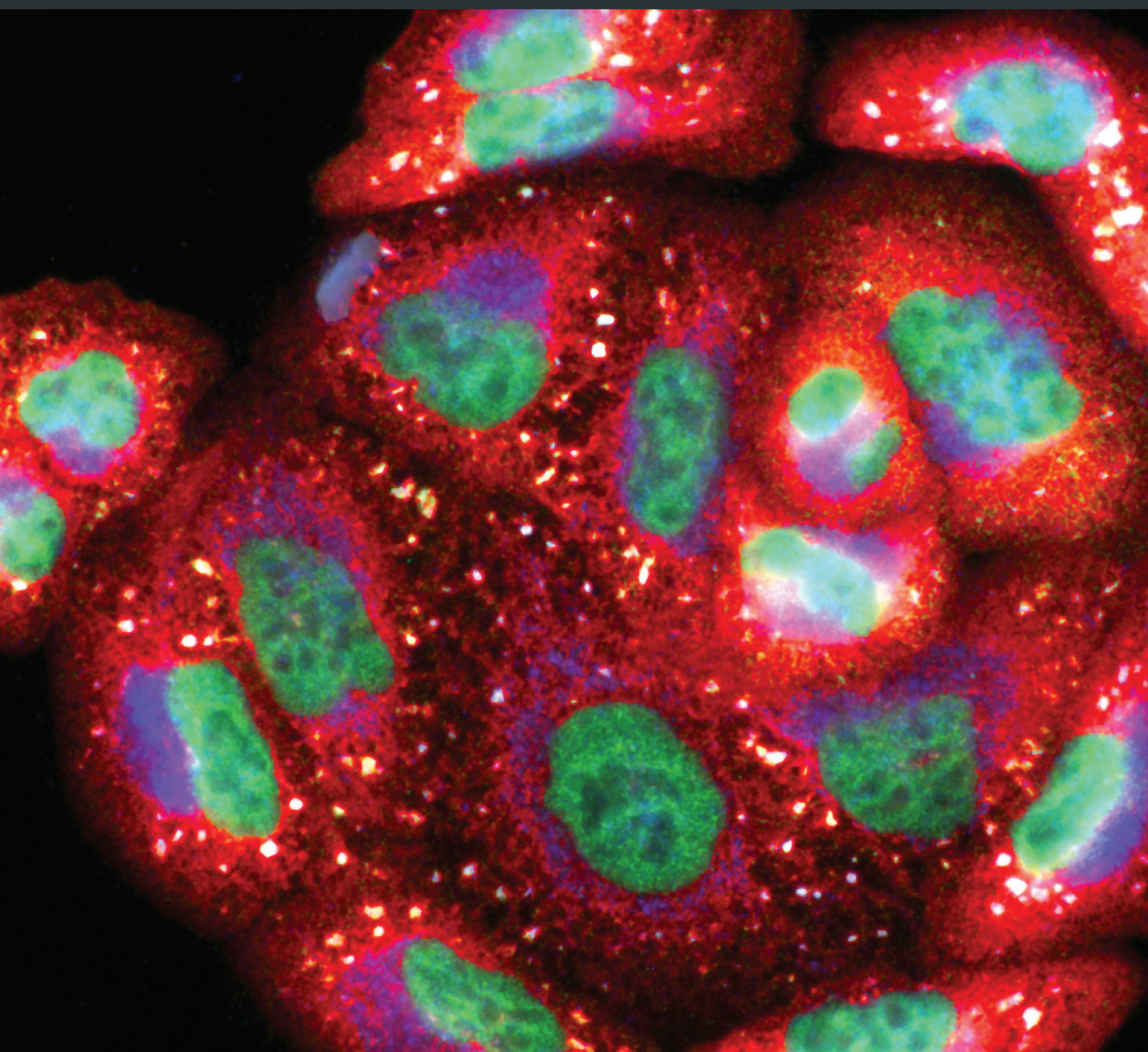
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Retinal Diseases

Guest Editors: Yuhei Nishimura, Hideaki Hara, Mineo Kondo, Samin Hong, and Takeshi Matsugi



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Editorial Board

Contents

Oxidative Stress in Retinal Diseases
Yuhei Nishimura, Hideaki Hara, Mineo Kondo, Samin Hong, and Takeshi Matsugi
Volume 2017, Article ID 4076518, 2 pages

Spermidine Oxidation-Mediated Degeneration of Retinal Pigment Epithelium in Rats
Koji Ohashi, Masaaki Kageyama, Katsuhiko Shinomiya, Yukie Fujita-Koyama, Shin-ichiro Hirai, Osamu Katsuta, and Masatsugu Nakamura
Volume 2017, Article ID 4128061, 13 pages

Targeting Oxidative Stress for Treatment of Glaucoma and Optic Neuritis
Atsuko Kimura, Kazuhiko Namekata, Xiaoli Guo, Takahiko Noro, Chikako Harada, and Takayuki Harada
Volume 2017, Article ID 2817252, 8 pages

Oxidative Stress-Related Mechanisms and Antioxidant Therapy in Diabetic Retinopathy
Cheng Li, Xiao Miao, Fengsheng Li, Shudong Wang, Quan Liu, Yonggang Wang, and Jian Sun
Volume 2017, Article ID 9702820, 15 pages

Nutritional Supplementation Inhibits the Increase in Serum Malondialdehyde in Patients with Wet Age-Related Macular Degeneration
Toshiyuki Matsuura, Kei Takayama, Hiroki Kaneko, Fuxiang Ye, Hiroshi Fukukita, Taichi Tsunekawa, Keiko Kataoka, Shiang-Jyi Hwang, Yosuke Nagasaka, Yasuki Ito, and Hiroko Terasaki
Volume 2017, Article ID 9548767, 9 pages

Sirtuins Expression and Their Role in Retinal Diseases
Sankarathi Balaiya, Khaled K. Abu-Amero, Altaf A. Kondkar, and Kakarla V. Chalam
Volume 2017, Article ID 3187594, 11 pages

Retinal Diseases Associated with Oxidative Stress and the Effects of a Free Radical Scavenger (Edaravone)
Tomomi Masuda, Masamitsu Shimazawa, and Hideaki Hara
Volume 2017, Article ID 9208489, 14 pages

Integrated Approaches to Drug Discovery for Oxidative Stress-Related Retinal Diseases
Yuhei Nishimura and Hideaki Hara
Volume 2016, Article ID 2370252, 9 pages

Nrf2 Is an Attractive Therapeutic Target for Retinal Diseases
Yasuhiro Nakagami
Volume 2016, Article ID 7469326, 8 pages

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 4076518, 2 pages
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Editorial

Oxidative Stress in Retinal Diseases

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The retina is exposed to chronic oxidative stress (OS) through several mechanisms, including constant exposure to light and reactive oxygen species, which are generated by visual signal transduction pathways triggered by high oxygen consumption, oxidization of polyunsaturated fatty acids, and phagocytosis of photoreceptor cells. In the healthy state, all cell types in the retina are able to maintain homeostasis under conditions of OS. However, when the balance between pro- and antioxidative signaling is compromised; excessive OS induces dysregulation of functional networks and deleterious changes that result in visual impairment. Age-related macular degeneration (AMD), diabetic retinopathy (DR), and glaucoma are leading causes of visual impairment. Owing to a combination of lifestyle changes, such as increased consumption of a high-fat diet and decreased physical activity, and extended life expectancy, an increasing number of people are at risk for retinal diseases, and the resulting economic burden imposed on health care systems is increasing accordingly. This special issue focuses on the role of OS in retinal diseases.

A. Kimura et al. review the contribution of OS to glaucoma and optic neuritis. Glaucoma is characterized by progressive degeneration of retinal ganglion cells (RGC) and their axons, which form the optic nerve. Optic neuritis is a demyelinating inflammation of the optic nerve that is usually associated with multiple sclerosis. In their review, the authors focus especially on the role of apoptosis signal-regulating kinase 1 (ASK1). They previously demonstrated that ASK1 deficiency significantly reduces OS in various animal models of glaucoma and optic neuritis. Moreover, the expression of toll-like receptor 4 (TLR4) is increased in the glaucoma model, which, in turn, activates ASK1. Interestingly, candesartan, an angiotensin II receptor antagonist, suppresses the increase in TLR4 expression in the glaucoma model. The renin-angiotensin system (RAS) is reportedly involved in OS-induced RGC death. These findings suggest that suppression of RAS-TLR4-ASK1 signaling may be a promising approach to reduce OS in glaucoma and optic neuritis.

T. Matsuura et al. focus on the role of OS in AMD, which is a leading cause of blindness in developed countries. AMD is classified as wet or dry: the wet form is characterized by choroidal neovascularization (CNV) and the dry form is characterized by atrophy of retinal pigment epithelium (RPE). Multiple risk factors, including obesity, hypertension, smoking, and light exposure, have been reported to contribute to the pathogenesis of AMD by increasing OS. Therefore, it is important to identify biomarkers that reflect the level of OS and the state of AMD. Matsuura et al. examined serum levels of malondialdehyde (MDA), a well-known marker of OS, in patients with AMD and in healthy subjects. They found not only that MDA levels were significantly higher in patients with wet AMD compared to healthy subjects but also that the MDA levels correlated significantly with the state of CNV. The authors also demonstrated that nutritional supplementation with anti-OS agents tended to

reduce MDA levels in patients with wet AMD. These findings suggest that MDA might be a valuable biomarker of the OS and the CNV status in wet AMD.

K. Ohashi et al. explore OS in the rat retina caused by the polyamine spermidine. Polyamines are metabolites of ornithine, and previous studies have suggested that ornithine accumulation is involved in the pathogenesis of dry AMD. In vitro studies found that high levels of spermidine induce RPE cell death; however, the underlying mechanisms are incompletely understood. K. Ohashi and colleagues examined the effects of intravitreal spermidine injection in the rat. They found that spermidine increased the permeability of the blood-retinal barrier and induced the vacuolation, atrophy, and death of RPE cells. However, these effects were profoundly inhibited by coinjection of N-acetylcysteine, an antioxidant, or of aldehyde dehydrogenase with spermidine. These results suggest that intravitreal injection of spermidine causes dry AMD by increasing OS.

C. Li et al. review the contribution of OS to DR, which is a major and potentially life-threatening complication of diabetes and is the main cause of blindness among working-age adults. Four classical pathways are involved in the pathophysiology of DR: increased polyol pathway flux, activation of the protein kinase C pathway, accumulation of advanced glycation end products, and activation of the hexosamine pathway. C. Li et al. demonstrate that hyperglycemia also affects epigenetic regulatory pathways, including promoter methylation, histone acetylation, and microRNA expression. Dysregulation of epigenetic pathways by hyperglycemia can affect the expression of genes related to OS in the retina and could thus play a critical role in the development of DR.

S. Balaiya et al. also review the involvement of OS-induced epigenetic changes in retinal diseases, focusing on the sirtuins, which are class III histone deacetylases. In mammals; the sirtuin family consists of seven proteins. In their review, S. Balaiya et al. discuss the roles of sirtuins in glaucoma, optic neuritis, and AMD, and they describe the potential of sirtuins as therapeutic targets for these diseases.

The therapeutic effects of free radical scavengers in various retinal diseases have been studied extensively. T. Masuda et al. provide a comprehensive review of the therapeutic potential of edaravone, a free radical scavenger, in glaucoma, AMD, DR, and retinal vein occlusion. Edaravone can protect against N-methyl-d-aspartate-induced retinal thinning by reducing OS and inhibiting activation of the JNK and p38 MAP kinase pathways, suggesting that edaravone may have therapeutic activity in glaucoma. Edaravone may also slow the progression of AMD, not only through its anti-OS and antiapoptotic effects but also through the suppression of VEGF-induced endothelial cell proliferation. T. Masuda et al. indicate that edaravone may slow the progression of DR by attenuating the suppression of brain-derived neurotrophic factor. In a clinical trial, edaravone following arteriovenous sheathotomy was effective against macular edema associated with a branch retinal vein occlusion and improved the best-corrected visual acuity. These findings suggest that edaravone may be useful for treating retinal diseases associated with OS.

Another important therapeutic strategy for OS-associated retinal diseases is elevation of antioxidant enzymes. Y. Nakagami reviews the therapeutic potential of small molecule compounds that can activate nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a redox-sensitive transcription factor that binds to antioxidant response elements located in the promoter region of genes encoding many antioxidant enzymes and phase II detoxifying enzymes. Y. Nakagami recently developed a novel Nrf2-activating small molecule compound; here, they demonstrate its therapeutic effects on various retinal diseases associated with OS.

