad Prism 5. t-test was used to detect any significant difference between two groups and one-way ANOVA was used to detect any significant difference between 3 or more groups followed by Tukey analysis. Results are shown as mean ± SEM and were considered significant when P value < 0.05. At least 4 dishes were prepared for each treatment group and each experiment was replicated with at least 3 different batches of retinal cells.

3. Results

3.1. Effect of High Glucose on Caspase-14 Expression. To test whether caspase-14 is implicated in hyperglycemia-induced RPE dysfunction, we first evaluated the changes

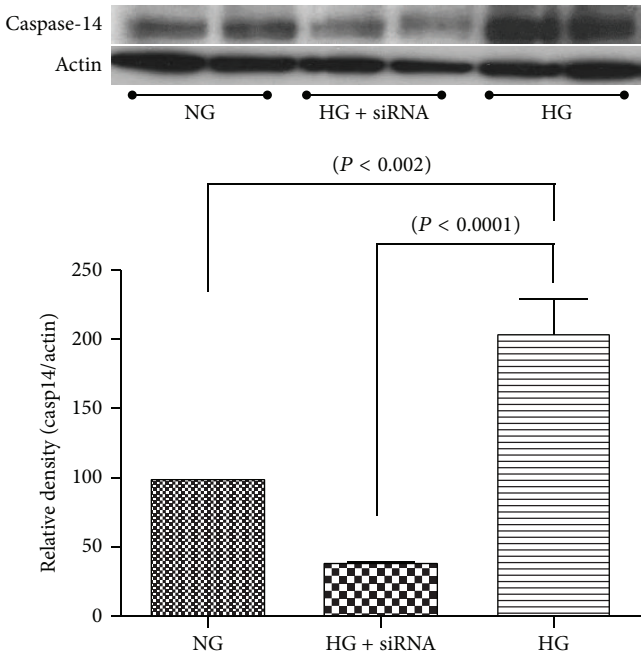

Figure 1: High glucose conditions increased caspase-14 expression in ARPE-19 cells. Western blot analysis of caspase-14 showed a significant increase in caspase-14 expression of RPE cells under high glucose (30 mM D-glucose) compared with normal glucose (5 mM D-glucose + 25 mM L-glucose) conditions. Transfection of ARPE-19 cells with caspase-14 siRNA significantly reduced caspase-14 in RPE cells under high glucose conditions ($n = 4$, $^{*}P < 0.05$).

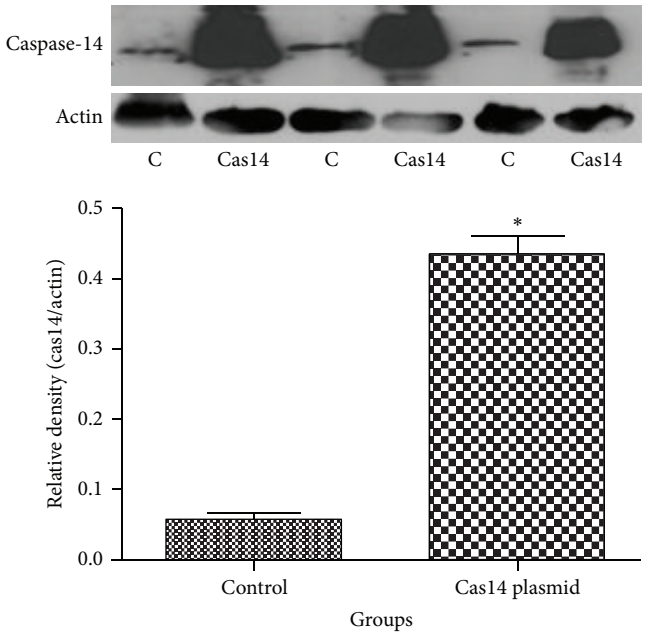

Figure 2: Overexpression of caspase-14 in ARPE-19 cells. Western blot analysis of caspase-14 in ARPE-19 cells transfected with pCMV plasmid encoding human caspase-14 cDNA showed a remarkable increase in the levels of caspase-14 compared with cells expressing the empty vector ($n = 4$; $^{*}P < 0.0001$).

in caspase-14 expression in ARPE-19 cells grown in HG (30 mM D-glucose) or in NG (5 mM D-glucose + 25 mM L-glucose) for 5 days. Western blot analysis of the cell lysates demonstrated that caspase-14 was significantly upregulated in ARPE-19 cells grown under HG conditions compared with NG. Transfection of the ARPE-19 cells with caspase-14 siRNA significantly abrogated caspase-14 expression under HG conditions (Figure 1). Caspase-14 expression was also evaluated in ARPE-19 cells transfected with caspase-14 plasmid or empty vector to assure the efficacy of the caspase-14 plasmid expression. Caspase-14 transfected ARPE-19 cells had significantly higher caspase-14 than cells transfected with empty vector ($P < 0.0001$; Figure 2).

3.2. Effect of Caspase-14 Expression on RPE Barrier Function. Breakdown of the RPE barrier function contributes to the development of DME, a major cause of vision loss in diabetic patients. Thus, we studied the effect of caspase-14 expression on the RPE cell barrier function by measuring the changes in the transcellular electrical resistance (TER) and stress fibers levels and organization. The zero time point is when the ARPE-14 cells reach full confluences and resistance was measured until the cells start detaching from the array. ARPE-19 cells transfected with caspase-14 plasmid had lower resistance compared with cells transfected with empty vector (Figure 3).

To further evaluate the impact of caspase-14 expression on RPE barrier function we examined the changes in the distribution and expression of ARPE-19 cell cytoskeleton protein F-actin, which is known to increase and become disorganized by hyperglycemia [30]. The relative distribution of ARPE-19 cell F-actin was monitored using phalloidin (red) staining, which binds to F-actin and provides details of the cellular cytoskeleton. There was a significant increase in the levels and the disorganization of F-actin stress fibers in caspase-14 expressing cells compared to control (Figure 4).

3.3. Effects of Caspase-14 Expression on RPE Cell Phagocytic Function. In addition to the role of RPE in maintaining the outer retinal barrier, RPE cells also play an important role in phagocytosis of the photoreceptor outer segments. Therefore, we tested whether caspase-14 effect is specific to the barrier function by examining the effects of caspase-14 expression on the phagocytic function of the ARPE-19 cells using a commercially available phagocytic assay kit. Our experiments demonstrated no significant differences in the phagocytic activity of caspase-14 overexpressing RPE cells compared with control cells (38 + 0.7 versus 37 + 1; Figure 5).

3.4. Effect of Caspase-14 Knockdown on HG-Induced RPE Hyperpermeability. To assess the direct effect of caspase-14 transfection on RPE permeability compared to the control, we first examined whether transfection with the caspase-14 vector induces changes in FITC dextran flux through a confluent monolayer of ARPE-19 cell. We noticed that ARPE-19 cells became significantly permeable to FITC-dextran

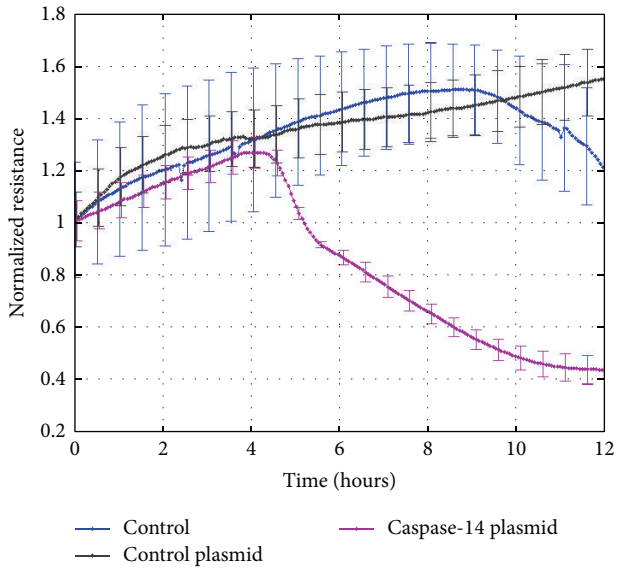

FIGURE 3: Effect of HG and caspase-14 expression on RPE barrier function. ECIS analysis of the transcellular electrical resistance (TER) demonstrated a significant decrease in the TER by caspase-14 expression compared to RPE cells transfected with or without the empty vector ($n = 4$, $P < 0.05$).

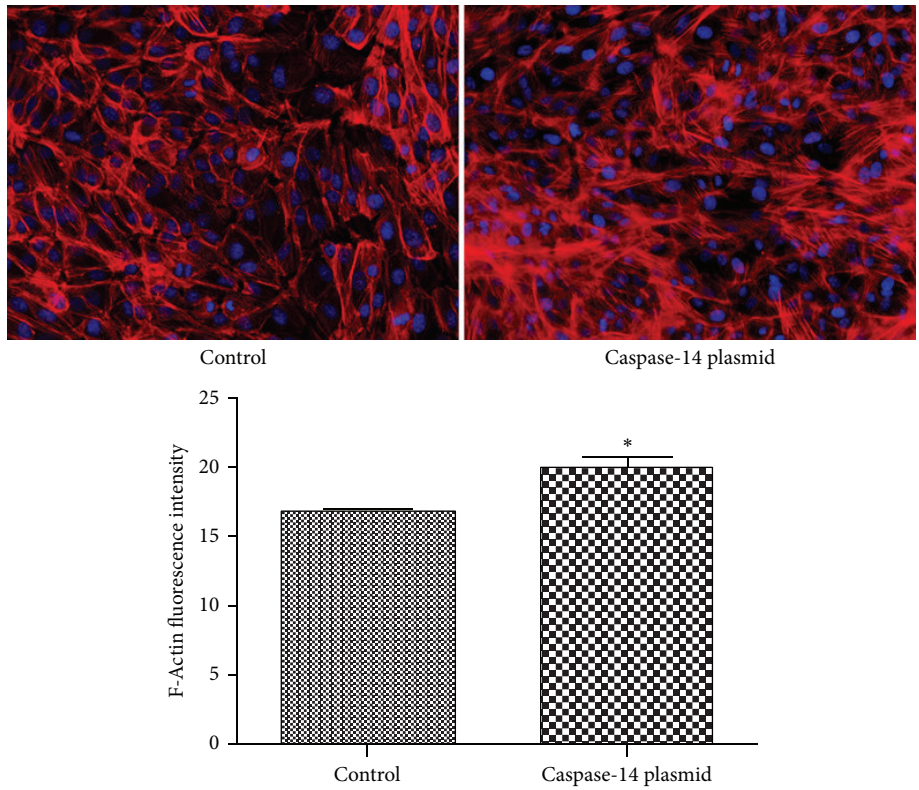

FIGURE 4: Immunofluorescence staining of RPE cell's cytoskeleton, F-actin (red). The nuclei were counterstained with DAPI (blue). Please note the marked increase and disorganization of the stress fibers (F-actin) immunoreactivity in caspase-14 expressing RPE cells compared with control cells ($^*P < 0.0008$ versus control).