Finally, Y. Nishimura and H. Hara focus on recent advances in high-throughput technologies that have facilitated the collection of multilevel omics data. Integration of the knowledge gained from omics databases can be used to generate disease-related biological networks and to identify potential therapeutic targets within the networks. The authors provide an overview of integrative approaches in the drug discovery process and provide simple examples of how the approaches can be exploited to identify OS-related targets for retinal diseases.

We hope that this special issue will stimulate further efforts to understand retinal diseases at the systems level, with the hope of finding new preconditioning and therapeutic strategies to prevent or treat retinal pathologies.

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Yuhei Nishimura
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Mineo Kondo
Samin Hong
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Research Article

Spermidine Oxidation-Mediated Degeneration of Retinal Pigment Epithelium in Rats

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Retinal pigment epithelium (RPE) degeneration is a crucial event in dry age-related macular degeneration and gyrate atrophy. The polyamine spermidine has been shown to induce RPE cell death in vitro. The present study aimed to establish a novel in vivo model of spermidine-induced RPE degeneration and to determine whether spermidine-induced RPE cell death involves oxidative mechanisms. In this study, spermidine caused ARPE-19 cell death in a concentration-dependent manner. This effect was prevented by removal of serum from the culture medium or treatment with amine oxidase inhibitors, N-acetylcysteine (NAC), or aldehyde dehydrogenase (ALDH). Intravitreal injection of spermidine into rats significantly increased the permeability of the blood-retinal barrier and decreased the amplitudes of scotopic electroretinogram a- and b-waves. Histological analysis revealed that spermidine induced vacuolation, atrophy, and dropout of RPE cells, leading to the disruption of photoreceptor outer segments. Simultaneous intravitreal administration of NAC and ALDH with spermidine prominently inhibited the functional and morphological changes induced by spermidine. In conclusion, this study demonstrated that the intravitreal administration of spermidine induced RPE cell dysfunction and death followed by photoreceptor degeneration in rats. These effects of spermidine are thought to be mediated by oxidative stress and a toxic aldehyde generated during spermidine oxidation.

1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of cells located between the sensory retina and the choroid. The RPE exerts a variety of important functions involved in maintaining sensory retina homeostasis, including the regulation of nutrient transport to the photoreceptors, phagocytosis of distal tips of rod outer segments, absorption of stray light, and secretion of growth factors [1]. RPE degeneration predisposes photoreceptor cells to secondary damage and death consequent to the loss of support from the RPE and thus causes vision-threatening diseases such as dry age-related macular degeneration (dry AMD) [2, 3] and gyrate atrophy with hyperornithinemia [4].

Previous studies have suggested that the RPE degeneration observed in dry AMD and gyrate atrophy is caused by various factors, including oxidative stress [5] and ornithine accumulation [6]. Several animal models of RPE degeneration, such as sodium iodate-induced mouse, rat, and rabbit models [7–9], the ornithine-induced rat model [10], and the ornithine delta-aminotransferase deficient mouse [11], have been established and used in studies of the mechanisms of dry AMD and gyrate atrophy. However, the precise mechanism(s) underlying the degeneration of RPE and photoreceptor cells in these diseases are still not fully understood, and currently there are no approved drugs for the treatment of these conditions. A novel in vivo model of RPE degeneration would be useful for the elucidation of these mechanisms.

Polyamines such as spermine, spermidine, and putrescine are metabolites of ornithine and ubiquitous cellular components [12]. These polyamines have been reported to regulate various functions of RPE cells, including proliferation [13] and migration [14]. However, a previous in vitro study found that excessive spermine and spermidine induced the death of bovine RPE cells, suggesting that polyamines might be involved in the RPE degeneration associated with gyrate atrophy [15]. Previous studies of other cell lines suggested that toxic metabolites, particularly hydrogen peroxide and

the toxic aldehyde acrolein, which are generated during polyamine oxidation, are involved in polyamine-induced cell death [16–19]. Therefore, the intravitreal administration of spermidine in vivo may induce RPE degeneration via spermidine oxidation.

The aims of this study were to establish a novel in vivo model of RPE degeneration, using spermidine as an inducer, and to determine whether oxidative mechanisms were involved in spermidine-induced RPE cell death. To achieve these aims, we examined the effects of intravitreal spermidine administration on the function and histology of the rat sensory retina and RPE and examined the effects of various inhibitors of the polyamine oxidation pathway on spermidine-induced RPE cell death in vitro and in vivo. We selected an intravitreal injection as an administration route of spermidine in in vivo studies, because it may be a suitable way to deliver an adequately high concentration of spermidine to the retina.

2. Methods

2.1. Materials. ARPE-19 cells were purchased from ATCC (Manassas, VA, USA). DMEM/F12 was obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) and penicillin-streptomycin were supplied by Thermo Fisher (Waltham, MA, USA). The CellTiter 96® Aqueous One Solution cell proliferation assay reagent (containing the tetrazolium compound MTS) was provided by Promega (Madison, WI, USA). Spermidine and spermine were purchased from Merck Millipore (Billerica, MA, USA). Aminoguanidine was provided by Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's phosphate-buffered saline (DPBS), pentamidine, N-acetylcysteine (NAC), and aldehyde dehydrogenase (ALDH) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Glutaraldehyde and formalin were obtained from Wako (Osaka, Japan). 0.5% Tropicamide, 0.5% phenylephrine hydrochloride (Mydrin-P®), 0.4% oxybuprocaine hydrochloride (Benoxil®), and 0.5% levofloxacin ophthalmic solution (Cravit®) were provided by Santen Pharmaceutical (Osaka, Japan). 10% Fluorescein (Fluorescite®) was purchased from Alcon Japan (Tokyo, Japan). Ten mg/mL Ketamine (Ketalar®) was supplied by Daiichi Sankyo (Tokyo, Japan). 2% Xylazine (Selactar®) was obtained from Bayer Health Care (Tokyo, Japan). Mouse monoclonal anti-acrolein antibody (5F6) was provided by NOF Corporation (Tokyo, Japan). Histofine Simple Stain Rat MAX-PO (MULTI) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). DAB substrate kit was supplied by Dako Japan (Tokyo, Japan).

2.2. Cell Culture. ARPE-19 cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, as previously described [20, 21]. Passage numbers 23–36 were used for the experiments.

2.3. Assessment of Cell Viability. ARPE-19 cell viability was assessed via an MTS assay; for this purpose, the CellTiter 96 Aqueous One Solution cell proliferation assay was used according to the manufacturer's protocol. In brief, ARPE-19 cells were seeded at a density of 10^4 cells per well into 96-well plates. Twenty-four hours after seeding, spermidine was added to the medium in the presence or absence of 10% FBS to obtain final medium concentrations of 100–500 μM. Twenty-four hours after the addition of spermidine, Aqueous One solution reagent was added to the medium, followed by a two-hour incubation. Next, the intensity of each colorimetric reaction was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). To examine the effects of aminoguanidine, pentamidine, NAC, or ALDH on spermidine-induced ARPE-19 cell death, these compounds were added simultaneously with spermidine.

2.4. Animals. Animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement concerning the use of animals in ophthalmic and vision research. The experimental procedure was approved and monitored by the Animal Care and Use Committee of the Nara Research & Development Center, Santen Pharmaceutical Co., Ltd. Six to eight-week-old female Brown Norway rats were purchased from Charles River Japan (Yokohama, Japan). Rats were housed under a 12-hour light/12-hour dark cycle and provided with food and water ad libitum.

2.5. Intravitreal Administration. Rats were anesthetized by an intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. DPBS, spermidine (1, 2, and 3 mM dissolved in DPBS), or spermine (1.5 mM dissolved in DPBS), each in a total injection volume of 10 μL, were administrated into the vitreous bodies of both eyes per animal using a microinjector (Hamilton, Reno, NV, USA) with the aid of a dissecting microscope. Next, 0.5% of levofloxacin ophthalmic solution was applied to the ocular surface to prevent infection. Eyes treated with DPBS were used as controls. To examine the effects of NAC and ALDH on spermidine-induced RPE degeneration, these drugs were coadministered with spermidine.

2.6. Vitreous Fluorophotometry. The permeability of the blood-retinal barrier (BRB) was evaluated using vitreous fluorophotometry, as previously described [21]. Rats were anesthetized as described above on days 1, 3, 7, 14, and 28 after the administration of either DPBS or spermidine (10, 20, or 30 nmol/eye). Next, fluorescein (10 mg/kg) was injected intravenously, and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. After fluorescein was allowed to circulate for 45 minutes, the concentrations of vitreous and plasma fluorescein were measured using a Fluorotron Master (OcuMetrics, Mountain View, CA, USA). The permeability of the BRB was calculated according to the following formula:

$$\text{Permeability} = \frac{\text{Vitreous fluorescein (ng/mL)}}{\text{Plasma fluorescein (ng/mL)}}. \quad (1)$$

In experiments to evaluate the effects of NAC and ALDH, we only measured the concentration of vitreous fluorescein to evaluate BRB permeability because the intravitreal administration of spermidine did not affect the concentration of plasma fluorescein in the time course experiment.

2.7. Electroretinogram. Scotopic electroretinogram (ERG) data were recorded on days 2, 6, 13, and 27 after the administration of either DPBS or spermidine (10, 20, or 30 nmol/eye), as previously described [20, 21]. For these recordings, rats were dark-adapted for at least 30 min. All manipulations were done under dim red light. Rats were anesthetized as described above, and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. Corneal anesthesia was achieved using topical 0.4% oxybuprocaine hydrochloride. A platinum electrode was placed in contact with the cornea, and a reference electrode and ground electrode were positioned on the nose and tail, respectively. Responses to a 3000 cd/m^2 white light flash (10 ms) were amplified, filtered, and recorded using a portable ERG&VFP LE-3000 device (Tomey, Nagoya, Japan). The a-wave amplitudes were measured from the baseline to the trough of the a-wave, and the b-wave was measured from the trough of the a-wave to the peak of the b-wave.

2.8. Histology. Rats were sacrificed by bleeding on days 1, 3, 7, 14, and 28 after the administration of either DPBS or spermidine (10, 20, or 30 nmol/eye). The eyes were enucleated and fixed in a mixture of 2.5% glutaraldehyde and 9% formalin for 2 hours, followed by 10% formalin overnight. Fixed eyes were embedded in paraffin. Three-micrometer-thick sections were cut with a microtome and stained with hematoxylin and eosin (HE). Photographs at the posterior region of retina were taken. In this experiment, we also examined the effect of spermine (15 nmol/eye), another polyamine, as reference.

2.9. Transmission Electron Microscopy. Rats were sacrificed by bleeding at 6 hours and 4 days after the administration of either DPBS or spermidine (20 nmol/eye). The eyes were enucleated and fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide for 2 hours at 4°C, dehydrated, and embedded in epoxy resin for electron microscopy. One-micrometer-thick sections were stained with toluidine blue and used to select suitable areas for electron microscopy. Finally, 80 nm ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with an H-7600 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

2.10. Immunohistochemistry. Rats were sacrificed by bleeding on days 1 and 3 after the administration of either DPBS or spermidine (20 nmol/eye). The eyes were enucleated and fixed at 4°C for several days in phosphate buffer containing 4% formaldehyde. Fixed eyes were embedded in paraffin. Following deparaffinization and blocking, mouse monoclonal anti-acrolein antibody which is specific for acrolein-modified proteins and the secondary antibody Histofine Simple Stain Rat MAX-PO (MULTI) were sequentially applied to 4-micrometer-thick sections for 1 hour each, at room temperature. Specific immunoreactivity was visualized using a DAB substrate kit. Finally, counter staining was performed using 3% Giemsa staining solution.

2.11. Statistical Analysis. EXSAS Version 6.10 (Arm, Osaka, Japan) was used for the statistical analysis. Data are expressed as means ± standard errors of the means. Student's t-test or the Aspin–Welch t-test was used to compare two groups. A one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, was used to compare more than three groups. Differences were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Protective Effects of Polyamine Oxidation Pathway Inhibitors on Spermidine-Induced ARPE-19 Cell Death. First, we confirmed the concentration-dependent effect of spermidine on ARPE-19 cell viability and the serum-dependence of this effect. In the presence of 10% FBS, spermidine (100–500 μM) induced ARPE-19 cell death in a concentration-dependent manner (Figure 1(a)). In contrast, in the absence of FBS, spermidine did not induce cell death (Figure 1(a)). These results suggest that a spermidine byproduct generated by serum components, but not spermidine itself, induced ARPE-19 cell death.

We next examined the effects of various polyamine oxidation pathway inhibitors on spermidine-induced ARPE-19 cell death. In the presence of 10% FBS, the amine oxidase inhibitors aminoguanidine (10–100 μM) and pentamidine (0.01–0.1 μM) inhibited spermidine (250 μM)-induced cell death (Figure 1(b)). In addition, the antioxidant NAC (100–1000 μM) and the aldehyde metabolizing enzyme ALDH (1 U/mL) significantly inhibited the loss of ARPE-19 cell viability (Figures 1(c) and 1(d)). These results suggest that serum amine oxidase, oxidative stress, and aldehyde are involved in spermidine-induced ARPE-19 cell death.

3.2. Spermidine-Induced Impairment of the Functions of BRB and Retina in Rats. To examine the effect of spermidine on retinal function, including the integrity of the BRB (both outer and inner), BRB permeability [21] and scotopic ERG a- [22] and b-waves [23, 24] were assessed.

Intravitreal administration of spermidine at doses of 20 and 30 nmol/eye significantly increased BRB permeability on day 7 in a dose-dependent manner (Figure 2). By days 14 and 28, however, this spermidine-induced increase in BRB permeability had been attenuated (Figure 2). In contrast, the BRB permeability change brought about by spermidine at 10 nmol/eye underwent recovery to a nonstatistically significant level (Figure 2).

Typical traces of scotopic ERGs recorded from a rat intravitreally injected with either DPBS or spermidine (10–30 nmol/eye) are shown in Figures 3(a)–3(d). Spermidine at 20 and 30 nmol/eye initially increased the ERG a-wave amplitude on day 2; this parameter gradually decreased on days 6 and 13 in a dose-dependent manner (Figure 3(e)). Spermidine at 20 and 30 nmol/eye significantly decreased the ERG b-wave amplitude from day 6 to day 13 and day 2 to day 27,

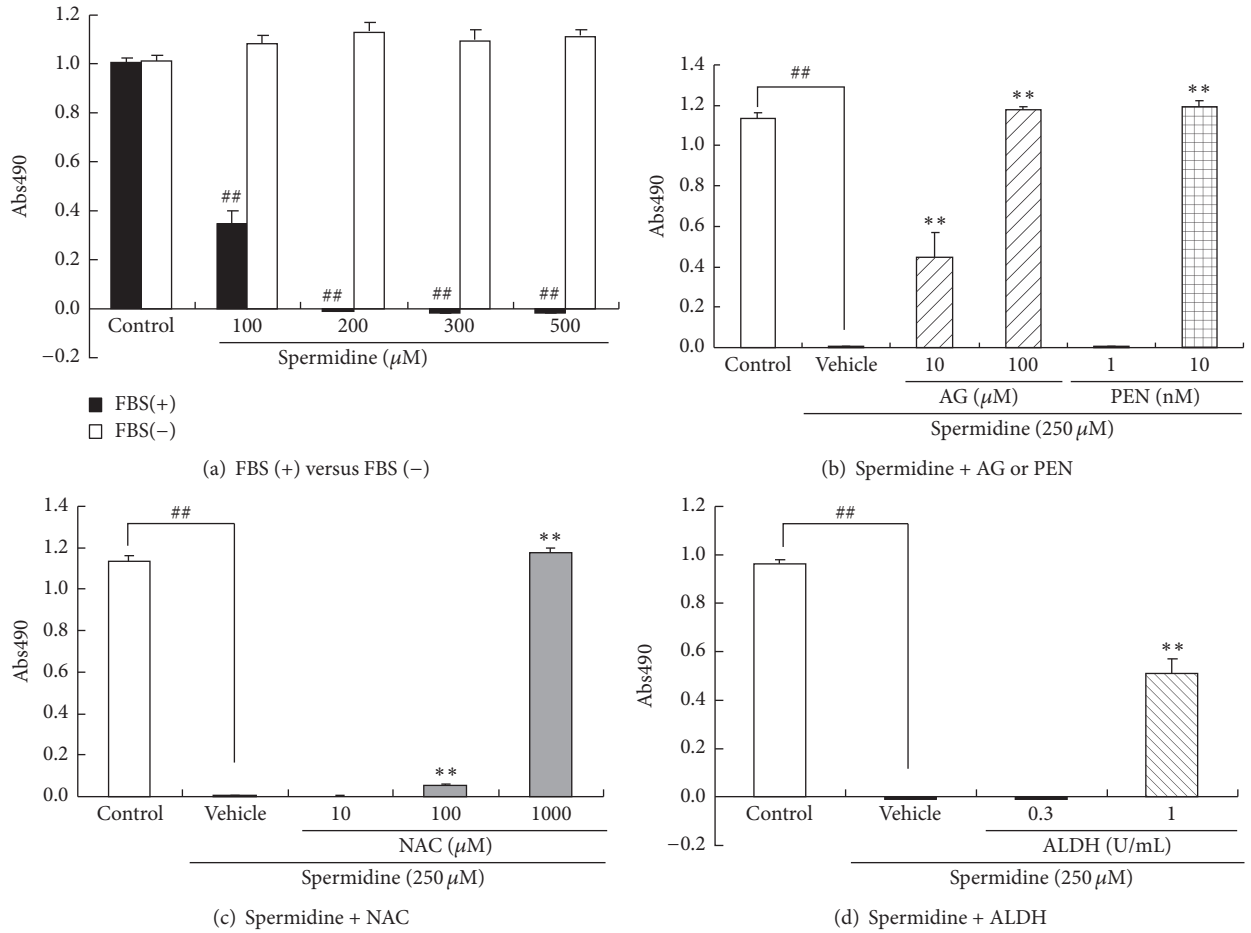


Figure 1: Effects of serum removal and treatment with amine oxidase inhibitors, an antioxidant compound, and aldehyde dehydrogenase (ALDH) on spermidine-induced ARPE-19 cell death. Cell viability was assessed with a MTS assay 24 h after the addition of spermidine. The effects of (a) serum removal and treatment with (b) amine oxidase inhibitors, (c) an antioxidant, and (d) ALDH when applied simultaneously with spermidine. Each column represents a mean ± standard error of the mean of four wells. $^{\#\#}P < 0.01$ versus control. $^{**}P < 0.01$ versus spermidine. AG: aminoguanidine. PEN: pentamidine.

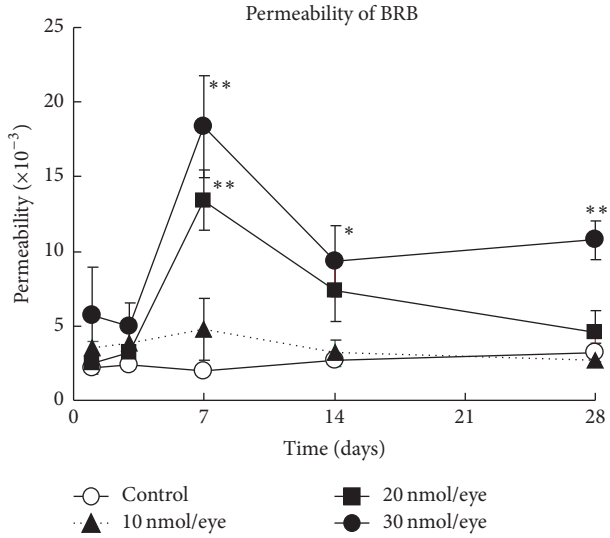


Figure 2: Spermidine-induced hyperpermeability of the blood-retinal barrier (BRB) in rats. BRB permeability was assessed by vitreous fluorophotometry on days 1, 3, 7, 14, and 28 after the intravitreal administration of spermidine. Each value represents the mean ± standard error of the mean of six to eight eyes. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

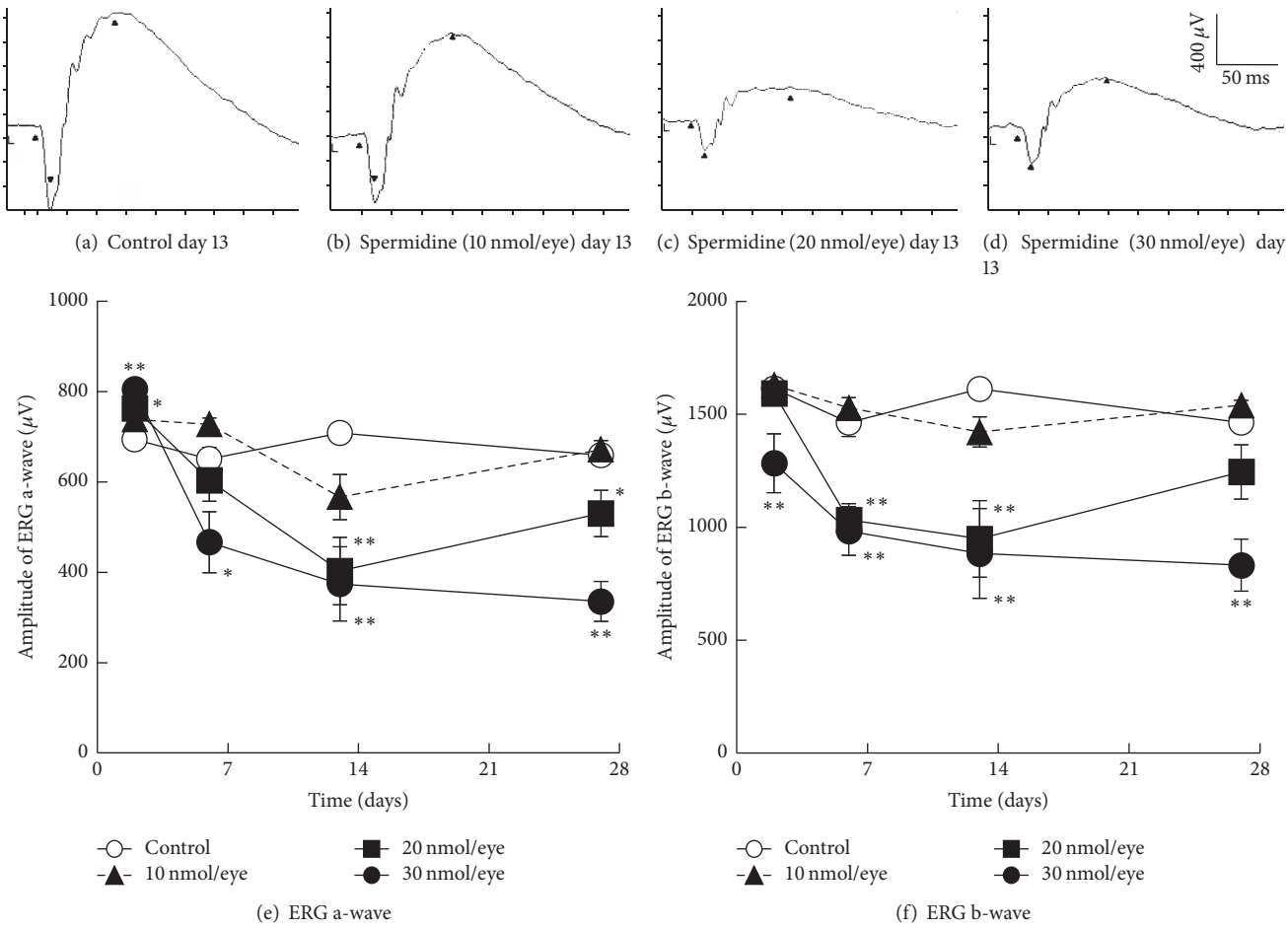
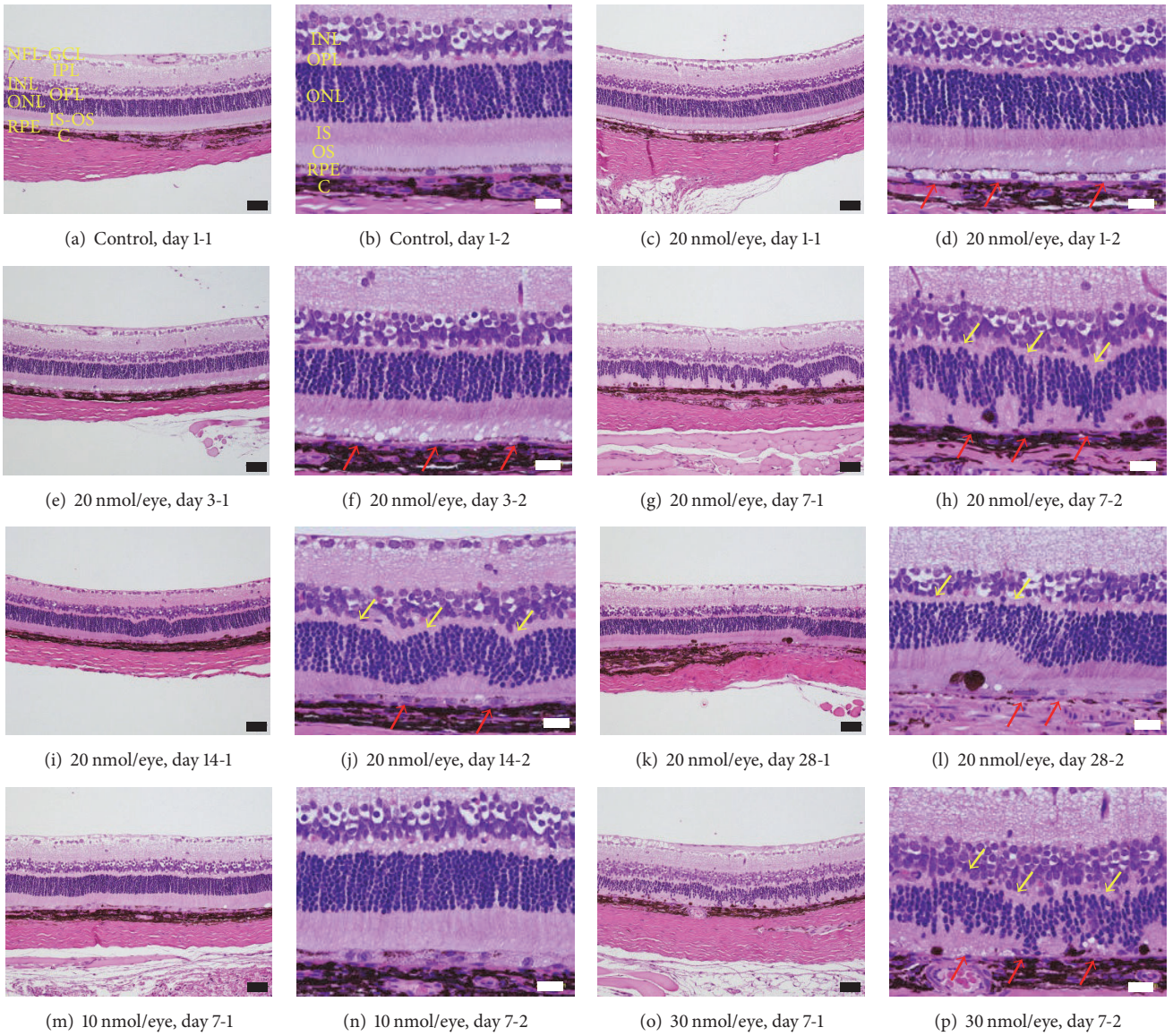