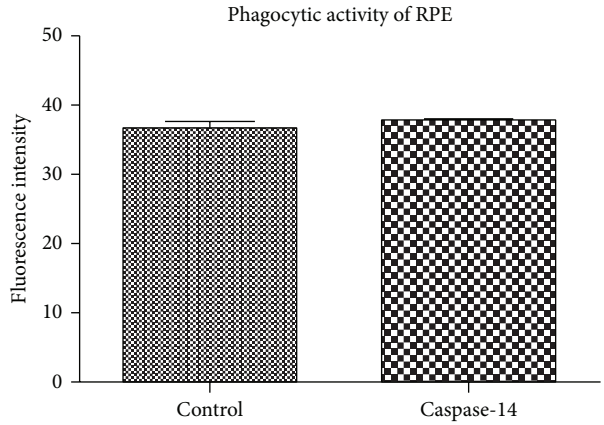

Figure 5: Effect of caspase-14 expression on ARPE-19 cell phagocytic activity. Assessment of phagocytic activity of the RPE cells was performed using a commercially available phagocytic assay kit. We observed no significant differences between the phagocytic activity of RPE cells expressing caspase-14 and the control ($P > 0.05$).

when transfected with caspase-14 compared with vector control cells after 1, 3, and 6 hr. HG treatment induced a similar effect as caspase-14 overexpression on the ARPE-19 cell permeability. However, this effect was significantly abrogated in ARPE-19 cells transfected with the caspase-14 siRNA compared with the scrambled control siRNA (Figure 6).

3.5. Caspase-14 Expression Is Implicated in HG-Induced Apoptosis of RPE Cells. The exact function of caspase-14 in various tissues other than skin has not yet been characterized. It is not clear if caspase-14 belongs to the proapoptotic or proinflammatory caspases. Therefore, it is important to examine if modulation of caspase-14 expression impacts these pathways in the RPE cells. For this purpose we measure the rate of apoptosis in ARPE-19 cells by TUNEL assay. High glucose conditions or caspase-14 overexpression significantly enhanced the apoptotic cell death of ARPE-19 cells compared to cells under NG or transfected with control plasmid, respectively ($P < 0.001$). Furthermore, caspase-14 knockdown reduced the number of RPE cells undergoing apoptosis under HG conditions ($P < 0.05$; Figure 7).

3.6. Effects of Caspase-14 Expression on the Activity of Other Caspases. Since there is no information regarding how caspase-14 interacts with other members of caspase family, we sought to test the impact of caspase-14 knockdown on HG-induced activation of various caspases in ARPE-19 cells. HG increased the activity of caspase-1 and caspase-9 out of several caspases examined including caspase-3, -4, -5, and -8. Caspase-14 knockdown significantly reduced the activation of caspase-1 and caspase-9 under HG conditions (Figure 8).

4. Discussion

To the best of our knowledge, the current study is the first to investigate the impact of caspase-14 expression on RPE cell barrier and phagocytic function. Our major findings are as follows: (1) hyperglycemia upregulates caspase-14 expression in human RPE cells, (2) caspase-14 expression impairs RPE barrier function with no effect on its phagocytic function, and (3) caspase-14 knockdown in RPE inhibits hyperglycemia-mediated RPE barrier disruption, activation of caspase-1 and -9, and enhanced apoptosis.

Our previous study demonstrated that caspase-14 is normally expressed in the retina and various retinal cells including RPE cells and was upregulated in human and mouse retina during diabetes. Additionally, the overexpression of caspase-14 demonstrated a proapoptotic effect in cultured retinal endothelial cells and pericytes suggesting caspase-14 as a potential player in the pathogenesis of DR via enhancing retinal vascular cell death and capillary degeneration, which causes loss of inner retinal barrier function [27, 31]. Here we investigated whether caspase-14 also contributes to hyperglycemia-induced RPE barrier dysfunction, which constitutes the outer retinal barrier.

The RPE is the major component of the outer BRB, playing important roles in the flow of metabolites and ions from the choroidal blood supply to the neural retina [22, 32], and contributes to the ocular vascular homeostasis through production of pro- and antiangiogenic factors. Therefore, RPE may provide good target for studying molecular basis of DR and diabetic macular edema (DME); in particular, hyperglycemia has been shown to induce inflammation and apoptosis and disrupts RPE tight junctions leading to breakdown of RPE barrier and finally DME [33, 34].

Caspase-14 was found to be expressed in tissues involved in barrier function such as epidermis and RPE and to preserve skin barrier and protect it against dehydration and ultraviolet light [34, 35]. However, the current study found that, similar to hyperglycemia, overexpression of caspase-14 leads to hyperpermeability of RPE cells. Transmembrane proteins and cytoplasmic protein of RPE cells are linked to the actin cytoskeleton and participate in many important cellular processes, such as cell motility, phagocytosis, and establishment and maintenance of cell junctions and cell shape [10]. In this study, we found that overexpression of caspase-14 transfection was accompanied by disorganization of RPE cytoskeleton including increased amounts of F-actin filaments.

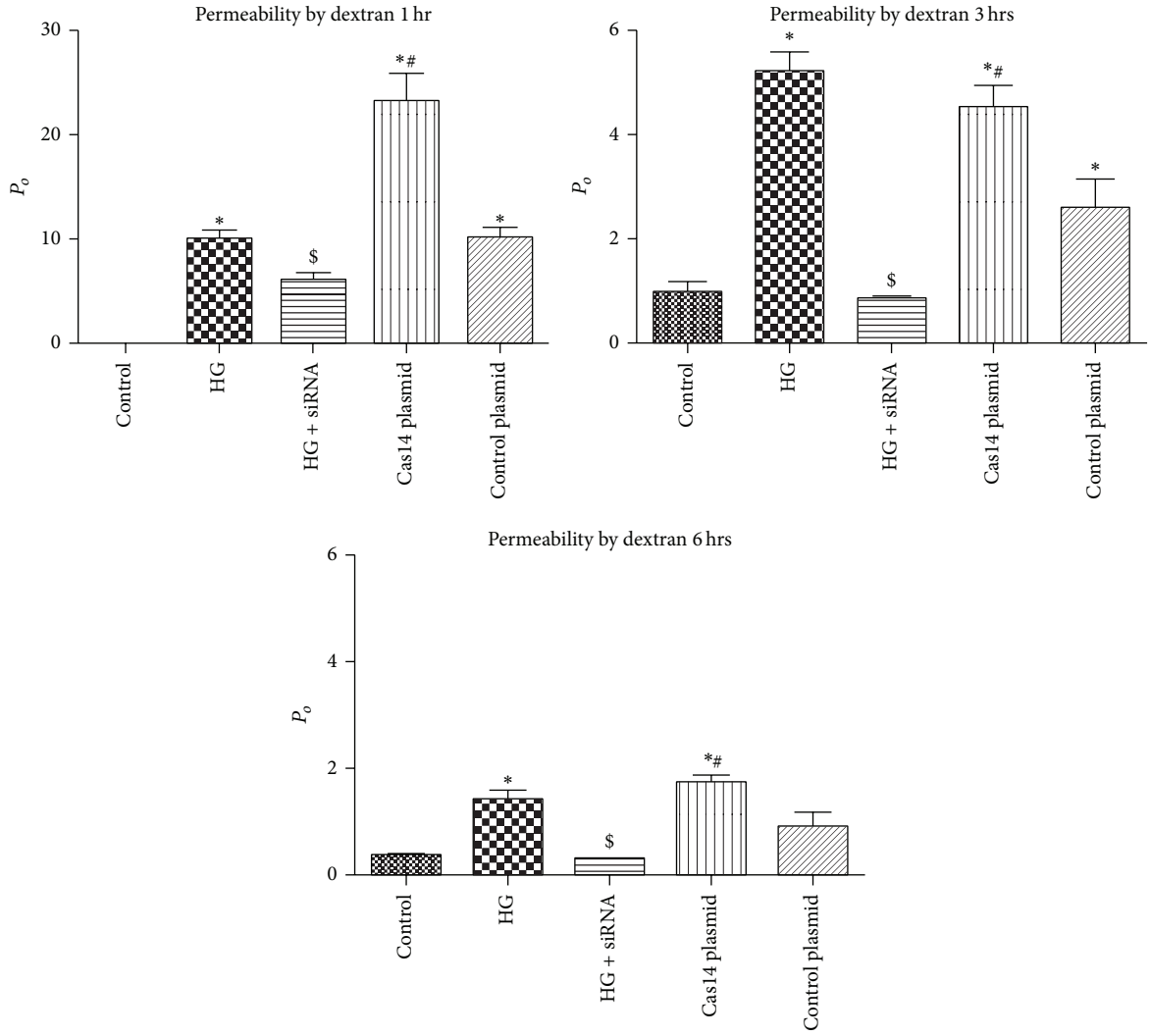

FIGURE 6: FITC-dextran flux assay. Permeability is defined by P_o (cm/s). There was a significant increase in permeability of cells cultured under high glucose (HG) conditions or transfected with caspase-14 plasmid at different time points (1, 3, and 6 h) compared with cells grown under normal glucose (NG) conditions or HG-treated cells transfected with caspase-14 siRNA ($^*P < 0.0001$). Caspase-14 siRNA is significantly lower than high glucose at 3 h and 6 h ($^\$P < 0.001$). Please note that caspase-14 plasmid has the same permeability effect as HG at 3 and 6 h ($n = 4$, $^*P < 0.05$ versus control and HG + caspase-14 siRNA, $^\#P < 0.05$ versus control plasmid).

These data suggest that caspase-14 is implicated in RPE barrier function and could be a potential molecular target to study the underlying mechanisms of retinal diseases associated with disruption of the outer retinal barrier such as DME. This led us to test the effects of caspase-14 knockdown in RPE cell's hyperglycemia-induced barrier dysfunction. Consistent with our hypothesis, knockdown of caspase-14 preserved RPE cell barrier function under HG conditions.

Caspase-14 is mainly involved in the epithelial differentiation, which shares some features with apoptosis including DNA fragmentation, nuclear condensation, and activation of caspase-3 [36]. The proteolytic process of procaspase-14 has been reported to increase in brain following reperfusion injury, and this was linked to increased number of neuronal cell deaths [25]. Our previous data showed that activation of caspase-14 under pathological conditions might influence retinal vascular function by promoting apoptosis of retinal microvascular cells [27]. RPE cells are believed to actively participate in progression of many inflammatory diseases including DR through caspase-mediated inflammatory and apoptotic pathway mechanisms [20, 31]. These include activation of various caspases including caspase-1, caspase-4, and caspase-3. Therefore, we determined how caspase-14 interacts with other caspases. For this purpose we examined the effect of caspase-14 knockdown on HG-mediated activation of other caspases in RPE cells, especially the known caspases which impact RPE cell function including caspase-1, -3, -4, -5, -8, and -9 [37, 38]. HG conditions significantly increased activities of caspase-1 and caspase-9 with no effect on other tested caspases. The effect of HG conditions on caspase-1 and