Figure 3: Spermidine-induced impairment of electroretinogram (ERG) a- and b-wave amplitudes in rats. Scotopic ERG a- and b-waves were measured on days 2, 6, 13, and 27 days after the intravitreal administration of spermidine. Traces typical of (a) control, (b) spermidine (10 nmol/eye), (c) spermidine (20 nmol/eye), and (d) spermidine (30 nmol/eye) treatments, 13 days after the injection are shown. Time course data of (e) ERG a-waves and (f) ERG b-waves are shown. Each value represents the mean ± standard error of the mean of seven to eight eyes. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

respectively (Figure 3(f)). On day 27, the effect of 20 nmol/eye spermidine was reduced, whereas that of 30 nmol/eye spermidine remained unchanged (Figures 3(e) and 3(f)). In contrast to these two higher doses, 10 nmol/eye spermidine did not have a significant effect on the ERG a- and b-wave amplitudes (Figures 3(e) and 3(f)). These results demonstrate that spermidine at doses of 20 and 30 nmol/eye impairs retinal electrophysiological and barrier functions. In addition, the BRB begins functional recovery on day 14 after administration of 20, but not 30, nmol/eye of spermidine.

3.3. Spermidine-Induced RPE and Photoreceptor Cell Degeneration in Rats. Histological analysis revealed that in rats intravitreal injection of 20 nmol/eye spermidine induced RPE cell vacuolization on day 1 (Figure 4(d); red arrows) and RPE cell degeneration on days 3 and 7 (Figures 4(f) and 4(h); red arrows), whereas the intravitreal injection of DPBS did not affect the structure of RPE cells on day 1 (Figures 4(a) and 4(b)). Spermidine at 20 nmol/eye also caused the disruption of photoreceptor outer segments from day 3 to day 28 (Figures 4(f), 4(h), 4(j), and 4(l)) and induced degeneration of the outer nuclear layer from day 7 to day 28 (Figures 4(h), 4(j), and 4(l); yellow arrows). On days 14 and 28, regeneration of RPE morphology was observed (Figures 4(j) and 4(l); red arrows). The spermidine-induced degenerative change of RPE and photoreceptors on day 7 was dose-dependent (Figures 4(h), 4(n), and 4(o)). In addition, spermidine at 20 nmol/eye slightly decreased the thickness of the inner plexiform layer (IPL) and the inner nuclear layer (INL) from days 7 to 28 and days 14 to 28, respectively (Figures 4(g)–4(l)), but spermidine at 10 and 30 nmol/eye did not affect the inner retinal structure on day 7 (Figures 4(m)–4(p)). These results demonstrate that the intravitreal administration of spermidine induced the degeneration of both RPE and photoreceptors. In addition, RPE cell regeneration following treatment with 20 nmol/eye spermidine appeared to begin on day 14.

On the other hand, the intravitreal injection of spermine at 15 nmol/eye induced the atrophy of all retinal layers on day 7 (data not shown). This result suggests that spermidine is



(a) Control, day 1-1 (b) Control, day 1-2 (c) 20 nmol/eye, day 1-1 (d) 20 nmol/eye, day 1-2

(e) 20 nmol/eye, day 3-1 (f) 20 nmol/eye, day 3-2 (g) 20 nmol/eye, day 7-1 (h) 20 nmol/eye, day 7-2

(i) 20 nmol/eye, day 14-1 (j) 20 nmol/eye, day 14-2 (k) 20 nmol/eye, day 28-1 (l) 20 nmol/eye, day 28-2

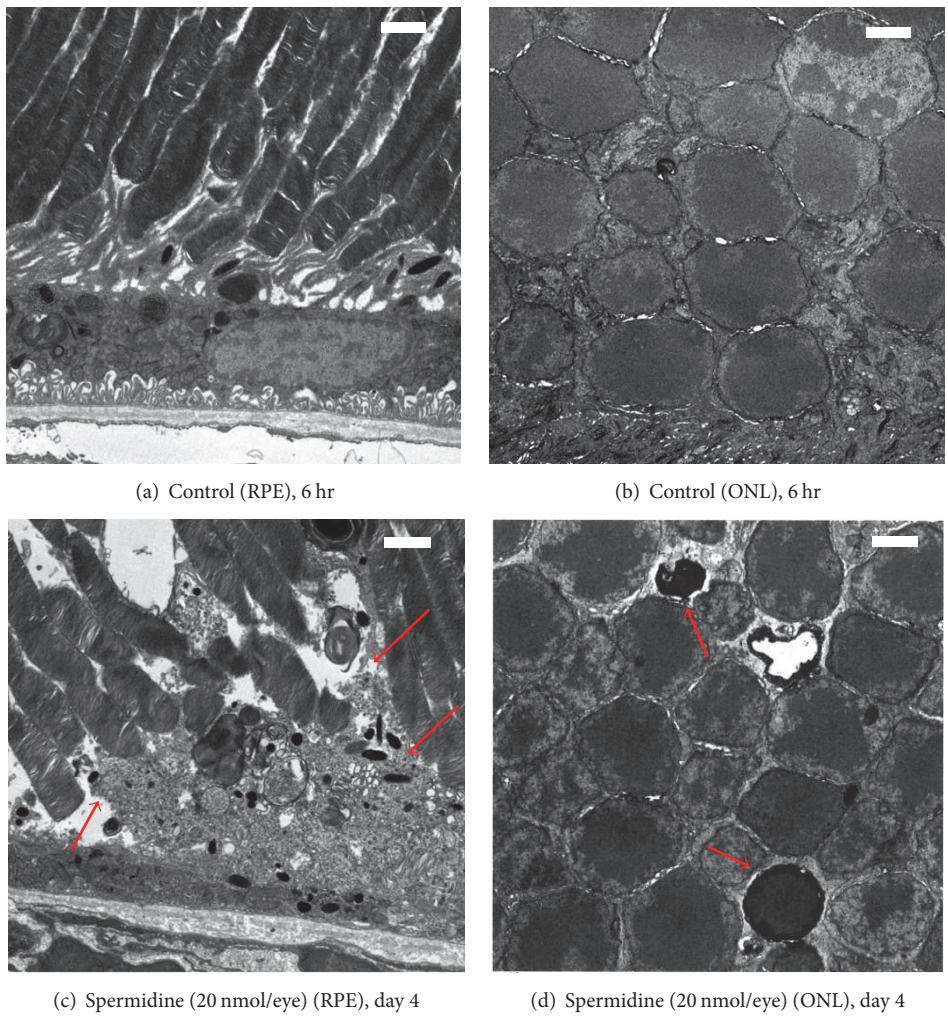
(m) 10 nmol/eye, day 7-1 (n) 10 nmol/eye, day 7-2 (o) 30 nmol/eye, day 7-1 (p) 30 nmol/eye, day 7-2

Figure 4: Spermidine-induced degeneration of retinal pigment epithelium (RPE) and photoreceptor cells in rats. Cross-sections of rat eyes were prepared (a, b) 1 day after the administration of DPBS, (c–l) 1, 3, 7, 14, and 28 days after the intravitreal administration of spermidine (20 nmol/eye), and (m–o) 7 days after the injection of spermidine (10 or 30 nmol/eye). Black and white scale bars indicate 50 and 20 μm, respectively. Red arrows in (d) indicate RPE cell vacuolization. Red arrows in (f), (h), and (p) indicate RPE cell degeneration. Red arrows in (j) and (l) indicate RPE cell regeneration. Yellow arrows in (h), (j), (l), and (p) indicate photoreceptor cell degeneration. NFL-GCL: nerve fiber layer and ganglion cell layer. IPL: inner plexiform layer. INL: inner nuclear layer. OPL: outer plexiform layer. ONL: outer nuclear layer. IS: inner segment. OS: outer segment. RPE: retinal pigment epithelium. C: choroid.

a more selective inducer of the degeneration of outer retina than spermine.

Ultrastructural analysis revealed that spermidine (20 nmol/eye) disrupted RPE cell plasma membranes on postinjection day 4 (Figure 5(c); red arrows), whereas the intravitreal injection of DPBS did not affect RPE or photoreceptor morphology at 6 hours (Figures 5(a) and 5(b)). In contrast, spermidine did induce chromatin condensation in photoreceptor nuclei by day 4 (Figure 5(d); red arrows). These results support our conclusion that spermidine induces necrosis in RPE cells and apoptosis in photoreceptor cells.

3.4. Protective Effects of NAC and ALDH against Spermidine-Induced RPE Degeneration in Rats. To clarify the involvement of oxidative stress and aldehyde toxicity in spermidine-induced RPE degeneration in this rat model, the effects of NAC and ALDH were examined. Both NAC (500 nmol/eye) and ALDH (1.5 U/eye) significantly inhibited spermidine-induced hyperpermeability of the BRB on day 7 (Figure 6) and impairment of the ERG a- and b-wave amplitudes on day 13 (Figure 7). Moreover, NAC and ALDH prominently inhibited the spermidine-induced degeneration of RPE and photoreceptors previously observed by day 13 (Figure 8). In



(a) Control (RPE), 6 hr

(b) Control (ONL), 6 hr

(c) Spermidine (20 nmol/eye) (RPE), day 4

(d) Spermidine (20 nmol/eye) (ONL), day 4

Figure 5: Spermidine-induced ultrastructural changes in the retinal pigment epithelium (RPE) and outer nuclear layer (ONL) of the rat retina. Ultrathin 80–nm sections were prepared from samples fixed 6 hours after the injection of (a, b) DPBS or 4 days after the intravitreal administration of (c, d) spermidine (20 nmol/eye). Scale bars indicate 2 μm. Red arrows in (c) indicate plasma membrane disruption in RPE cells. Red arrows in (d) indicate chromatin condensation in photoreceptor cells.

addition, the immunodetection of acrolein-modified proteins (which are highly reactive and longer-lived than free acrolein [25]) was observed only within the RPE of a spermidine injected eye (Figure 9(b)). These results, which are consistent with those of our in vitro findings, suggest that oxidative stress and toxic aldehyde byproducts are involved in spermidine-induced RPE degeneration in rats.

4. Discussion

A major finding of the present study is that the intravitreal administration of spermidine induced the dysfunction and death of RPE cells in association with the degeneration of photoreceptors in rats. Although our results do not rule out spermidine also affecting the structure and function of the inner retina under our experimental conditions, the most prominent histological changes induced by this molecule were observed in the outer retina. This is, to our knowledge, a novel animal model of RPE degeneration mediated by spermidine, and more specifically by oxidation products of spermidine.

To elucidate the mechanisms underlying spermidine-induced RPE cell death, we examined the effects of various polyamine oxidation pathway inhibitors on spermidine-induced RPE cell death in both in vitro and in vivo studies. Aminoguanidine and pentamidine (amine oxidase inhibitors), as well as NAC (an antioxidant) and ALDH (an aldehyde-quenching enzyme), suppressed spermidine-induced ARPE-19 cell death in our in vitro study, and the latter two agents also ameliorated RPE dysfunction and death in our in vivo experiments. In addition, the immunolocalization of acrolein-modified macromolecules was observed in RPE cells of a spermidine-treated eye. These results are consistent with previous studies which showed that amine oxidase inhibitors, NAC, and/or ALDH suppressed the polyamine-induced cell death of cancer cell lines [16, 17] and microglia [18]. NAC

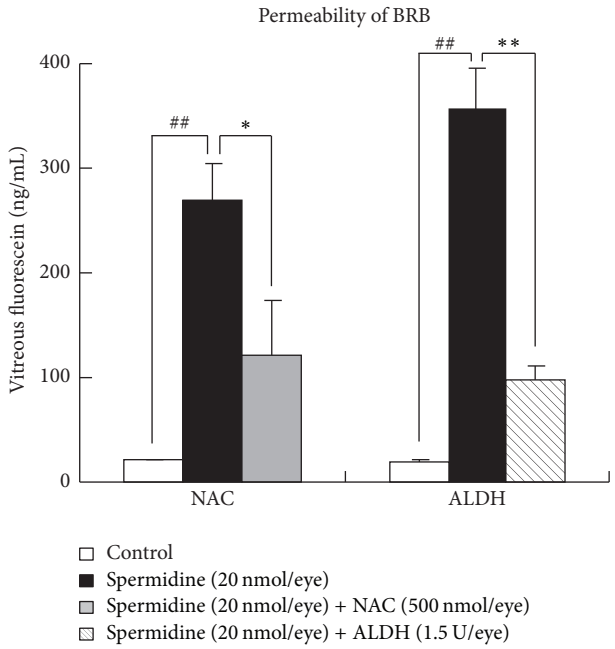


Figure 6: Protective effects of N-acetylcysteine (NAC) and aldehyde dehydrogenase (ALDH) on spermidine-induced hyperpermeability of the blood-retinal barrier (BRB). (a) BRB permeability was assessed by vitreous fluorophotometry 7 days after the intravitreal administration of (a) spermidine (20 nmol/eye) alone, (b) spermidine (20 nmol/eye) plus NAC (500 nmol/eye), and (c) spermidine (20 nmol/eye) plus ALDH (1.5 U/eye). Each column represents a mean ± standard error of the mean of seven to eight eyes. $^{\#\#}P < 0.01$ versus control and $^{*}P < 0.05$ and $^{**}P < 0.01$ versus spermidine (20 nmol/eye).

is believed to suppress oxidative stress resulting from either polyamine oxidation [26] and/or as a result of increased production of acrolein [18, 27]. ALDH is an aldehyde-quenching enzyme and known to metabolize acrolein [28]. Therefore, these results and previous evidence suggest that spermidine-induced RPE cell death is mediated by hydrogen peroxide and the toxic aldehyde acrolein, both generated by spermidine oxidases both in vitro and in vivo. Importantly, both hydrogen peroxide and acrolein have been reported to induce the oxidative stress-mediated death of ARPE-19 cells [29–31].

In this study, the intravitreal administration of spermidine induced hyperpermeability of the BRB in rats, as assessed by vitreous fluorophotometry. The BRB is comprised of inner and outer components, corresponding to intercellular tight junctions between retinal endothelial, and RPE cells, respectively [32], and the breakdown of either or both the inner and/or the outer BRB may contribute to the observed retinal hyperpermeability. Based on the results of our histological analysis, the disruption of outer BRB contributes at least in part to the hyperpermeability of BRB which we documented in spermidine-treated animals. However, the added contribution of changes in the inner BRB in spermidine-treated eyes cannot at this point be ruled out. Further study is needed to elucidate this.

We found that the intravitreal administration of spermidine had a biphasic effect on the functions of photoreceptor cells, as determined through an evaluation of ERG a-wave amplification in rats. The reason for this spermidine-induced initial increase in ERG a-wave amplitudes on day 2 is unclear, although a similar early enhancement effect on ERG a-waves was reported in another RPE degeneration model [33, 34]. Therefore, further studies are needed to elucidate this phenomenon. On the other hand, the later spermidine-induced decrease in ERG a-wave amplitude from days 6 to 27 is attributable to the impaired function and, in some cases, loss of photoreceptor cells because spermidine induced disruption of photoreceptor outer segments from day 3 to day 28, and outer nuclear layer degeneration was evident from day 7 to day 28.

Our histological analysis revealed that spermidine initially induced RPE cell damage and degeneration, whereas photoreceptor cell death was a secondary outcome. This phenomenon could be explained by the loss of support provided by RPE cells to photoreceptor cells [1]. In fact, similar changes were reported in other animal models of RPE degeneration, including sodium iodate [7] and ornithine-induced models [10] and the ornithine delta-aminotransferase deficient mouse [11], and a similar scenario also has been invoked as a mechanism in human dry AMD [2, 3] and gyrate atrophy [4].

Spermidine is suggested to impair the function of inner retinal elements such as bipolar and Müller cells, since spermidine at 20 and 30 nmol/eye decreased the ERG b-wave amplitude, without decreasing ERG a-wave amplitude, on day 6 and day 2 following injection, respectively. Since spermidine is reported to block the potassium channels of retinal Müller cells [35], spermidine may suppress the function of Müller cells without spermidine oxidation. In addition, the slight degeneration of the inner retina by spermidine might also contribute to this effect, since spermidine at 20 nmol/eye slightly decreased the thickness of IPL and INL in this study. However, we think that further precise studies are needed to determine whether this slight histological change in the inner retina induced by spermidine is a significant degeneration or an artifact, because photographs of HE-stained sections were not taken at exactly the same position of the eye for all histological samples, and there was no evidence of the dose-dependency of spermidine relative to this change.

It is important to elucidate the mechanism underlying the prominent degenerative effect of spermidine on RPE, since excess polyamines are known to induce cell death of neurons [36] and glia [18, 37] as well as RPE [15]. In fact, intravitreal administration of spermine (15 nmol/eye), a stronger inducer of cell death than spermidine [15, 37], in our hands induced the atrophy of all retinal layers. Although there were different degrees of retinal degeneration induced by spermine and spermidine, the changes observed in the outer retina indicate that RPE cells are more directly sensitive to the toxic effects of polyamines than other retinal cells. Moreover, our immunohistochemical analysis showed that acrolein was detected only in RPE cells following spermidine treatment. This result suggests that the higher production of acrolein due to the higher activity of spermidine oxidation in RPE than other retinal cells may be one of the reasons for the higher

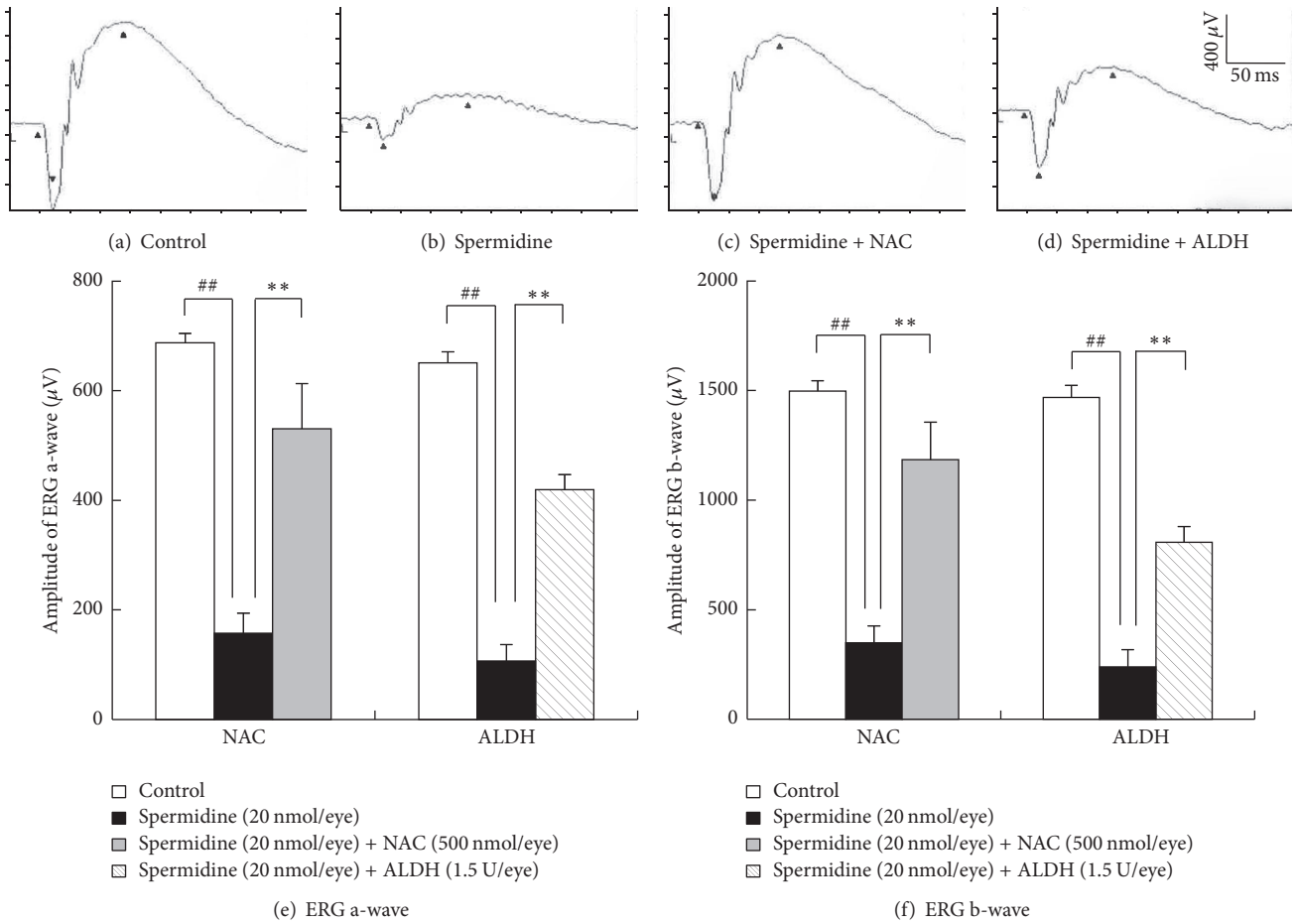
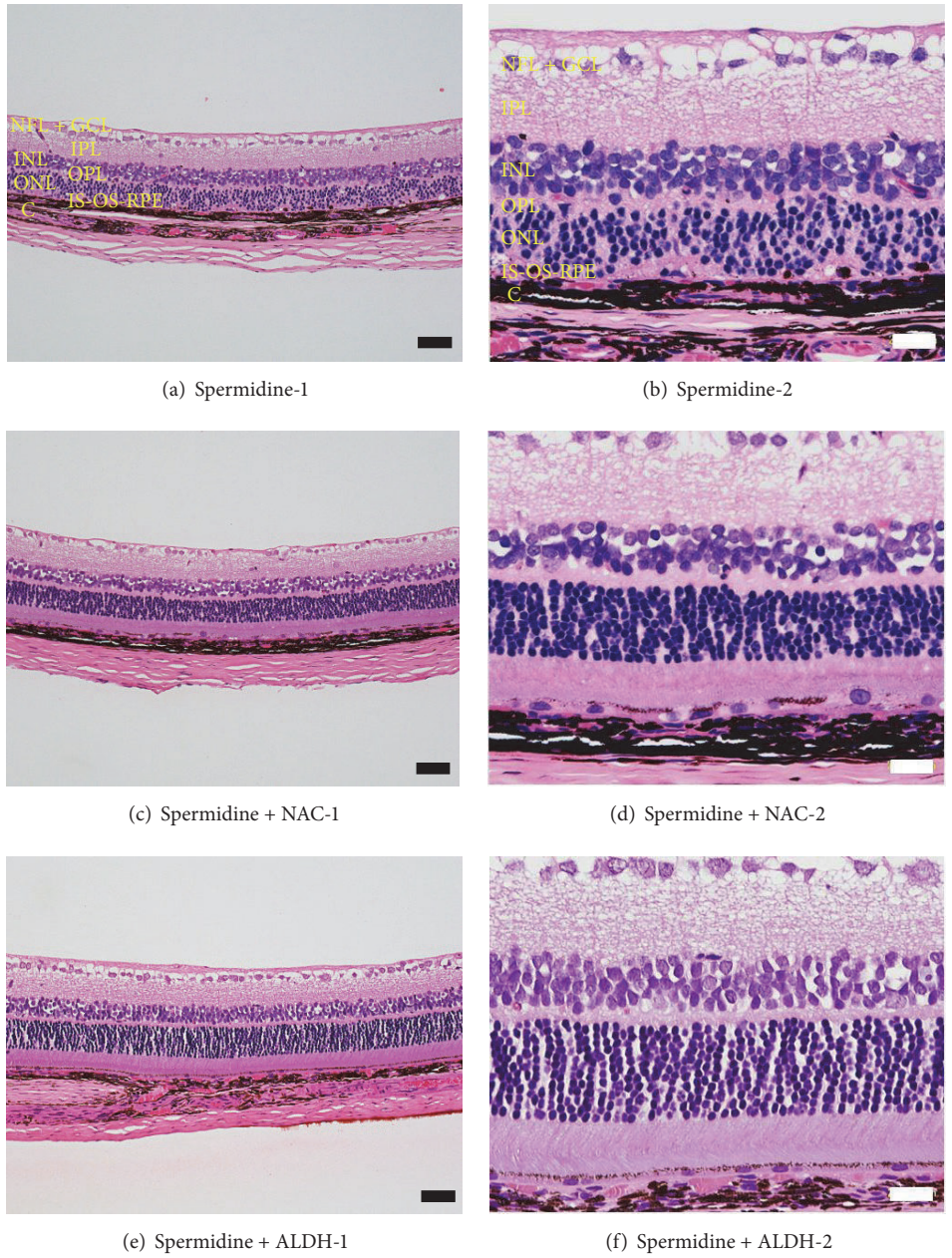