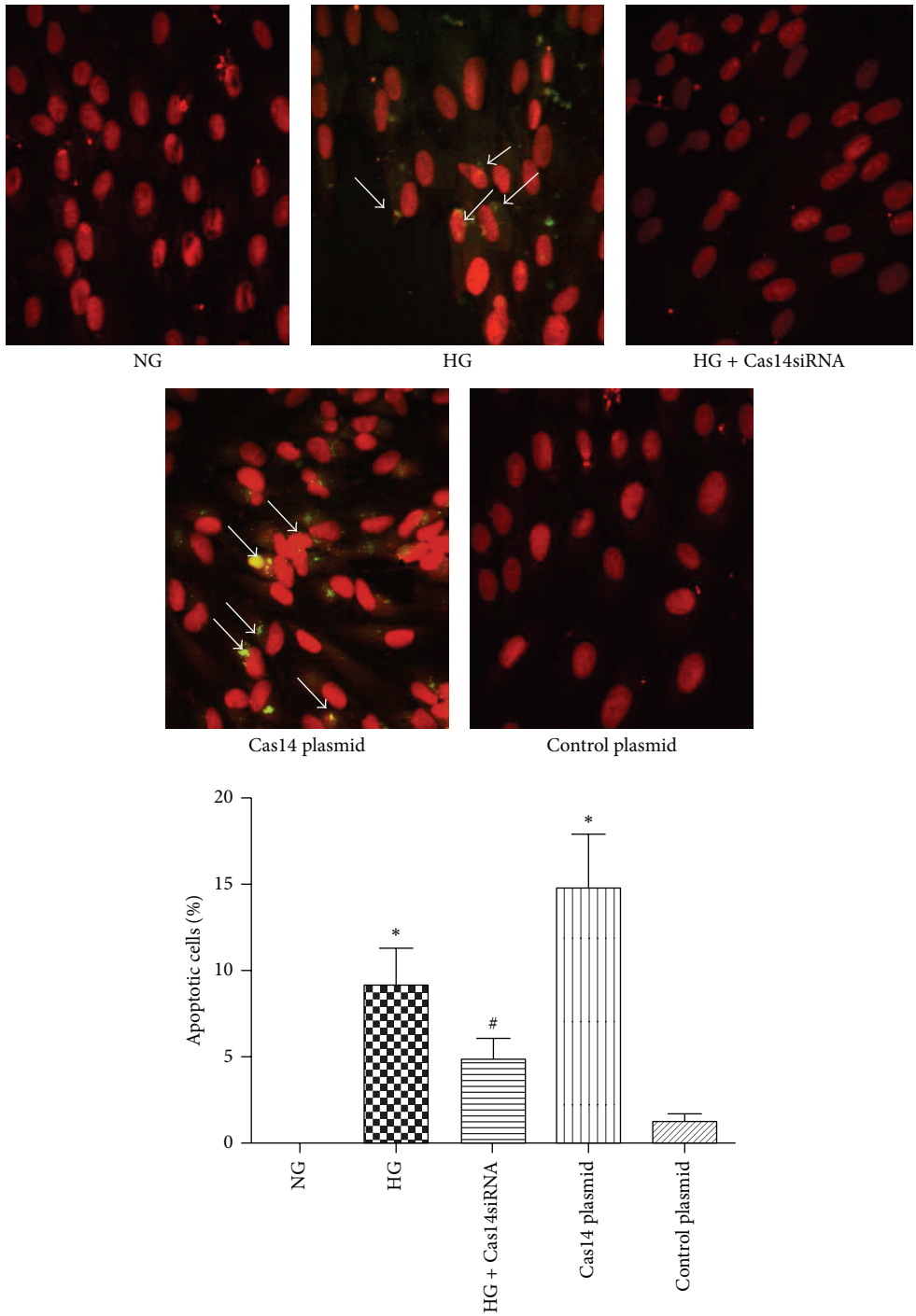

Figure 7: Increased apoptosis in RPE cells cultured under HG conditions or overexpressing caspase-14. Both high glucose treatment and caspase-14 transfected cells showed significantly increased levels of apoptosis compared with cells under NG or expressing control vector ($^{*}P < 0.001$). Caspase-14 knockdown by siRNA reduced the number of apoptotic RPE cells under HG conditions. However, the number of apoptotic cells was higher than the control ($^{\#}P < 0.05$ versus HG). Cell transfected with control plasmid had no significant difference in apoptosis compared with control ($n = 4$; $P > 0.05$).

caspase-9 activities was attenuated by caspase-14 knockdown. These results suggest that caspase-1 and caspase-9 are the primary caspases implicated in hyperglycemia-induced RPE dysfunction, and caspase-14 is upstream of caspase-1 and caspase-9 in these processes. Pyroptosis is a proinflammatory mode of cell death, whereas apoptosis is noninflammatory programmed cell death which occurs in RPE cells and mediated by the caspase-1 rather than apoptotic caspases such as caspase-3 [39–41]. On the other hand, caspase-9 is involved in the activation cascade of other caspases [42, 43] and apoptosis

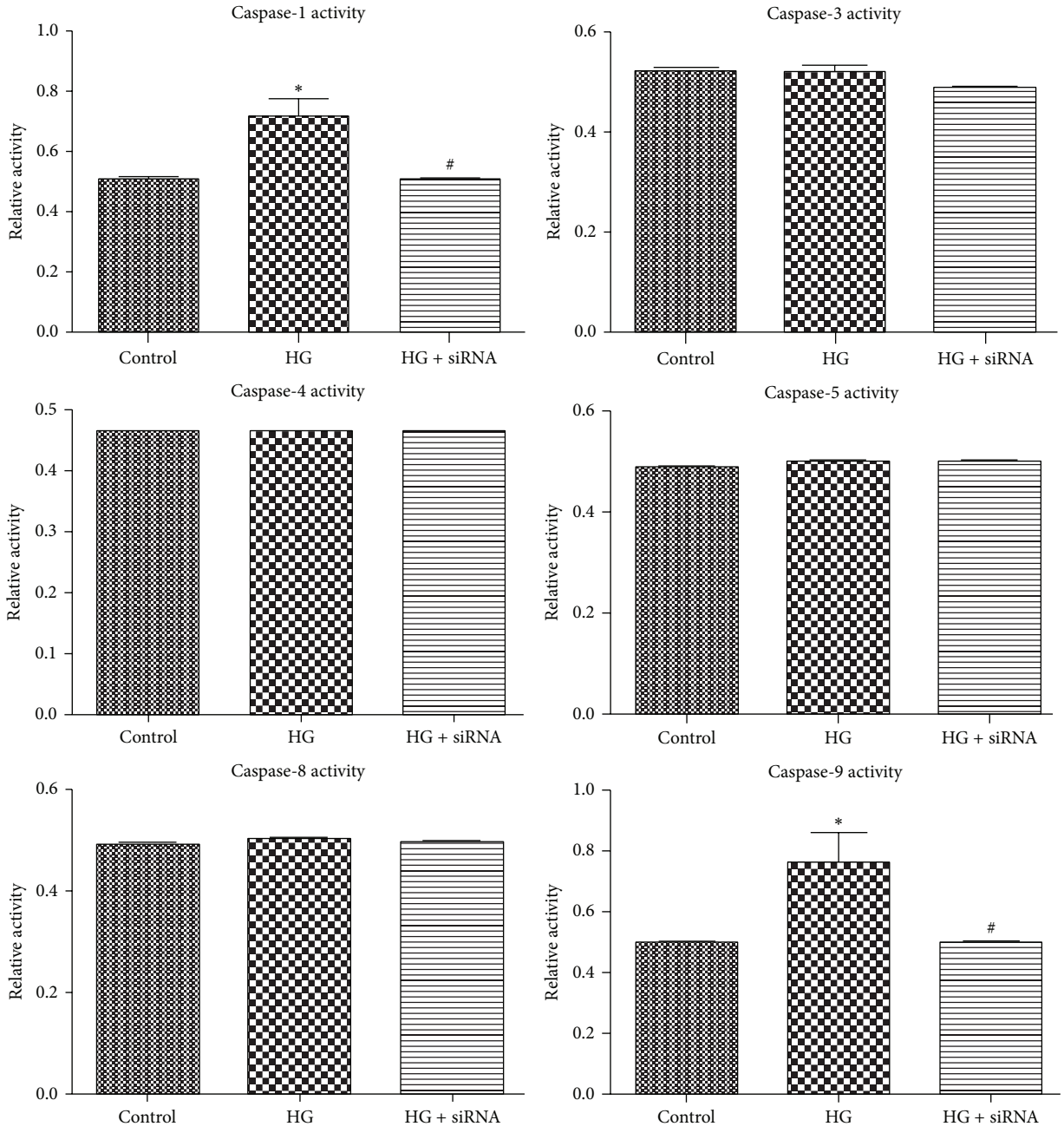

Figure 8: The effects of high glucose conditions on caspases activity. High glucose conditions increased the activity of caspase-1 and caspase-9 compared with NG. The siRNA knockdown of caspase-14 prevented the effect of HG conditions on activity of these caspases ($^{*}P < 0.05$ versus control and caspase-14 siRNA, $^{\#}P < 0.05$ versus high glucose). There were no significant changes in the levels of caspase-3, -4, -5, or -8 activity under the experimental conditions utilized here ($n > 3$; $^{*}P > 0.05$).

[44, 45]. Thus, our data implicate caspase-14 in hyperglycemia mediated RPE barrier dysfunction and apoptosis. This occurs in a caspase-3 independent manner and may involve activation of caspase-1 and caspase-9 dependent inflammatory and proapoptotic pathways, respectively (Figure 9).

To evaluate whether the function of caspase-14 is limited to the barrier function of the RPE cells, we also tested the impact of caspase-14 on their phagocytic activity. Interestingly, we noticed no changes in the phagocytic activity of RPE cells by caspase-14 expression. Collectively, our findings indicate caspase-14 as a potential player in retinal diseases associated with RPE barrier dysfunction such as DME. This process may constitute a novel mechanism for the pathogenesis of DME and, in turn, a novel therapeutic target to treat these pathological conditions. Further studies are required to identify the mechanisms of caspase-14 regulation and its coordinated interactions with other caspases and apoptotic proteins in retina.

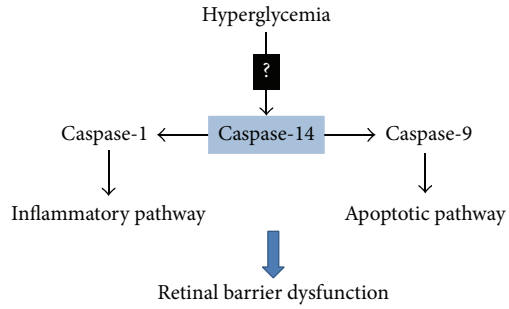

Figure 9: Schematic diagram demonstrates the proposed role of caspase-14 in the hyperglycemia-induced RPE barrier dysfunction and its potential role in DME. Hyperglycemia upregulates caspase-14 level/activity in the RPE cells modulating the activity of both caspase-1 and caspase-9 and promoting the proinflammatory and proapoptotic responses to hyperglycemia, respectively.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Molecular Mechanisms of Diabetic Retinopathy, General Preventive Strategies, and Novel Therapeutic Targets

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The growing number of people with diabetes worldwide suggests that diabetic retinopathy (DR) and diabetic macular edema (DME) will continue to be sight threatening factors. The pathogenesis of diabetic retinopathy is a widespread cause of visual impairment in the world and a range of hyperglycemia-linked pathways have been implicated in the initiation and progression of this condition. Despite understanding the polyol pathway flux, activation of protein kinase C (KPC) isoforms, increased hexosamine pathway flux, and increased advanced glycation end-product (AGE) formation, pathogenic mechanisms underlying diabetes induced vision loss are not fully understood. The purpose of this paper is to review molecular mechanisms that regulate cell survival and apoptosis of retinal cells and discuss new and exciting therapeutic targets with comparison to the old and inefficient preventive strategies. This review highlights the recent advancements in understanding hyperglycemia-induced biochemical and molecular alterations, systemic metabolic factors, and aberrant activation of signaling cascades that ultimately lead to activation of a number of transcription factors causing functional and structural damage to retinal cells. It also reviews the established interventions and emerging molecular targets to avert diabetic retinopathy and its associated risk factors.