Figure 7: Protective effects of N-acetylcysteine (NAC) and aldehyde dehydrogenase (ALDH) on spermidine-induced impairment of electroretinogram (ERG) a- and b-wave amplitudes. Representative traces are shown of scotopic ERG a- and b-waves measured 13 days after the administration of (a) control, (b) spermidine (20 nmol/eye) alone, (c) spermidine (20 nmol/eye) plus NAC (500 nmol/eye), and (d) spermidine (20 nmol/eye) plus ALDH (1.5 U/eye). The amplitude data for ERG a- and b-waves, from eyes treated as above are depicted in (e) and (f), respectively. Each column represents the mean ± standard error of the mean of seven to eight eyes. $^{\#\#}P < 0.01$ versus control and $^{**}P < 0.01$ versus spermidine (20 nmol/eye).

sensitivity of RPE cells to the more localized, immediate toxic effect of spermidine.

We found that spermidine (20 nmol/eye)-induced RPE degeneration was transient and observed RPE recovery on days 14 and 28. One recent study showed that rat RPE cells could enter the cell cycle and complete cellular division [38]. In fact, several reports have shown that RPE regeneration occurs after low-dose but not high-dose sodium iodate-induced RPE degeneration in mice [39, 40]. Therefore, RPE regeneration is believed to occur after modest RPE degeneration.

It is noteworthy that RPE cells and photoreceptors experienced different modes of cell death in response to spermidine-induced retinal degeneration. In our hands, spermidine appeared to induce RPE cell death by necrosis following disruption of the plasma membrane in these cells, whereas spermidine has been reported previously to induce cell death by both necrosis and apoptosis [11, 13]. The mode of spermidine-induced cell death might depend on experimental conditions such as the cell type and spermidine concentration. According to a recent review article [41], although there is still some controversy regarding the mechanism(s) of RPE cell death in human dry AMD, ultrastructural and histopathological studies support necrosis as the major mechanism of RPE cell death in dry AMD. Although there are no reports about the mode of RPE cell death in human gyrate atrophy with hyperornithinemia, necrosis of RPE cell was observed in ornithine-induced retinopathy in rats [10]. Therefore, there are likely to be some equivalent RPE cell death pathways between the spermidine model presented here and human dry AMD and gyrate atrophy. In contrast, the secondary loss of photoreceptor cells overlying damaged RPE cells might be caused by apoptosis, as spermidine caused chromatin condensation in photoreceptor cells. This phenomenon is therefore not unique to our model and might be common to other types of retinal degeneration, given that similar results were reported in the contexts of sodium iodate-induced RPE degeneration in mice [42], ornithine-induced retinopathy in rats [10], and human dry AMD [43].



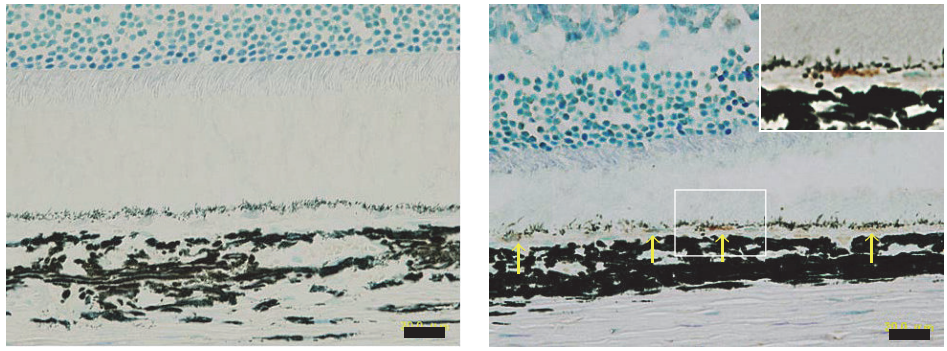
(a) Spermidine-1 (b) Spermidine-2

(c) Spermidine + NAC-1 (d) Spermidine + NAC-2

(e) Spermidine + ALDH-1 (f) Spermidine + ALDH-2

Figure 8: Protective effects of N-acetylcysteine (NAC) and aldehyde dehydrogenase (ALDH) on spermidine-induced degeneration of the retinal pigment epithelium (RPE) and photoreceptors in rats. Cross-sections of rat eyes were prepared 13 days after the intravitreal injection of (a, b) spermidine (20 nmol/eye) alone, (c, d) spermidine (20 nmol/eye) plus NAC (500 nmol/eye), and (e, f) spermidine (20 nmol/eye) plus ALDH (1.5 U/eye). Black and white scale bars indicate 50 and 20 μm, respectively. NFL-GCL: nerve fiber layer and ganglion cell layer. IPL: inner plexiform layer. INL: inner nuclear layer. OPL: outer plexiform layer. ONL: outer nuclear layer. IS-OS-RPE: inner segment, outer segment, and retinal pigment epithelium. C: choroid.

In contrast to our study, Noro et al. showed that spermidine has a protective effect on retinal ganglion cells in mouse models of optic nerve injury and normal tension glaucoma [44, 45]. In Noro's reports, spermidine was administrated by drinking water at a concentration of 30 mM [44, 45]. The effective retinal concentration of spermidine by administration via drinking water containing this agent would be expected to be much lower than what we obtained by intravitreal injection of this compound (1–3 mM). At different concentrations in the retina, spermidine indeed may demonstrate pleiotropic effects. At an optimal, permissive concentration, spermidine is known to be necessary for RPE proliferation [13], but excess spermidine is shown to cause RPE cell death [15]. Therefore, the contrasting effects



(a) Control, day 1 (b) Spermidine (20 nmol/eye), day 3

Figure 9: Immunohistochemistry of acrolein in spermidine-treated rat retina. Immunohistochemical localization of acrolein was performed using cross-sections of rat eyes which were prepared (a) 1 day and (b) 3 days after the intravitreal injection of DPBS or spermidine (20 nmol/eye). Scale bars indicate 20 μm. Inserted photograph in (b) is an enlarged image of the same slide.

of spermidine between Noro's studies and our own may be explained by the different concentrations of spermidine effectively delivered to the retina.

The clinical significance of spermidine oxidation in the posterior segment of the eye is currently unclear, as increased spermidine levels have not been reported in human diseases involving RPE degeneration. Further studies to evaluate spermidine concentrations and amine oxidase activity in patients with dry AMD and gyrate atrophy are needed to clarify this point. However, the phenotype of spermidine-induced retinal degeneration described herein shares some common features with the phenotypes of dry AMD [2, 3] and gyrate atrophy [4]. Additionally, the involvement of oxidative stress in this model has been proposed as a general mechanism of RPE degeneration in dry AMD [5]. Moreover, spermidine might be involved in the pathogenesis of gyrate atrophy with hyperornithinemia, as spermidine is an ornithine metabolite [7]. Therefore, an animal model of spermidine-induced RPE degeneration could be potentially useful as a model of both dry AMD and gyrate atrophy.

In conclusion, the results of this study demonstrate that the intravitreal administration of spermidine induces rat RPE cell dysfunction and death, leading to photoreceptor cell degeneration. These effects of spermidine are thought to be mediated by oxidative stress and a toxic aldehyde generated by amine oxidase as a consequence of spermidine oxidation. Although the effect of spermidine on the inner retina was not fully clarified, following further validation, this novel animal model will likely be not only useful for elucidating the pathophysiology of dry AMD and gyrate atrophy, but also useful for screening potential therapies for these diseases.

Competing Interests

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

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Review Article

Targeting Oxidative Stress for Treatment of Glaucoma and Optic Neuritis

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Glaucoma is a neurodegenerative disease of the eye and it is one of the leading causes of blindness. Glaucoma is characterized by progressive degeneration of retinal ganglion cells (RGCs) and their axons, namely, the optic nerve, usually associated with elevated intraocular pressure (IOP). Current glaucoma therapies target reduction of IOP, but since RGC death is the cause of irreversible vision loss, neuroprotection may be an effective strategy for glaucoma treatment. One of the risk factors for glaucoma is increased oxidative stress, and drugs with antioxidative properties including valproic acid and spermidine, as well as inhibition of apoptosis signal-regulating kinase 1, an enzyme that is involved in oxidative stress, have been reported to prevent glaucomatous retinal degeneration in mouse models of glaucoma. Optic neuritis is a demyelinating inflammation of the optic nerve that presents with visual impairment and it is commonly associated with multiple sclerosis, a chronic demyelinating disease of the central nervous system. Although steroids are commonly used for treatment of optic neuritis, reduction of oxidative stress by approaches such as gene therapy is effective in ameliorating optic nerve demyelination in preclinical studies. In this review, we discuss oxidative stress as a therapeutic target for glaucoma and optic neuritis.

1. Introduction

Glaucoma is a neurodegenerative disease of the eye and it is one of the major causes of irreversible blindness. It is estimated that, by 2020, more than 80 million people will be affected worldwide, with at least 6 to 8 million of them becoming bilaterally blind [1]. Glaucoma is characterized by damage to the optic nerve and progressive degeneration of retinal ganglion cells (RGCs), which are critical elements for vision loss. The factors associated with pathogenesis of glaucoma include high intraocular pressure (IOP), increased oxidative stress, aging, glutamate neurotoxicity, and susceptibility genes such as optineurin and myocilin [2–4].

Optic neuritis is a demyelinating inflammation of the optic nerve and it typically affects young adults ranging from 18 to 45 years of age. Patients usually present with an acute reduction of visual acuity, orbital pain exacerbated by eye movements, dyschromatopsia, and an afferent papillary defect, with or without swelling of the optic nerve head. There is a strong association between optic neuritis and multiple sclerosis (MS), an acute inflammatory demyelinating disease of the central nervous system (CNS), in which optic neuritis is the initial presentation of MS for approximately 20% of MS patients and a risk of developing MS by 15 years after the onset of optic neuritis is 50% [5]. Research into optic neuritis is somewhat limited compared with MS research, but it is an important area of research that is continuously making progress.

In this review, we discuss the role of oxidative stress in the pathogenesis of glaucoma and optic neuritis and how we can target oxidative stress for treatment of these two disease conditions.

2. Oxidative Stress and Glaucoma

Oxidative stress reflects an imbalance between the production of reactive oxygen species (free radicals) and antioxidant defenses, in which oxidative processes exceed antioxidant systems. Oxidative stress is an important risk factor in human

glaucoma [6] and consistently, the plasma level of glutathione (GSH), an important antioxidant, is decreased in glaucoma patients [7, 8]. Normal tension glaucoma (NTG) is a subtype of glaucoma that does not present with high IOP and there is an unexpectedly high prevalence of NTG in Japan and other Asian countries [9, 10]. Previously, we reported spontaneous mouse models of NTG; these mice lacked the glutamate transporter genes *EAAC1* and *GLAST*, in which EAAC1 is expressed in neurons and GLAST is expressed in Müller glia in the retina [11]. These mice exhibit spontaneous RGC death and optic nerve degeneration without an increase in IOP, a pathology that is similar to NTG. Glutamate transporters clear excess glutamate from the synapse, thus preventing excitotoxic damage on surrounding retinal neurons [12]. In addition, glutamate that is transported into cells by the glutamate uptake process, together with cysteine and glycine, is converted to GSH, a major antioxidant in the retina [13]. Therefore, glutamate transporters play important roles in reducing excitotoxic and oxidative stress damage to cells. To this end, GLAST KO mice and EAAC1 KO mice exhibit the key pathological features of NTG as a result of increased glutamate neurotoxicity and oxidative stress. These mice have been useful in providing important information on therapeutic targets for NTG [14–23].