1. Introduction

The number of people with diabetes worldwide was 382 million in 2013 and nearly 592 million people are estimated to be diabetic by 2035 [1]. Diabetes is one of the most common metabolic disorders, characterized by defective secretion of insulin. Immune mediated destruction of pancreatic b-cells leads to insulin deficiency and eventually to type I diabetes, while type II diabetes is characterized by insulin resistance and relative deficiency in insulin signaling [2]. Hyperglycemia is recognized as a major responsible factor for the development of diabetic complications. Diabetes involves many overlapping and interrelated pathways that results in potentially blinding complications like diabetic retinopathy and macular edema [3]. Diabetic retinopathy (DR) is the most widespread microvascular complication of diabetes and a major cause of vision loss worldwide. Globally, there are approximately 93 million people with DR, 17 million with proliferative DR, 21 million with diabetic macular edema, and 28 million with VTDR [4]. A new systematic review of 35 population-based studies has revealed that the prevalence of diabetic retinopathy, proliferative diabetic retinopathy (PDR), and diabetic macular edema (DME) among diabetic patients is 34.6%, 7.0%, and 6.8%, respectively [5, 6]. It is characterized by the increased development of distinct morphological abnormalities in the retinal microvasculature that either remains stable or progresses to diabetic macular edema or proliferative diabetic retinopathy, which are leading causes of severe visual impairment in working-age adults especially in industrialized countries [7]. The severity of diabetic retinopathy ranges from nonproliferative and preproliferative to more severely proliferative diabetic retinopathy, in which the abnormal growth of new vessels occurs [8]. A number of clinical trials on the prevention or treatment of diabetic retinopathy and diabetic macular edema (DME) are in progress (Table 1).

Multiple cellular pathways and potential molecular mechanisms have been proposed to explain diabetes induced complications. In diabetic retinopathy some of the most studied mechanisms are increased polyol pathway flux, increased

Table 1: Diabetic retinopathy: clinical trials [9].

Number	Title	Target sample size	Study type	Country
1	Continuous Positive Airway Pressure (CPAP) in Patients with Impaired Vision due to Diabetic Retinopathy and Concurrent Obstructive Sleep Apnoea (OSA): ROSA trial	150	Interventional	UK
2	Screening intervals for diabetic retinopathy	24,000	Observational	UK
3	Computer Detection of Diabetic Retinopathy Compared to Clinical Examination	600	Observational	USA
4	Computer-based Screening for Diabetic Retinopathy	10,000	Observational	USA
5	Prompt Panretinal Photocoagulation Versus Ranibizumab + Deferred Panretinal Photocoagulation for Proliferative Diabetic Retinopathy	316	Interventional	USA
6	Treatment for CI-DME in Eyes With Very Good VA Stud	702	Interventional	USA
7	The Role of Prostaglandins in the Progression of Diabetic Retinopathy	100	Interventional	USA
8	Comparison of Phase-variance Optical Coherence Tomography and Fluorescein Angiography in Retinovascular Imaging	78	Observational	USA
9	The Use of Alpha Lipoic Acid for the Treatment and Prevention of Diabetic Retinopathy	200	Interventional	USA
10	NSAID Phase II for Non-central Involved Diabetic Macular Edema (DME)	120	Interventional	USA
11	Rapid, Non-invasive, Regional Functional Imaging of the Retina. (Diabetic Retinopathy Diagnosis Device)	315	Observational	USA
12	A Safety and Efficacy Study of Vitreosolve for Non-Proliferative Diabetic Retinopathy Subjects	160	Interventional	India
13	Intravitreal Bevacizumab for Retinal Disorders	150	Interventional	India
14	A study to Find out Whether Vitrectomy Is Better Than Laser for Diabetic Patients with Partial Bleeding into the Vitreous Jelly	64	Interventional	India
15	Anterior and Posterior Segment Vascular Changes Following Laser and Anti-Vascular Endothelial Growth Factor (VEGF) Treatment of Diabetic Retinopathy	64	Interventional	Canada
16	Prospective Study Phase: Retinal Oxygen Saturation, Blood Flow, Vascular Function and High Resolution Morphometric Imaging in the Living Human Eye	381	Observational	Canada
17	A Comparison of Islet Cell Transplantation With Medical Therapy for the Treatment of Diabetic Eye Disease	40	Interventional	Canada
18	Prospective Study Phase: Retinal Oxygen Saturation, Blood Flow, Vascular Function and High Resolution Morphometric Imaging in the Living Human Eye	381	Observational	Canada
19	Safety and Efficacy of Low-Fluence PRP for PDR	60	Interventional	Mexico
20	Topic Antiinflammatory Therapy Added to Selective Photocoagulation in Macular Edema	84	Interventional	Mexico
21	Standard versus Intensive Statin Therapy for Hypercholesterolemic Patients with Diabetic Retinopathy	5,000	Interventional	Japan
22	Japan Public Health Center-based Prospective Study-NEXT on Glaucoma, Age-Related Macular Degeneration and Diabetic Retinopathy	4,000	Observational	Japan
23	Research on Age-Related Macular Degeneration and Diabetic Complications Using Non-invasive AGEs Measurement Equipment	500	Observational	Japan

Table 1: Continued.

Number	Title	Target sample size	Study type	Country
24	Choroidal Structure of Diabetic Retinopathy Eye on OCT Image After the Treatment	50	Interventional	Japan
25	Diabetes-Related Eye Disease Study	400	Observational	Japan
26	The Effect of NSAID for the Cystoid Macular Edema After Panretinal Photocoagulation in Diabetic Retinopathy	140	Interventional	Japan
27	To Investigate the Effects of Panretinal Photocoagulation by a Multicolor laser Photocoagulator with a Scan Delivery System in Eyes with Proliferative Diabetic Retinopathy	60	Interventional	Japan
28	A Pilot Study on the Effects of ILARIS on Patients With Proliferative Diabetic Retinopathy (PDRP)	10	Interventional	Switzerland
29	Panretinal Photocoagulation for Diabetic Retinopathy With PASCAL Laser	60	Interventional	Brazil
30	Bevacizumab as Adjunctive Treatment to Laser Panretinal Photocoagulation for Proliferative Diabetic Retinopathy	30	Interventional	Brazil
31	Thiazolidinedione (TZD) on the Diabetic Retinopathy and Nephropathy	200	Interventional	Taiwan
32	Genetic Association of Diabetic Retinopathy-1	200	Observational	Taiwan
33	Retinal Adaptation to Intensified Insulin Therapy and Bariatric Surgery in Patients With Diabetes	100	Observational	Denmark
34	Topical Application of Latanoprost in Diabetic Retinopathy	50	Interventional	Denmark
35	A Phase 2 Clinical Study to Investigate Effects of Darapladib in Subjects With Diabetic Macular Edema	54	Interventional	Australia
36	Trial of Switching Between Intravitreal Bevacizumab (Avastin) & Intravitreal Dexamethasone (Ozurdex) for Persistent Diabetic Macular Oedema	50	Interventional	Australia
37	Intravitreal Triamcinolone Acetonide for Diabetic Macular Edema	60	Interventional	Germany
38	Multicenter 12 Months Clinical Study to Evaluate Efficacy and Safety of Ranibizumab Alone or in Combination With Laser Photocoagulation vs. Laser Photocoagulation Alone in Proliferative Diabetic Retinopathy (PRIDE)	120	Interventional	Germany
39	Effects of Fenofibrate on Endothelial Progenitor Cells in Type 1 Diabetes	38	Interventional	Italy
40	Effect of Folic Acid, Vitamin B6 and Vitamin B12 in Diabetic Retinopathy	160	Interventional	Italy
41	Intravitreal Ozurdex After Pars Plana Vitrectomy for Proliferative Diabetic Retinopathy	100	Interventional	Sweden
42	Vitreous Analysis in Proliferative Diabetic Retinopathy	200	Observational	Sweden
43	Different Interventions Promoting Diabetic Retinopathy Screening Among Chinese Type 2 diabetes: A Randomized Trial	300	Interventional	China
44	Clinical Investigation on Early Lesions in Diabetic Retinopathy	500	Observational	China
45	Laser Photocoagulation in Patients with Diabetic Retinopathy Derived from New International Clinical Classification	180	Observational	China
46	Effect of Berberine on Diabetic Retinopathy	100	Interventional	China
47	Study of Evaluation on the Clinical Efficacy of Tradition Chinese Medicine in the Treatment of Non-Proliferative Diabetic Retinopathy	60	Interventional	China

Table 1: Continued.

Number	Title	Target sample size	Study type	Country
48	Clinical Study of Treatment with Kudiezi Injection in Nonproliferative Diabetic Retinopathy Patients	80	Interventional	China
49	Hemodynamics of Ocular Artery in Ischemic Ocular Diseases with TCD Study	180	Diagnostic test	China
50	Morphological and Functional Retinal Changes Following Retinal Photocoagulation	50	Interventional	Austria
51	Study Investigating the Levels and Effects of Low-grade Inflammation in Diabetic Retinopathy of Type 1 Diabetes	50	Observational	Austria
52	Choroidal Blood Flow Changes During Dark/Light Transitions in Patients With Insulin-Dependent Diabetes Mellitus (IDDM)	80	Interventional	Austria
53	Autologous Plasmin and Fibrinolytic System in Diabetic Retinopathy	40	Interventional	Korea, Republic of Korea
54	Incidence of Macular Edema After Panretinal Photocoagulation (PRPC) Performed in a Single Session Versus Four Sessions in Diabetic Patients.	90	Interventional	France
55	Trial of Yellow 577 nm Laser Versus Green 532 nm Laser for Proliferative Diabetic Retinopathy	120	Interventional	Malaysia
56	Preoperative Injection of Bevacizumab Prior to Vitreoretinal Surgery in Diabetic Tractional Retinal Detachment	50	Interventional	Iran, Republic of Iran
57	Effect of Intravitreal Bevacizumab on Early Post-Vitrectomy Hemorrhage in Diabetic Patients	80	Interventional	Iran, Republic of Iran
58	Intravitreal Adalimumab in Refractory Diabetic Retinopathy, Choroidal Neovascularization or Uveitis: A Pilot Study	15	Interventional	Lebanon

advanced glycation end-products (AGE) formation, abnormal activation of signaling cascades such as activation of protein kinase C (PKC) pathway, increased oxidative stress, increased hexosamine pathway flux, and peripheral nerve damage. All these pathways in one way or another end in increased oxidative stress, inflammation, and vascular occlusion, causing upregulation of factors such as insulin-like growth factor (IGF), stromal derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), angiopoietins (Ang-2), tumor necrosis factor (TNF), and basic fibroblast growth factor-2 (bFGF) that eventually contribute to the pathogenesis of diabetic retinopathy [10, 11].

A number of candidate genes have been identified which are directly or indirectly involved in diabetic retinopathy. Aldose reductase (ALR2), endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), receptor for advanced glycation end products (RAGE), paraoxonase1 (PON1), angiotensin converting, and plasminogen activator inhibitor1 (PAI) are some of the genes that are shown to be associated with diabetic retinopathy. Several polymorphisms at the regulatory regions of these genes have been characterized and evaluated as risk alleles for the susceptibility or progression of diabetic retinopathy in different populations of the world [12, 13]. Hypertension, hyperglycemia, and diabetes duration are the established risk factors of diabetic retinopathy. The diabetes control and complications trial (DCCT) conducted a study in 1993 to see whether intensive or conventional method is more efficient. According to their report the intensive treatment and improved glucose control delayed the onset of retinopathy and slowed down its progression in comparison to conventional method of treatment [14]. Intensive glucose and blood pressure control can lessen the progression of diabetic retinopathy but long-term management of these risk factors could be difficult to manage. Laser photocoagulation and Focal/grid photocoagulation have been shown to be effective in treating and reducing further vision loss [15]; however, these procedures are associated with potential complications, affecting visual field, color vision, and contrast sensitivity [16]. To avoid all these complications, new drugs and therapeutic targets must be identified which can disrupt the chain of events that lead to vision loss and weakening of the retina. In this paper we have tried to review molecular mechanisms regulating cell survival and apoptosis of retinal cells and discuss new and exciting therapeutic targets with comparison to the old and inefficient preventive strategies.

2. Molecular and Biochemical Mechanisms of Diabetic Retinopathy and Its Pathogenesis

A range of studies have described the biochemical mechanisms in the development and progression of diabetic retinopathy; however, no mechanism can be regarded as established. All forms of diabetes are characterized by

hyperglycemia, insulin resistance, relative or absolute lack of insulin action, and the development of diabetes specific pathology in the retina [17]. Diabetic retinopathy has been one of the major factors of vision impairment in the world. The basic hallmarks of this disease include loss of pericytes, basement membrane thickening, microaneurysms, neovascularization, and blood retinal barrier breakdown [18]. Molecular and biochemical mechanisms that have been implicated in diabetic retinopathy are increased flux of glucose through the polyol and hexosamine pathways, activation of protein kinase C, and increased advanced glycation end product formation [19] (Figures 1 and 2).

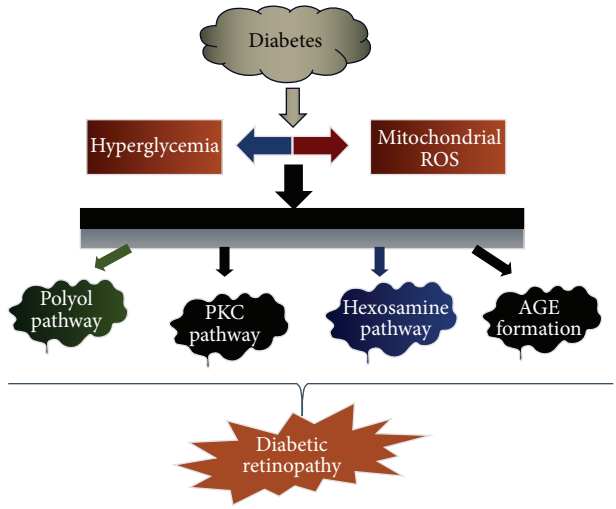

Figure 1: The four major mechanisms involved in DR are increased polyol pathway flux, increased AGE formation, activation of PKC, and polyol pathways.

2.1. Increased Polyol Pathway Flux. Detrimental effects of hyperglycaemia-induced increase in polyol pathway flux could be explained by a number of proposed mechanisms including decreased (Na^+&K^+) ATPase activity, sorbitol-induced osmotic stress, decrease in cytosolic NADPH, and increase in cytosolic NADH/NAD^+. The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol, which is then converted to fructose (Figure 3). Several biochemical and molecular studies implicate the polyol pathway as a reasonable and significant contributor to diabetic retinopathy and other complications of diabetes. Retinal endothelial cells of both rat and human showed aldose reductase immunoreactivity and human retinas exposed to high glucose in organ culture increased the production of sorbitol by a degree comparable to that observed in the rat. Such excess aldose reductase activity can be a mechanism for human diabetic retinopathy [20].

The polyol pathway of glucose metabolism becomes active when intracellular glucose levels are elevated. Aldose reductase (AR), the first and rate-limiting enzyme in the pathway, reduces glucose to sorbitol using NADPH as a cofactor; sorbitol is then metabolized to fructose by sorbitol dehydrogenase, which uses NAD^+ as a cofactor [21, 22]. Under euglycemic conditions, the higher affinity of hexokinase for the glucose substrate ensures the formation of sorbitol at a low level. However, in hyperglycemic conditions, there is a substantial increase in intracellular sorbitol levels. Aldose reductase has a high capacity and a low affinity for glucose, but sorbitol dehydrogenase (SDH) has a high affinity and a low capacity for sorbitol. Thus, glucose flux mediated by Aldose reductase is very low in this pathway except during hyperglycemia, and sorbitol oxidation is relatively independent of the sorbitol concentration within the physiological range [23, 24]. In diabetes, the sorbitol pathway increases in activity in tissues like retina, kidney, peripheral nerves, and blood vessels where insulin is not required for cellular glucose uptake. Sorbitol does not easily diffuse through cell membranes; as a result, it accumulates and causes osmotic damage [25].

The polyol pathway is by all criteria an extremely attractive target for the treatment of diabetic retinopathy; however, we cannot exclude the possibility of other mechanisms of polyol pathway-induced damage only in few particular types of retinal cells. For example, osmotic stress seemingly cannot be invoked from data in the whole retina because retinal levels of sorbitol increase in diabetic rats only 3–8-fold above control [26, 27]. Although animal data persuasively shows that aldose reductase has an early role in the pathogenesis of diabetic retinopathy, studies of inhibition of the polyol pathway in vivo have yielded inconsistent results. The long-term sorbinil trial [28] also indicated that sorbinil did not prevent the worsening of the disease except for a slower progression rate. The failure in clinical trials of therapeutic agents based on these putative pathogenic mechanisms may not rule out the mechanisms as important to the development or progression of diabetic retinopathy.

2.2. Accumulation of AGEs. It has become established that chronic exposure of the retina to hyperglycemia gives rise to accumulation of advanced glycation end products (Figure 2) that play an important role in retinopathy [29]. Advanced glycation end products (AGEs) are proteins or lipids that become nonenzymatically glycated and oxidized after exposure to aldose sugars [30]. Some of the best chemically characterized AGEs in human are carboxyethyllysine (CEL), carboxymethyllysine (CML), and pentosidine, which are shown to play a crucial role in the formation and accumulation of AGE in hyperglycemia. CML and other AGEs have been localized to retinal blood vessels of diabetes patients and were found to correlate with the degree of retinopathy suggesting the pathophysiological role of AGEs in diabetes [31]. Early glycation and oxidation processes result in the formation of Schiff bases and Amadori products. Further glycation of proteins and lipids causes molecular rearrangements that lead to the generation of AGEs [32]. These AGEs contribute to a variety of microvascular and macrovascular complications through the formation of cross-links between molecules in the basement membrane of the extracellular matrix and receptor for advanced glycation end products (RAGE) [33]. After cellular attachment, AGEs have been shown to increase procoagulant activity, vascular

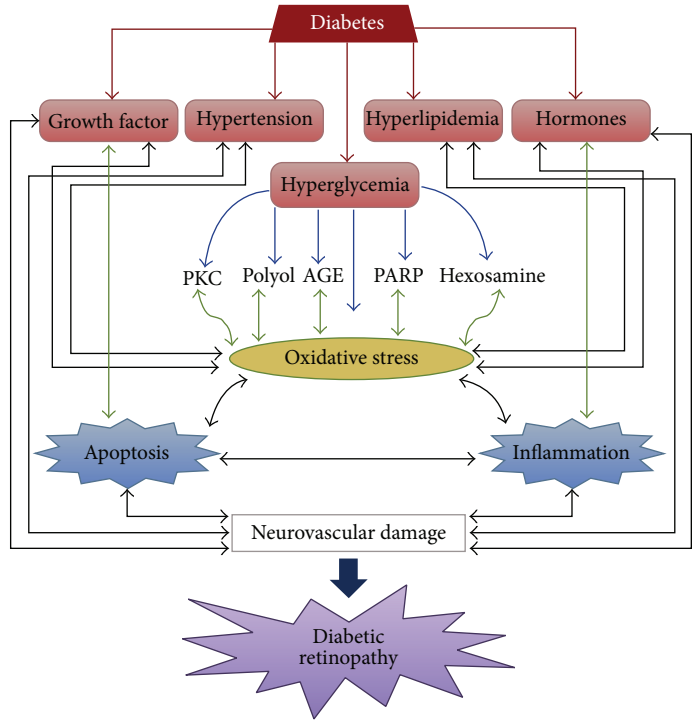

Figure 2: Hyperglycemia-induced biochemical alterations precipitated by mitochondria-driven oxidative stress leading to diabetic complications including apoptosis, inflammation, and ultimately diabetic retinopathy.

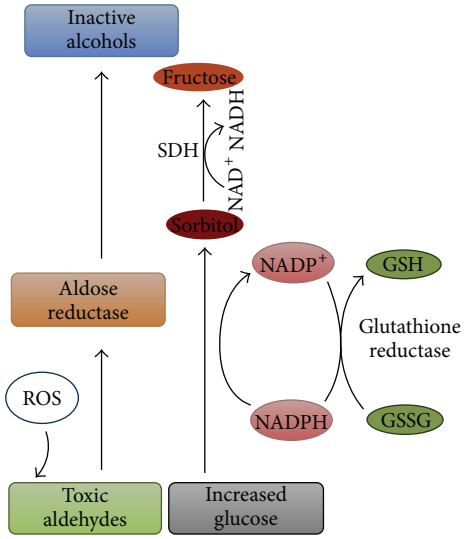

Figure 3: Aldose reductase and the polyol pathway. Aldose reductase reduces aldehydes generated by reactive oxygen species (ROS) to inactive alcohols, and glucose to sorbitol, using NADPH as a cofactor. Glutathione (GSH), glutathione disulfide (GSSG), and sorbitol dehydrogenase (SDH).

permeability, adhesion molecule expression, and monocyte influx actions that may contribute to vascular injury [34].

A number of studies have reported that oxidative stress is increased in diabetic patients and that it plays an important role in the pathogenesis of diabetic complications, including diabetic retinopathy [35]. It has been reported that overproduction of mitochondrial superoxide dismutase (SOD) [36] and inhibition of superoxide with antioxidants [37, 38] can protect against capillary degeneration during diabetic retinopathy in experimental diabetes, although how this influences AGE accumulation in the retina has not been studied. It is also possible that some chelators may shift the redox potential of iron or copper, affecting their catalytic activity in a way that could exacerbate oxidative stress and diabetes complications. The involvement of inflammatory processes in the initiation of neurovascular lesions during diabetic retinopathy has received recent attention. Global mRNA expression profiling has highlighted altered expression of proinflammatory cytokines and interrelated pathways, not only in the retinal vessels, but also in the neuroglia [39]. A study in which they investigated the correlation between age, metabolism, and AGEs found that older adults had higher serum concentrations than younger adults; however, the authors also reported that higher AGE concentrations were directly related to dietary intake across all age groups [35].

Increasing evidence suggests that AGE receptor binding can initiate important signaling pathways involving tyrosine phosphorylation of Janus kinase (JAK)/signal transducers and activators of transcription (STAT) [36], recruitment of phosphatidylinositol 3′ kinase to Ras [37], activation of protein kinase C [38], and oxidative stress through NFkB and AP-1 transcription [39]. AGEs interact with cells through several routes. AGE-modified serum proteins, such as CML, may interact with vascular endothelium via RAGE, which can activate nuclear factor kappa B (NF-κB), leading to enhanced expression of adhesion molecules and secretion

of cytokines such as tumor necrosis factor alpha (TNF-alpha) and VEGF. Similarly sedentary cells like endothelium encounter AGEs such as pentosidine-derived cross-links within basement membrane proteins where they may disrupt integrin signaling. These reactions, together with intra- and intermolecular cross-link formation, are able to modify structure and function of target molecules in such a way that they do not respond anymore to biological signals [40, 41]. AGEs also inhibit prostacyclin production and stimulate plasminogen activator inhibitor-1 (PAI-1) through an interaction with RAGE [42]. The molecular mechanisms of VEGF overexpression induced by AGEs are not fully understood; however, recent investigations have shown that the AGE-RAGE interaction might increase VEGF gene transcription by NADPH oxidase-mediated ROS generation and the subsequent nuclear factor-κB (NF-κB) activation via Ras-mitogen activated protein kinase (MAPK) pathway [43, 44]. In another recent study it has been shown that knocking down of integrin-linked kinase (ILK) gene expression with siRNA inhibited the elevation of VEGF and intercellular adhesion molecule 1 (ICAM-1). These results suggest that ILK has been involved in the response of cells to high glucose and may therefore play a role in the pathogenesis of diabetic retinopathy [45].