Although glaucoma therapy that is currently available focuses on reduction of IOP, some patients do not respond to this type of treatment and research into neuroprotection of RGCs as a novel therapeutic strategy is advancing. One of such strategies is reduction of oxidative stress [4]. For example, an antioxidant α-lipoic acid protects RGCs in the glaucomatous retina in DBA/2J mice, an animal model that recapitulates the slow and progressive nature of human glaucoma [24, 25], and administration of another antioxidant, tempol, reduces RGC death in an experimental glaucoma model [26]. Furthermore, geranylgeranylacetone (GGA), which is used for treatment of gastric ulcers, can act as an antioxidant by directly inducing the cytoprotective heat shock protein 70 (Hsp70) expression and inhibits cell apoptosis caused by H_2O_2 in cultured hepatocytes [27]. It also reduces oxidative stress levels following light-induced retinal damage [28] and increases survival of retinal neurons in an ischemic retinal injury model [29]. Oral administration of GGA induces Hsp70 expression in the retina and suppresses RGC death in GLAST KO mice, a mouse model of NTG [22]. Therefore, targeting to reduce oxidative stress in the retina may be a novel therapeutic strategy for glaucoma.

3. Inhibition of Oxidative Stress for Treatment of Glaucoma

The summary of this section is shown in Table 1.

3.1. Apoptosis Signal-Regulating Kinase 1 (ASK1). Apoptosis signal-regulating kinase 1 (ASK1) is a member of mitogen-activated protein kinase kinase kinase (MAP3K) that plays key roles in cellular responses to oxidative stress and endoplasmic reticulum stress [30, 31]. ASK1 acts downstream of tumor necrosis factor alpha (TNF-α) signalling and is a key regulator of stress- and cytokine-induced apoptosis [32]. It has been reported that stress such as serum withdrawal or TNF-α generates ROS that activates ASK1 by removing a physiological inhibitor of ASK1, thioredoxin, and initiates the ASK1-mediated apoptotic pathway [33]. ASK1 is strongly activated in response to various oxidants such as H_2O_2 and the activation of the ASK1-JNK/p38 pathway plays an essential part in oxidative stress-induced apoptosis [34]. We have previously reported that deletion of the *ASK1* gene prevents RGC death in various mouse models of glaucoma, including retinal ischemia, optic nerve injury (ONI), and GLAST KO mice (GLAST/ASK1 double KO mice) [15, 35, 36]. In all the models we have used, ASK1 deficiency reduced oxidative stress levels that led to increased RGC survival, indicating that targeting oxidative stress is an effective approach for treatment of glaucoma. It is important to note that the therapeutic effect of ASK1 deletion may also involve reduction of factors that cause oxidative stress, such as TNF-α [37, 38], which mediates neurodegeneration in glaucoma [39]. Currently, we are examining if a therapeutic effect is achieved by oral administration of an ASK1 inhibitor in EAAC1 KO mice, a spontaneous mouse model of NTG [11], to further confirm that ASK1 inhibition is a promising target for treatment of glaucoma.

Table 1: Possible genes and drugs targeting oxidative stress for the treatment of glaucoma and optic neuritis.

Therapeutic target	Glaucoma	Optic neuritis
ASK1	References [15, 35, 36, 68]	References [37, 68, 69]
Dock3	References [16, 42, 43, 47]	
VPA	References [19, 50, 53]	Reference [69]
Spermidine	References [20, 59]	Reference [70]
Candesartan	References [17, 61]	
Nrf2	References [64–66]	
Brimonidine	References [18, 71, 72]	Reference [73]

3.2. Dedicator of Cytokinesis 3 (Dock3). Dedicator of cytokinesis 3 (Dock3) belongs to a family of atypical guanine exchange factors (GEFs). It is specifically expressed in the CNS and regulates actin cytoskeleton dynamics causing cellular morphological changes by activating the small GTPase Rac1 [40, 41]. Recent studies have indicated that Dock3 acts downstream of the brain-derived neurotrophic factor-(BDNF-) TrkB pathway [42] and possesses functions that are independent of its GEF activity: for example, it directly binds to GSK-3β and stimulates microtubule dynamics to promote optic nerve regeneration [43, 44]. Interestingly, Dock3 also binds to GluN2B, one of the subunits for *N*-methyl-D-aspartate (NMDA) receptors, and reduces the NMDA receptor expression leading to RGC protection from NMDA-induced cell death and in GLAST KO mice [16]. Stimulation of NMDA receptors leads to superoxide production and neurotoxicity in neurons [45, 46]. Therefore, it is possible that Dock3 reduces oxidative stress indirectly by attenuating NMDA receptor activation. In addition, overexpression of Dock3 in cultured RGCs increases cell survival following

H_2O_2 stimulation and in vivo, the activation of the ASK1-p38 pathway is decreased in mice with Dock3 overexpression following ONI [16, 47]. These results suggest the possibility that Dock3 prevents oxidative stress-induced RGC death by suppression of the ASK1 pathway. Further studies are required to confirm this.

3.3. Valproic Acid (VPA). Valproic acid (VPA) is a short chain fatty acid that has been used clinically worldwide for treatment of epilepsy since the 1970s. It exerts multiple pharmacological actions and one of the recently identified effects is inhibition of histone deacetylases, which is distinct from its therapeutic antiepileptic activity [48, 49]. Recently, we reported that VPA prevents glaucoma-like retinal degeneration in mouse models of glaucoma, by inhibition of the oxidative stress level in the RGCs and by stimulation of the BDNF-TrkB pathway [19, 50]. In addition, VPA has been shown to exert antioxidant properties in the brain following ischemia/reperfusion injury [51] and in motor neurons following spinal cord injury [52]. Since VPA has been reported to increase activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase in the retina following ischemia/reperfusion injury [53], it can be postulated that VPA acts on RGCs as an HDAC inhibitor resulting in increased expression of antioxidant enzymes such as SOD and catalase in glaucoma. Interestingly, clinical studies have reported that oral administration of VPA improves visual function in patients with retinitis pigmentosa, which is a group of hereditary eye disease that is characterized by selective degeneration of photoreceptors [54–56]. VPA is a drug that is already established for use in treatment of conditions other than retinal diseases, like epilepsy, with relatively minor side effects. Together with the data to indicate its therapeutic efficacy in glaucoma and retinitis pigmentosa, VPA is a suitable candidate for "drug repurposing," which is an application of known drugs to new conditions. VPA is a promising therapeutic candidate for glaucoma and retinitis pigmentosa and further studies are required to assess its efficacy and safety for retinal diseases.

3.4. Spermidine. Spermidine is a naturally occurring polyamine that is essential for life. There is an association between decline of spermidine concentration and human aging, and exogenous application of spermidine extended the lifespan of yeast, flies, worms, and human immune cells by promoting autophagy, which leads to enhanced resistance to oxidative stress and decreased cell death [57]. Indeed, spermidine-treated yeast cells and mouse fibroblast cells are more resistant to damage induced by H_2O_2 treatment than nontreated cells, and feeding mice with spermidine increases the serum level of free thiol groups, indicating that spermidine reduces oxidative stress both in vitro and in vivo [57, 58]. We previously reported that spermidine prevents RGC death and visual impairment following ONI and in EAAC1 KO mice, by reducing oxidative stress levels in the retina (Figure 1) [20, 59]. Interestingly, spermidine inhibits activation of the ASK1-p38 pathway in RGCs and suppresses inducible nitric oxide synthase (iNOS) expression in microglia following ONI [59]. These findings indicate that oral intake of spermidine and its antioxidative effects are beneficial in treatment of glaucoma and traumatic optic neuropathy. Spermidine is a natural component of our diet and evidence shows that eating food that is rich in spermidine, such as soybeans and mushrooms, results in increased blood spermidine levels [60], suggesting that beneficial effects of spermidine can be easily attained by making a conscious choice of food.

3.5. Candesartan. Candesartan is an angiotensin II receptor antagonist that is clinically used for treatment of hypertension. It modulates the renin-angiotensin system, which regulates the arterial blood pressure and thus plays a major role in the cardiovascular system. The renin-angiotensin system has been reported to be involved in oxidative stress-induced RGC death [61]. Indeed, we reported that suppression of the renin-angiotensin system by candesartan led to RGC protection and preservation of visual function in EAAC1 KO mice [17], suggesting that this drug may be a good drug repurposing candidate for glaucoma therapy. In the EAAC1 KO mouse retina, the expression of Toll-like receptor 4 (TLR4) is increased, which stimulates the ASK1 signalling pathway and upregulates iNOS expression in Müller glia leading to RGC death (Figure 2). Candesartan demonstrates neuroprotective effects by suppression of TLR4 upregulation in EAAC1 KO mice [17]. TLR4 polymorphisms are associated with NTG [62, 63]. Therefore, targeting TLR4 may be a promising strategy for treatment of glaucoma.

3.6. Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by oxidative stress and is a master regulator of various antioxidant pathways. Consequently, Nrf2 KO mice are susceptible to a wide range of toxicity and disease conditions associated with oxidative stress. Following ONI, RGC death is significantly increased in Nrf2 KO mice [64], and gene therapy with Nrf2 reduces RGC death [65]. In addition, agents such as α-lipoic acid and VPA exert neuroprotective effects by inhibition of ROS generation through activation of the Nrf2/HO-1 pathway [66, 67]. These findings suggest that activation of Nrf2 may be an effective therapeutic target for glaucoma.

4. Oxidative Stress and Optic Neuritis

In preclinical studies, experimental autoimmune encephalomyelitis (EAE), which is an animal model of MS, is often used to study optic neuritis. There are accumulating data that indicate oxidative stress plays a major role in the pathogenesis of MS and EAE [74]. Indeed, studies demonstrate that antioxidants are effective in suppressing inflammation in the optic nerve. For example, lipoic acid, a natural antioxidant, ameliorates inflammation and protects the optic nerve in EAE mice [75], and spermidine reduces oxidative stress in the optic nerve as well as in the RGCs in EAE mice leading to reduced optic nerve demyelination, RGC death, and visual impairment [70]. Another drug of interest is GGA, which reduces oxidative stress and is effective in protection of RGCs in glaucoma models as mentioned earlier.