In a study when AGE-modified albumin was administered to nondiabetic rats for 4 weeks, it caused glomerular hypertrophy and increased extracellular matrix production in association with activation of the genes for collagen, laminin, and TGFb [46]. Other observations in diabetic animals are compatible with a pathogenetic role for AGEs in microvascular disease. Similarly AGEs alone are given to achieve plasma concentrations equivalent to those seen in diabetic animals [47]. After 5 months, the renal AGE content in AGE-treated rats was 50% above that in controls, while the plasma concentration was 2.8 times greater than that of controls. It is well established that AGEs are involved in the pathogenesis of diabetic complications. However, more studies are needed to elucidate the exact role of AGE in this area.

2.3. Increased Flux through the Hexosamine Pathway. Hexosamine content has been found to be increased in retinal tissues of humans and rats with diabetes [48]. Recent in vitro and in vivo studies have revealed that the increased flux of glucose via the hexosamine pathway has been implicated in insulin resistance, diabetic vascular complications (Figure 2), and stimulation of the synthesis of growth factors [49, 50]. In particular, it was demonstrated that hyperglycemia-induced production of transforming growth factor-b (TGF-b1), a prosclerotic cytokine, was causally involved in the development of diabetic nephropathy. In the hexosamine pathway, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT). N-Acetylglucosamine-6-phosphate is then converted to N-acetylglucosamine-1, 6-phosphate, and UDP-GlcNAc. UDP-GlcNAc is a substrate for O-linked glycosylation, which is catalyzed by O-GlcNAc transferase. UDP-GlcNAc, the major product, is the unique donor for the O-linkage of a single N-acetylglucosamine molecule (O-GlcNAc) to many cytoplasmic and nuclear proteins [51, 52]. Glucose is rapidly phosphorylated to glucose-6-phosphate after entering the cell which can then oxidize via glycolysis or the pentose phosphate shunt or stored as glycogen. Before the pathway proceeds, G6P is isomerized to fructose-6-phosphate (F6P) during glycolysis. Fructose-6-phosphate-amidotransferase (GFAT) catalyzes the formation of glucosamine-6-phosphate with glutamine as an amine donor and F6P as an acceptor substrate in the first and rate-limiting step of the pathway [53, 54].

Inhibition of the rate-limiting enzyme in the conversion of glucose to GFAT blocks hyperglycaemia-induced increases in the transcription of TGF-b1 [55] and plasminogen activator inhibitor-1 (PAI-1) [56]. Chen et al. observed that binding sites for the transcription factor Sp1 regulate hyperglycaemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells which suggests that covalent modification of Sp1 by N-acetylglucosamine (GlcNAc) might explain the link between activation of the hexosamine pathway and hyperglycaemia-induced changes in transcription of the gene for PAI-1 [57]. Thus, activation of the hexosamine pathway by hyperglycaemia results in many changes in both gene and protein levels, which together contribute to the pathogenesis of diabetic retinopathy. Another study [58] suggests that the increased glucose flux by the hexosamine pathway may direct retinal neurons to undergo apoptosis in a bimodal fashion, that is, via induction of apoptosis possibly by altered glycosylation of proteins and via perturbation of the neuroprotective effect of insulin mediated by Akt. This report emphasizes that hexosamine pathway may be involved in retinal neurodegeneration in diabetes. Clinically, the increased GFAT activity has been well interrelated with HbA1c levels in diabetic patients [59]. Distinct and high expression of GFAT was also demonstrated in diabetic nephropathy and other complications [52]. Despite a number of studies and data documentation saying that most of the tissues express GFAT, in eye specific tissues the data on GFAT is still lacking [51].

2.4. PKC Pathway. Increased vascular permeability and excessive neovascularization are the hallmarks of endothelial dysfunction, which can lead to diabetic macular edema and proliferative diabetic retinopathy. Many of the microvascular alterations in the eyes of patients with diabetes are thought to arise from hyperglycemia-induced activation of protein kinase C (PKC). PKC consists of a family of multifunctional serine/threonine kinases, which are involved in the control of other proteins. So far, at least 12 PKC isoforms have been identified and can be subdivided into three groups: classical, novel, and atypical. The activities of the classical isoforms (PKC-α, -β1/2, and PKC-δ) are greatly enhanced by DAG and have been linked to vascular dysfunctions and pathogenesis of DR [51]. Being important signaling transducers, PKCs are activated when second messengers bind to their regulatory domain, usually at the plasma membrane [60]. Similar vascular pathological conditions are observed in diabetic animals and those with diet-induced hypergalactosemia. Both diabetes and hypergalactosemia are believed to cause vascular

dysfunction via a common biochemical mechanism. Out of twelve PKC isoforms so far identified, nine are activated by the lipid second messenger DAG, and this implies that altered DAG-PKC pathway may have an important role in diabetic complications [61]. In cultured microvascular cells and in the retina and renal glomeruli of diabetic animals, intracellular hyperglycaemia increases the amount of DAG and increased de novo synthesis of DAG results in activation of PKC [62]. PKC activation can lead to mitogen-activated protein kinase (MAPK) activation and phosphorylation of several important transcription factors that increase gene expressions of various stress related genes like c-Jun kinases and heat shock proteins [63]. PKC-b has been shown to have a role in the form of a signaling component for VEGF and a regulator of endothelial cell permeability [64]. Furthermore, PKC activation contributes to the overexpression of plasminogen activator-1 (PAI-1), the activation of NADPH oxidase, and the activation of NFkB in many vascular cells including endothelial cells, smooth muscle cells, pericytes, mesangial cells, and others [65]. Thus, PKC activation involving several isoforms is likely to be responsible for some of the pathologies in diabetic retinopathy.

2.5. Relationship of These Pathways. All these mechanisms and events act in conjunction in several ways. Hyperglycemia activates polyol pathway, where a part of excess glucose is metabolized to sorbitol which is then changed to fructose. Aggregated sorbitol within retina may cause osmotic stress and also the byproducts of polyol pathway, fructose-3-phosphtae, and 3-deoxyglucosone are powerful glycosylating agents that result in the formation of AGEs [66]. Similarly AGE formation leads to activation of PKC pathway and poly (ADP-ribose) polymerase that may lead to initiation of inflammation and growth factor imbalances [67]. An increased flux through hexosamine pathway is associated with TGF-β expression, PKC activation, and ECM production, all of which are linked with the pathogenesis of diabetic retinopathy [68].

3. General Preventive Strategies

General strategies for the prevention of diabetic retinopathy should be aimed at identifying risk factors in the patient and counseling for the same. Patients should be encouraged to keep a close eye on their blood pressure, duration of the disease, hyperlipidemia, metabolic control, hypertension, and family history. For reducing and restoring the vision loss, scatter (panretinal) laser photocoagulation or vitrectomy surgery can also be employed.

3.1. Primary Prevention. The initial approach in diabetes management is lifestyle modifications. For instance, modifications before administrating medication, a healthy dietary pattern and physical activity program are the mainstay of diabetes treatment. In type diabetes, healthy eating habits, along with healthy weight, normal lipids level, and good control of blood glucose, are the basic goals to be considered. Regular exercise, healthy food choices, and weight loss are the basic lifestyle modifications in type 2 diabetes. The diabetes control and complications trial (DCCT) from 1983 to 1993 [14] established that intensive control of blood glucose levels for type 1 diabetes considerably reduces the risk of onset and progression of retinopathy and the need for laser surgery. It was demonstrated by reduction of glycated hemoglobin readings. Intensive control also had a beneficial effect in reducing the risk of kidney disease, neuropathy, and to a lesser degree, large vessel disease. Although intensive glucose and blood pressure control can reduce diabetic retinopathy (DR) progression, the long-term management of these risk factors over decades of diabetes duration can be difficult to maintain. Evidence from randomized controlled trials (RCTs) indicates that tight control of blood pressure is a major modifiable factor for the incidence and progression of DR. According to several observational studies, dyslipidemia also increases the risk of DR, particularly DME [69, 70] (Figure 4(a)).

3.2. Secondary Prevention. Current treatment modalities, precise clinical algorithms for diagnosis, management, follow-up, and understanding of diabetic retinopathy have largely reduced the risk of vision loss from both diabetic retinopathy and diabetic macular edema. Laser photocoagulation and vitrectomy have improved the quality of life for patients with diabetic retinopathy and prevented the visual loss [71]. In the 1970s and 1980s, randomized controlled clinical trials (RCTs) in diabetic retinopathy began to evaluate the effects of laser photocoagulation [72, 73]. The Early Treatment Diabetic Retinopathy Study (ETDRS) (1979–1990) also elucidated the natural history of diabetic retinopathy and provided new insights into the optimization of scatter laser photocoagulation for diabetic retinopathy [74]. These studies have helped in defining and fixing the natural history of diabetic retinopathy but at the same time highlighted the wide intersubject and intrasubject variability in retinal appearances and clinical progression. The Diabetic Retinopathy Study (DRS) (1971–1975) established the benefits of scatter (panretinal) laser photocoagulation for reducing the risk of vision loss from proliferative diabetic retinopathy [75]. From 1977 to 1987, the Diabetic Retinopathy Vitrectomy Study (DRVS) demonstrated the value of vitrectomy surgery for restoring useful and optimal timing for vitrectomy and for eyes with nonresolving vitreous hemorrhage or traction retinal detachment [76]. However, laser photocoagulation and vitrectomy are implicated only when DR has progressed to a measurably advanced stage in which some visual loss has already occurred. Because of these limitations of current management strategies, new pharmacological therapies are being developed, targeting the fundamental pathogenic mechanisms that initiate or sustain the progression of DR (Figure 4(a)).

4. Emerging Therapeutic Therapies

Early detection of retinal abnormalities is essential in preventing diabetic retinopathy (DR) and consequently loss of vision. Options for treating diabetic retinopathy are limited and display poor efficacy. So far the recommended

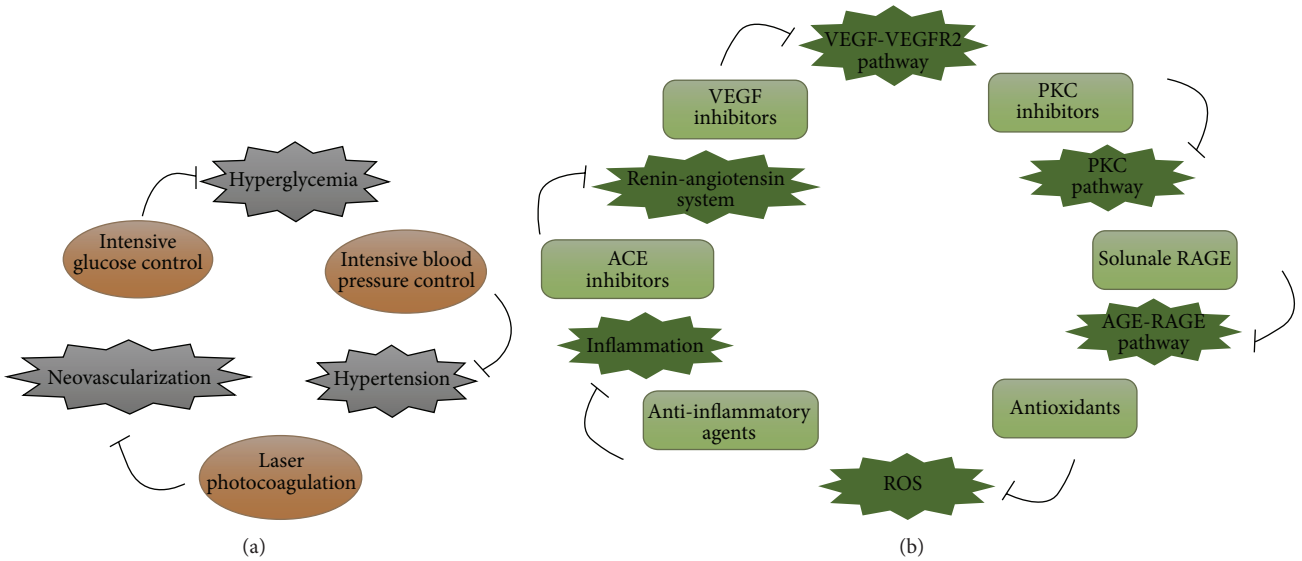

Figure 4: (a) The established preventive measures including general, primary, and secondary preventive strategies. (b) Novel and emerging therapeutic targets including PKC inhibitors, VEGF inhibitors, ACE inhibitors, and drugs as antioxidants.

treatment for DR is laser photocoagulation; however, this procedure also destroys the neural tissues [77]. Therefore, we need to establish clinical, biochemical, and molecular research methodologies to develop novel therapeutic strategies for diabetic retinopathy. Newer therapeutic mechanisms are under investigation including PKC pathway inhibitors, VEGF antagonists, AGE pathway inhibitors, and hormone antagonists. These new therapeutic options should result in better management and outcome of diabetic retinopathy (Figure 4(b)).

4.1. AGE Inhibitors and Prevention of Retinopathy. In hyperglycemic conditions, carbohydrates interact with protein side chains in a nonenzymatic way to form Amadori products, which consequently form AGEs. Excessive formation of AGEs is considering a biochemical link between diabetes and the development of diabetic retinopathy [78]. The chronic interaction of these products with AGE-specific receptors may perpetuate a proatherosclerotic state and proinflammatory signaling process in vascular tissues. Interaction of AGE-AGE-specific receptors has also been linked with the activation of nuclear factor-κB and oxidative stress, which leads to hyperexpression of lymphocyte adhesion molecules, proinflammatory cytokines, procoagulant factors, and vasoactive mediators [30]. Current treatments focus on preventing the formation of advanced glycosylation end products, breaking established crosslinks and reducing their receptor-ligand interactions.

A variety of different compounds that inhibit advanced glycation end products (AGEs) have been under investigation. Several approaches seeking to reduce AGE interactions, either by inhibiting AGE formation, blocking AGE action, or breaking preexisting AGE cross-links, have been explored. Recently Park et al. [30, 79, 82] have identified a novel inhibitor of the Wnt pathway that is a pigment epithelium-derived factor (PEDF), a multifunctional serine proteinase inhibitor. They have reported that overexpression of PEDF in transgenic mice as well as administration of PEDF protein attenuated Wnt signaling induced by retinal ischemia. In another recent study, Sheikpranbabu et al. investigated the PEDF and reported that PEDF abrogates AGE-induced oxidative stress and apoptosis in retinal pericytes by suppressing nicotinamide adenine dinucleotide phosphate-(NADPH-) oxidase mediated ROS generation and subsequently VEGF expression [83]. The work by Yamagishi and his group has shown that injection of AGEs to normal rats increases RAGE and ICAM-1 expression that induced retinal leukostasis and hyperpermeability; however, the process was blocked by simultaneous treatment with PEDF that completely inhibited superoxide generation and NFkB activation in AGE-exposed endothelial cells [81]. Combination of these studies suggests that PEDF can be an effective therapeutic agent for the treatment of diabetic retinopathy, by abrogating Wnt signaling and AGE-induced oxidative stress in retinal pericytes.

A pharmacologic strategy for AGE-inhibition commenced with the small nucleophilic hydrazine compound called aminoguanidine. This drug is a potent inhibitor of AGE-mediated cross-linking and has been shown to prevent diabetic vascular complications, including diabetic retinopathy, in experimental animals [82]. Blockade of the AGE/RAGE interaction by soluble RAGE has been shown to suppress nephropathy in diabetic animals [83]. sRAGE can prevent Muller cell dysfunction [84] during diabetes and retinal capillary leukostasis in AGE-infused normal mice. The clinical potential for reducing RAGE signaling in the diabetic retina is further underscored by development of new RAGE-regulating agents. One such agent is TTP488, which is an orally delivered small molecule, for which phase II studies have been completed [85].

Diabetes drugs, such as thiazolidinediones and nifedipine or (calcium channel blockers), have been investigated to reduce the expression of RAGE in endothelial cells and could serve to limit the proinflammatory effects of AGEs [86, 87]. Consequently the downregulation of RAGE and associated decrease in oxidative stress can act as PPARγ selective regulators, which suggests some cross-talk between the AGE-RAGE axis and PPARγ modulation. Therefore, a composite interaction between suppressed antioxidant status, enhanced levels of AGEs, and upregulation of the RAGE axis may together play a key role in the progression of diabetic retinopathy [88]. In diabetic animals, AGE inhibitors such as aminoguanidine can attenuate the formation of retinal microvascular lesion; however, recent studies indicate that this drug can significantly alleviate diabetic retinopathic lesions without affecting the formation of the AGE pentosidine in retinal basement membrane collagen [89]. This effect might be linked with aminoguanidine's antioxidative property rather than AGE inhibitory properties [90] as oxidative imbalances in diabetic retinopathy have also been extensively reported [91, 92]. Interestingly, some studies have reported no significant change of retinal vascular lesions after treatment with antioxidants [93]. Therefore, the exact role played by oxidative stress with advanced glycation reactions needs to be examined more intimately.

4.2. PKC Inhibitors as Potential Therapeutics. PKC isoform and selective inhibitors are likely new potential therapeutics, which can delay the onset or stop the progression of diabetic vascular diseases. First and second generation PKC inhibitors, for example, isoquinolinesulphonamides and staurosporine, were not even specific for PKC, but as the biochemical and functional profiles of individual PKC isoforms developed there has been renewed interest in the therapeutic opportunities for isoform selective blockade of PKC activation [94]. In 1996, the chemical characterization and in vivo pharmacological profile of orally active and highly selective PKC-β inhibitor, ruboxistaurin mesylate (LY333531), were reported in science [95]. The highly selective activation of PKCβ and its inhibition by ruboxistaurin mesylate have been studied extensively [96] and demonstrated through clinical studies in the prevention of vision loss [97]. In 2009, Geraldes et al. demonstrated that hyperglycemia persistently activated PKCδ and p38α MAPK to increase the expression of a novel target, SHP-1, leading to PDGF receptor-β dephosphorylation and actions, and increased pericyte apoptosis, independent of NF-κB [98]. These findings can be further demonstrated in patients with disease progression, as inhibition of this pathway attenuates the blood retinal barrier breakdown, which is the basis of diabetic retinopathy pathophysiology. Vitamin E is another type of PKC inhibitor: it can inhibit PKC activity probably by decreasing DAG contents via the activation of DAG kinase [99]. High dose vitamin E supplementation has been reported to normalize retinal blood flow in patients with type 1 diabetes [100].

VEGF is a potent proangiogenic factor in a very wide range of pathological conditions. Activation of PKCβ seems to be an essential step in VEGF mediated endothelial cell migration and replication. Inhibition by ruboxistaurin reduces the mitogenic response to VEGF, in contrast with PKCα inhibition [101]. Aiello et al. focused on vascular effects of PKC, while studies in rats confirmed a reduction of VEGF-induced permeability by ruboxistaurin [102]. A specific mechanism for reduction in vascular permeability is suggested by Harhaj et al. [103]. It demonstrated that VEGF-induced tight junction protein phosphorylation, tight junction disassembly, and endothelial cell permeability are mediated by PKC. In a study it was concluded that puerarin exerts significant protective effects against DR in rats, likely regulating angiogenesis factors expressions, and thus may be an effective and promising medicine for treatment of DR [104].

In the presence of diacylglycerol, both novel and conventional PKC isoforms translocate to the membranes of the cells to start crucial biological events. Being an essential enzyme, inhibition of all PKC isoforms with general approaches will originate severe consequences that can put the survival of animals in danger. Therefore, proper selectivity for inhibition is very crucial in the development of a clinically constructive therapeutic PKC inhibitor [105]. An earlier study, using a nonselective PKC inhibiter (PKC412), reported severe toxicity that excludes its clinical applications in diabetic patients [106]. However, such toxic effects are not surprising because PKC activation is indispensable for a range of functions in the heart and kidneys. Therefore, success with PKCβ inhibitor therapy for established and relatively severe diabetic retinopathy would also raise questions of its adverse effects. Tuttle et al. have also demonstrated in their study that PKC isoform selective inhibiter can be used for chronic clinical treatment with nominal side effects [107]. Ruboxistaurin is an orally active β-specific PKC inhibitor and appears to be well tolerated in large phase II and phase III clinical trials of intermediate duration. Despite the encouraging results from animal models, the therapeutic approaches of such drugs for the treatment of diabetic retinopathy raise both hopes and challenges. In the next few years, we are cautiously optimistic, but clearly, more large studies are needed to establish its efficacy for treatment of diabetic retinopathy and other vascular complications in diabetic patients.

4.3. Inhibition of Increased Polyol Pathway. The polyol pathway is comprised of two enzymes. Aldose reductase (AR) reduces glucose to sorbitol with the aid of its cofactor NADPH, and sorbitol dehydrogenase (SDH), with its cofactor NAD^+, converts sorbitol to fructose. In animal models, treatment with AR inhibitors (ARI) was shown to be effective in preventing the development of various diabetic complications, including diabetic retinopathy [108]. Although the exact mechanism is unknown, AR appears to be the possible link between increased polyol pathway activity and the development of some diabetic complications. Therefore, based on the polyol pathway, preventive and therapeutic approaches are used to develop potent inhibiters for diabetic complications [109]. Only new drugs that inhibit aldose reductase with higher efficacy and safety than older drugs will make possible to learn if the resilience of the polyol pathway

means that it has a role in human diabetic retinopathy that should not have gone undiscovered. The rate-limiting enzyme of the pathway, aldose reductase, acts on the glucose molecule at the most upstream possible site in the cascade.

Studies on animal models suggest that AR inhibitor, fidarestat, is active in the treatment of diabetic retinopathy. The use of fidarestat, an inhibitor of aldose reductase neutralizes diabetes-associated retinal oxidative stress and poly (ADP-ribose) polymerase formation [110]. This shows an important role for aldose reductase in diabetes and rationale for the development of aldose reductase inhibitors for counteraction of polyol pathway [111]. Similar results were obtained in the rat model with retinal ischemia-reperfusion injury. Fidarestat treatment caused increased cell death and elevated AR expression, coupled with the prevention or alleviation of sorbitol pathway intermediate accumulation [112]. Also in the streptozotocin-diabetic rats, fidarestat treatment significantly decreased concentrations of sorbitol and fructose in the rat retinas. The expression of ICAM-1 mRNA and leukocyte accumulation in the retinas were significantly reduced. Immunohistochemical study also revealed the suppressive effect of fidarestat on the expression of ICAM-1 [113].

In 2009 Drel et al. demonstrated an increase in PARP activity in streptozotocin-induced diabetic rats and PARP inhibitors reduced retinal oxidative-nitrosative stress, glial activation, and cell death in palmitate exposed pericytes and endothelial cells [114]. A double-blind study in patients with diabetic neuropathy by Sima et al. [115] gave exciting evidence of the efficacy of sorbinil, an aldose reductase inhibitor, against morphological signs of degeneration accompanied by a decrease in the nerve sorbitol level and an increase in the nerve conduction velocity. A similar observation was reported by Greene et al. [116] using another aldose reductase inhibitor, FK-366. In addition, the study using Zucker diabetic fatty rats, an animal model of type 2 diabetes, showed that the administration of a combination of four plant extracts inhibited the development of diabetic cataract through the inhibition of AR activity and protein expression in diabetic lenses [117]. However, the retinal microangiopathy developed by dogs fed a 30% galactose diet was just delayed or not prevented at all [118] by the AR inhibitor sorbinil. One group of researchers revealed that sorbinil was metabolized more abruptly in dogs as compared to rats, yielding unpredictably a shorter plasma half-life [119]. Results of the sorbinil retinopathy trial indicated that sorbinil had no clinically important effect on the course of human diabetic retinopathy [28]. Such negative results dampened the enthusiasm in pursuing the polyol pathway as a major player and target in diabetic retinopathy; however, the positive effect of aldose reductase inhibition on diabetic neuropathy with zenarestat [120] provides vested hopes in the use of these compounds in diabetic retinopathy which needs to be tested and validated by future studies.

4.4. Inhibition of ROS, Antioxidants, and Hexosamine Pathway as Emerging Therapeutics. Glutamine, fructose-6-phosphate amidotransferase, is the rate-limiting step of the hexosamine biosynthesis pathway, which activates as an alternative pathway to glycolysis for the utilization of hyperglycemia-induced overproduction of fructose-6-phosphate, resulting in excess of N-acetylglucosamine and irregular alteration of gene expression of plasminogen activator inhibitor-1 and TGF-β. This overexpression causes a spectrum of adverse metabolic diabetic derangements and endothelial cell and retinal neuron apoptosis [56, 121, 122]. The use of appropriate compounds has been described that can potentially alleviate the metabolic and functional abnormalities in diabetic retinopathy. WAS-406 (2-acetamido-1,3,6-tri-O-acetyl-2,4-dideoxy-α-D-xylo-hexopyranose) and Azaserine reduce cardiovascular effects caused by hyperglycemia as antioxidants rather than by inhibiting only the hexosamine pathway [123, 124]. Rhein, an anthraquinone compound isolated from rhubarb, decreases hexosamine pathway and is helpful in treatment of experimental diabetic nephropathy [125]. Benfotiamine, which converts fructose-6 phosphate into pentose-5 phosphates, is another compound that reduces flux through the hexosamine pathway [126]. The ability of benfotiamine, a lipid soluble thiamine, to inhibit simultaneously the hexosamine pathway along with AGE formation and PKC pathways might be clinically useful in preventing the development and progression of diabetic pathogenesis arising due to hyperglycemia-induced vascular damage. In a study, Hammes et al. have shown that benfotiamine can also inhibit hyperglycemia-associated NF-kappaB activation by activating the pentose phosphate pathway enzyme transketolase, which converts glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphates and other sugars [126].

There are several lines of evidence to suggest that antioxidant defenses may be lower in diabetes. These include reports of reduced plasma/serum total antioxidant status or free radical scavenging activity and increased plasma oxidisability in type 2 diabetics, together with reduced levels of specific antioxidants such as ascorbic acid and vitamin E [127]. Lipoic acid is an antioxidant capable of thiol-disulfide exchange. It is able to scavenge ROS and reduce metabolites such as glutathione to maintain a healthy cellular redox state [128]. This antioxidant attenuates the apoptosis of rat retinal capillary cells and decreases the levels of 8-OHdG and nitrotyrosine. Lipoic acid supplementation completely prevents diabetes-induced increase in nitrotyrosine and activation of NF-κB while decreasing the levels of VEGF and oxidatively modified proteins in the rat retina [129]. Apart from lipoic acids other experiments have also been tried in animal models, such as vitamin C and vitamin E. All of them have shown improved biological and pathological changes and prevented or slowed the progression of diabetic complications [130]. The potential benefit of vitamin E has been shown in DR by its free radical scavenger activity outside the cell through nonenzymatic mechanisms [100]. Trolox is a water soluble analog of vitamin E with potent antioxidant properties. Trolox is shown to partially prevent the loss of pericytes in diabetic rats via reducing membrane lipid peroxidation [131]. Another antioxidant, calcium dobesilate, decreased retinal permeability, stabilized BRB, and reduced overexpression of VEGF in diabetic rats [132]. Carotenoids are some of the powerful antioxidants, and diabetes

decreases lutein and zeaxanthin levels in the serum and retina. Kowluru et al. investigated the effect of carotenoid containing nutritional supplements which prevented diabetic retinopathy and also maintained normal retinal function, mitochondrial homeostasis, and inflammatory mediators [133].

In bovine endothelial cells, hyperglycemia induced a significant increase in the hexosamine pathway which was blocked by an inhibitor of electron transport, a mitochondrial uncoupling agent (CCCP), and the expression of either UCP1 or SOD2 [126]. A second new class of mechanism-based potential therapeutic agents is PARP inhibitors. In cultured arterial endothelial cells, a specific PARP inhibitor prevents hyperglycemia-induced activation of PKC, NF-κB, intracellular AGE formation, and the hexosamine pathway. In animal models of diabetes, PARP inhibition prevents arterial endothelial cell injury and podocyte apoptosis, ameliorates nephropathy, and alleviates sensory neuropathy [60].

4.5. Cannabidiol as an Emerging and Novel Therapeutic Modality. Diabetic retinopathy is characterized by the breakdown and neurotoxicity of blood-retinal barrier (BRB) which have been associated with oxidative stress and proinflammatory cytokines that may operate by activating their downstream target p38 MAP kinase. Cannabinoids are known to possess therapeutic properties including NMDA receptor-activation [134], inhibition of oxidation [135], and inflammation (Buckley NE). (−)-Δ9-tetrahydrocannabinol (THC) and (−)-cannabidiol (CBD) are the marijuana-derived cannabinoids which possess antioxidative and immunosuppressive effects [136]. It has already been established that nonpsychotropic CBD causes a decrease in interleukin-1, TNF-α, and interferon-γ in murine collagen-induced arthritis and prevents central nervous system neuronal damage in gerbils [137]. El-Remessy et al. demonstrated the neuroprotective role of both THC and CBD through antioxidant action in NMDA-induced retinal neurotoxicity in rats [138]. They also demonstrated the BRB-preserving effects of blocking oxidative stress in diabetic rats. These studies emphasize the need for rational and conceptual considerations on the mode of action of CBD in the treatment of diabetic retinopathy, which can serve as a potential biomarker and novel therapeutic agent for diabetic retinopathy.

4.6. Role of the Renin-Angiotensin System, Its Inhibitors, and Use of Fenofibrate in Diabetic Retinopathy. The renin-angiotensin system (RAS) has an important role in regulation of electrolyte balance, vasoconstriction, and vascular remodeling. Local renin-angiotensin regulation is present in the eye [139]. The role of the RAS in diabetic retinopathy has not been as well studied as that in the kidney and nephropathy; however, several studies including the Renin-Angiotensin System Study (RASS), Daily-Dose Consensus Interferon and Ribavirin: Efficacy of Combined Therapy (DIRECT) trial, and the ADVANCE Retinal Measurements (AdRem) have provided evidence that RAS inhibition may also be beneficial in diabetic retinopathy [140, 141]. Studies have reported that ACE inhibitor improves endothelial function and stimulates vascular remodeling, in addition to attenuating progression of arteriosclerosis and the occurrence of cardiovascular events in humans [142, 143]. EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus (EUCLID) study group reported a reduction in proliferative diabetic retinopathy via ACE inhibition, providing a potential clinical role for suppression of the renin-angiotensin system (RAS) in preventing and treating retinal neovascularization [144]. Satofuka et al. investigated receptor-associated prorenin system (RAPS) which dually activates the tissue renin-angiotensin system (RAS) and RAS-independent intracellular signaling. Their results showed a significant contribution of the RAPS to the pathogenesis of diabetes-induced retinal inflammation, suggesting the possibility of the (pro) renin receptor as a novel molecular target for the treatment of diabetic retinopathy [145]. From these studies we conclude that RAS blockade has an additional significant impact on slowing or stopping diabetic nephropathy and a more modest but still clinically relevant impact on diabetic retinopathy.

Fenofibrate is a peroxisome proliferactor-activated receptor- (PPAR-) α agonist that is used to treat high triglycerides and low HDL or as adjunct to statin therapy. It regulates the expression of many genes that work against lipids, inflammation, angiogenesis, and cell apoptosis. Diabetic patients not only need to maximize glycemic control, but also to closely monitor and treat other systemic conditions including diabetic retinopathy. Studies have shown consistent beneficial effects with fenofibrate in slowing the progression of DR [146]. Fenofibrate treatment among patients with type 2 diabetes reduced the need for laser treatment for diabetic eye disease. The mechanism by which this happens remains unclear; however, it does not appear to be related to plasma lipid concentrations. The ACCORD Eye Study group involved a subset of 2,856 participants and analyzed the effects of the treatment strategies on blood vessels in the eye by identifying diabetic retinopathy progression over four years. According to their report, rates of progression of diabetic retinopathy were significantly reduced in the intensive glycaemic control group and in the fenofibrate group, but not in the intensive blood pressure control group [147]. Keech et al. in FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study have reported that fenofibrate could reduce the need for laser treatment in a large cohort of 9,795 type 2 diabetic patients. Fenofibrate reduced the frequency of laser treatment for macular edema by 31% and for proliferative retinopathy by 30%. In addition, in a substudy performed on patients in whom retinal status was graded by fundus photography, fenofibrate was able to reduce the progression of existing retinopathy [148]. Although this study has some limiting factors, the substantial benefits obtained from reducing the need for laser treatment argue for consideration of using fenofibrate in the management of diabetic retinopathy [149]. The FIELD findings are clearly important therapeutically, but the trial's lasting contribution might be to provoke further research into underlying mechanisms of action of fenofibrate to improve endothelial function and reducing local inflammatory processes which could lead to new treatments of diabetic retinopathy.

5. Conclusion

The intent of this review is to provide better understanding of the complex molecular mechanisms and treatment modalities. At primary stage patients should be encouraged to keep a close eye on their blood pressure, duration of the disease, hyperlipidemia, metabolic control, and hypertension. To reduce and restore the vision loss, scatter laser photocoagulation or vitrectomy surgery can be employed. At the proliferative stages of the disease, the therapeutic interventions are effective in reducing visual loss; however, once DR develops, additional mechanisms, including hypoxia-induced VEGF production, contribute to retinal disease progression. Vascular endothelial growth factor is the most well-studied component of the mechanisms involved in the diabetic retinopathy and anti-VEGF therapies. Systemic therapeutic interventions targeting VEGF would be intravitreally administered so as to avoid the development of impaired angiogenesis in other organs. Hyperglycaemia induced de novo synthesis of DAG in vascular cells leads to selective activation of PKC isozymes, especially PKC-b, which in turn phosphorylate proteins involved in endothelial function and neovascularisation. These changes activate intracellular signaling proteins such as PKC, PKB, AGE, and MAPK which are finally culminating in pathological induction of transcription factors such as NF-κB and AP-1. After reviewing these as well as data in the literature, it is evident that each of the four main mechanisms implicated in the pathogenesis of diabetic complications reflects a single hyperglycaemia-induced process and pathogenetic mechanisms involved in diabetic retinopathy are interactive and interdependent. Therefore, within the near future pharmacologic treatment strategies may include multiple pharmacological agents, targeted simultaneously to block multiple pathways. This will probably be available for treating and preventing the progression of DR after further potential clinical trials.

Conflict of Interests

There is no conflict of interests among the contributing authors.

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