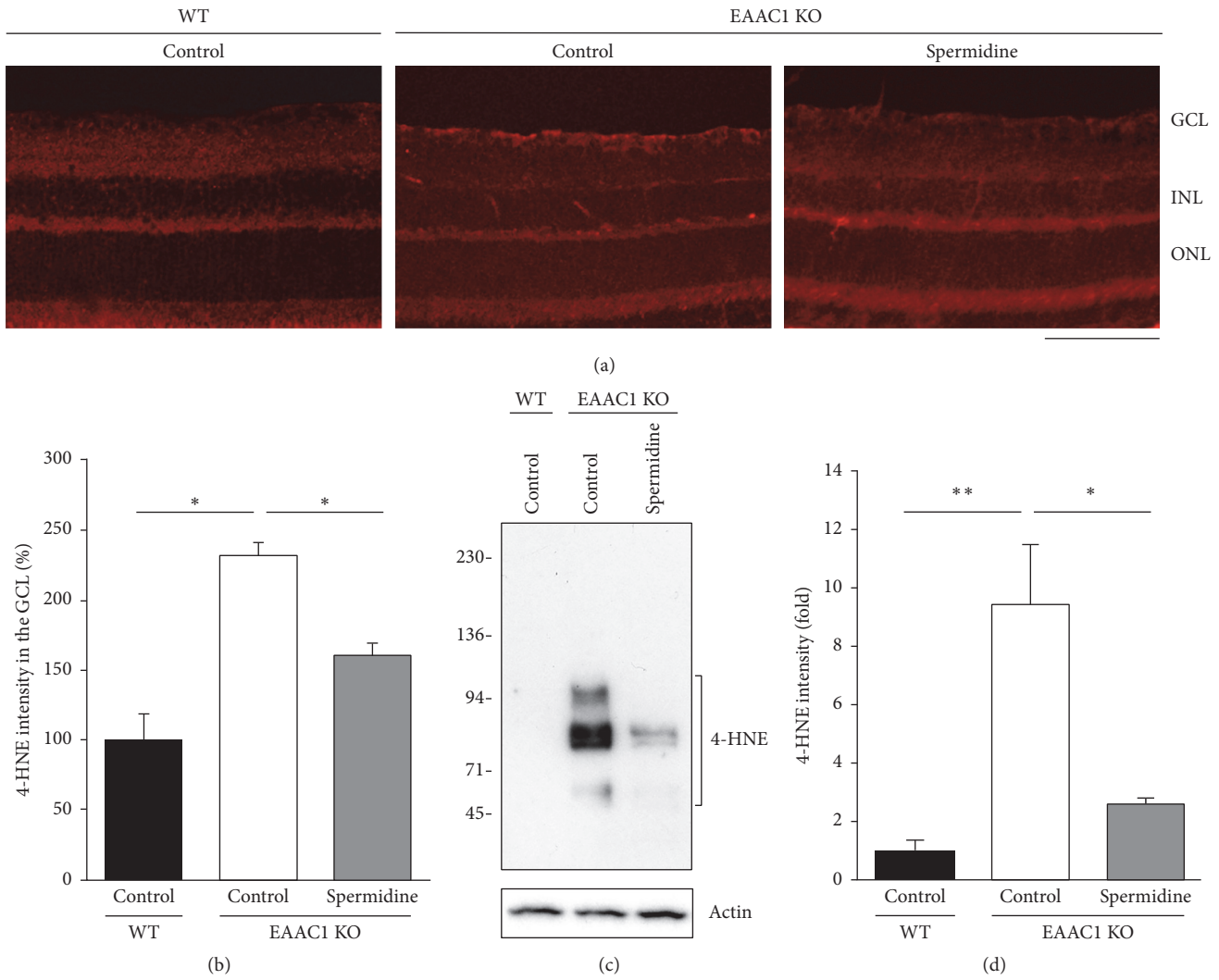

Figure 1: Spermidine reduces oxidative stress levels in the EAAC1 KO mouse retina. (a) Representative images of 4-HNE in the retina at 8 weeks old. Scale bar: 100 μm. (b) Quantitative analyses of (a). Data are normalized to the 4-HNE intensity at the GCL in control WT mice (100%). $n = 6$ in each group. (c) Representative images of immunoblot analyses of 4-HNE in the retina at 8 weeks old. (d) Quantitative analyses of (c). Data are normalized to the 4-HNE intensity in control WT mice (1.0). $n = 6$ in each group. $^{**}P < 0.01$; $^{*}P < 0.05$. Reproduced from Noro et al. [20].

We previously reported that oral administration of GGA suppresses demyelination of the optic nerve, RGC death, and visual impairment in EAE mice [76], suggesting that GGA is a good therapeutic candidate for optic neuritis. Furthermore, gene therapy with antioxidant genes, namely, *SOD2* and *catalase*, was effective in reducing optic nerve demyelination, axonal loss, and RGC loss in EAE mice [77, 78]. These findings suggest that oxidative stress is associated with the pathogenesis of optic neuritis and is an effective target for its treatment.

5. Inhibition of Oxidative Stress for Treatment of Optic Neuritis

The summary of this section is shown in Table 1.

5.1. ASK1 and Optic Neuritis. In addition to immune cells such as T cells and dendritic cells, glial cells play important roles in demyelinating neuroinflammation [79, 80]. Indeed, the ASK1-p38 pathway in astrocytes and microglia plays essential roles in release of key cytokines including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), regulated on activation, normal T cell expressed and secreted (RANTES), and TNF-α during neuroinflammation [37]. In EAE ASK1 KO mice, reduction of such proinflammatory cytokines as well as decrease in upregulation of iNOS leads to suppression of neuroinflammation and demyelination of the optic nerve, suggesting oxidative stress plays a part in degeneration of the optic nerve in this model [37]. Since VPA ameliorates inflammation of the spinal cord in EAE mice by suppressing

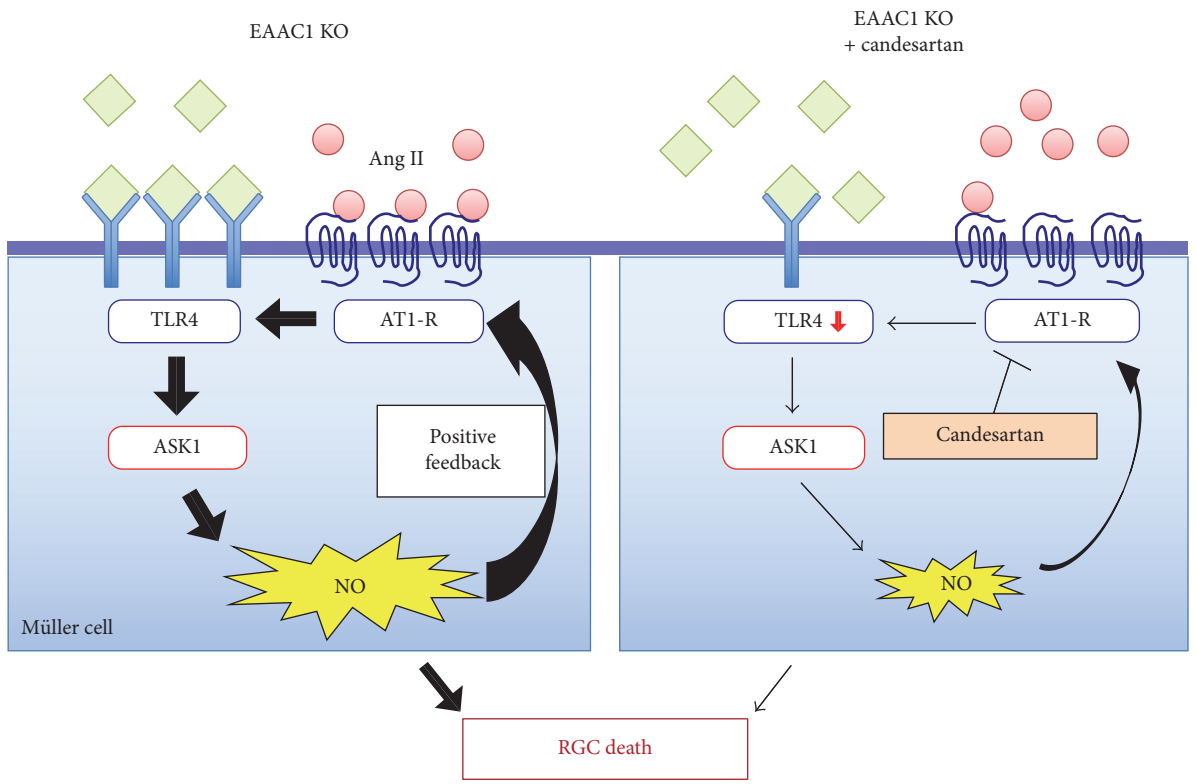

Figure 2: The proposed model of the effect of candesartan in EAAC1 KO mice. Increased oxidative stress in EAAC1 KO mice induces the upregulation of AT1-R and TLR4, resulting in increased NO expression via the ASK1 signalling pathway, which leads to RGC death. NO further stimulates AT1-R expression levels through a positive feedback loop. Candesartan blocks AT1-R and exerts neuroprotective effects by suppressing the upregulation of TLR4 and thus reducing ASK1-mediated NO production. This also results in inhibition of the positive feedback loop between NO and AT1-R. Reproduced from Semba et al., [17].

the activation of T cells [81], we applied VPA to EAE ASK1 KO mice and found that VPA and ASK1 inhibition have synergistic therapeutic effects during EAE [69]. In addition, EAE induces reduction in visual function, which can be assessed by electroretinogram, but ASK1 deficiency ameliorates this visual impairment [37], suggesting that inhibition of ASK1 is effective both histologically and functionally. We have previously demonstrated that oral administration of an ASK1 inhibitor, MSC2032964A, is effective in suppressing neuroinflammation and demyelination in EAE mice [37]. These results suggest that inhibition of ASK1 is a promising strategy for treatment of optic neuritis. In fact, suppression of oxidative stress with inhibition of the ASK1 activity holds therapeutic potential for various neurodegenerative diseases such as MS and glaucoma [68].

5.2. Brimonidine. Brimonidine is an α_2-adrenergic receptor agonist that is clinically used to lower IOP in glaucoma patients. Recent studies indicated that the therapeutic effect of brimonidine does not solely depend on reducing IOP. For example, brimonidine increases cultured RGC survival from oxidative stress damage [71], it increases glial expression of neurotrophic factors that are important for RGC survival and decreases phosphorylation of the GluN2B subunit in the retina, thereby reducing activation of the NMDA receptors leading to reduced RGC death [18]. These data suggest that pharmacological actions of brimonidine may include suppression of oxidative stress directly and indirectly. In EAE mice, daily treatment with brimonidine eyedrops led to reduction in RGC death and visual function, suggesting that brimonidine is an effective agent for preventing RGC loss and visual impairment in optic neuritis [73]. In this study, brimonidine eyedrops did not have any effect on demyelination of the optic nerve, but this may not be surprising as the route of administration was topical, directly to the eye. Since brimonidine exerts neuroprotective effects against glutamate excitotoxicity-induced oxidative stress [72] and oligodendrocytes in the optic nerve are vulnerable to glutamate neurotoxicity [82], it would be interesting to investigate if brimonidine can indeed prevent demyelination if administered *via* a different route, such as systemically or directly into the optic canal.

6. Conclusions

Oxidative stress plays an important part in the pathogenesis of neurodegenerative disease and neuroinflammation. Furthermore, increased oxidative stress is associated with aging [83] and with drastic increase in life expectancy worldwide [84], there is an urgent need to cure or manage age-related chronic neurodegenerative conditions such as glaucoma, Alzheimer's disease, and Parkinson's disease.

Currently, established treatment for glaucoma and optic neuritis does not involve targeting oxidative stress. However, preclinical data indicate that suppression of oxidative stress is a promising strategy for many eye diseases including glaucoma and optic neuritis.

Competing Interests

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

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Review Article

Oxidative Stress-Related Mechanisms and Antioxidant Therapy in Diabetic Retinopathy

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Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes and is the leading cause of blindness in young adults. Oxidative stress has been implicated as a critical cause of DR. Metabolic abnormalities induced by high-glucose levels are involved in the development of DR and appear to be influenced by oxidative stress. The imbalance between reactive oxygen species (ROS) production and the antioxidant defense system activates several oxidative stress-related mechanisms that promote the pathogenesis of DR. The damage caused by oxidative stress persists for a considerable time, even after the blood glucose concentration has returned to a normal level. Animal experiments have proved that the use of antioxidants is a beneficial therapeutic strategy for the treatment of DR, but more data are required from clinical trials. The aims of this review are to highlight the improvements to our understanding of the oxidative stress-related mechanisms underlying the development of DR and provide a summary of the main antioxidant therapy strategies used to treat the disease.

1. Introduction

Diabetes, a chronic metabolic disease, includes types I (lack of insulin) and II (insulin resistance). Globally, approximately 415 million people suffer from diabetes, and one person dies every six seconds with this disease. Moreover, a new patient is diagnosed every two seconds [1]. Diabetic retinopathy (DR), a sight-threatening microvasculature impairment, is one of the complications that seriously threaten the life of diabetic patients. DR is acknowledged as the main cause of blindness among working-age adults throughout the world [2]. In 2010, there were 126.6 million patients with DR, and it is predicted that this figure will increase to 191.0 million by 2030; consequently, the number of those with sight-threatening DR will increase from 37.3 million to 56.3 million during this period [3].

The development of DR is associated with sustained metabolic disorders caused by hyperglycemia and increased levels of inflammatory cytokines in the blood. Systemic inflammation caused by these metabolic alternations leads to hemodynamic changes, blood-retinal barrier (BRB) damage, the leakage of retinal microvessels and edema, a gradual thickening of the retinal vascular basement membrane, and a loss of pericytes. These lesions continue with the progression of diabetes. During the first few years of diabetes, patients may barely be aware of, or exhibit, retinal injury. However, the obvious symptoms of DR will be present in nearly all patients with type I diabetes for 20 years and in nearly 80 percent of those with type II diabetes for the same duration [4]. Therefore, an understanding of the mechanisms by which these pathogenic alterations are induced will improve the development of effective strategies to prevent and postpone the progression of DR.

Oxidative stress is thought to be one of the crucial factors in the pathogenesis of DR. Abnormal metabolism induced by hyperglycemia can result in the overproduction of free radicals such as hydroxyl and superoxide radicals, which are known as reactive oxygen species (ROS) [5]. The accumulation of ROS can lead to oxidative stress, which damages the tissue in and around retinal vessels, ultimately

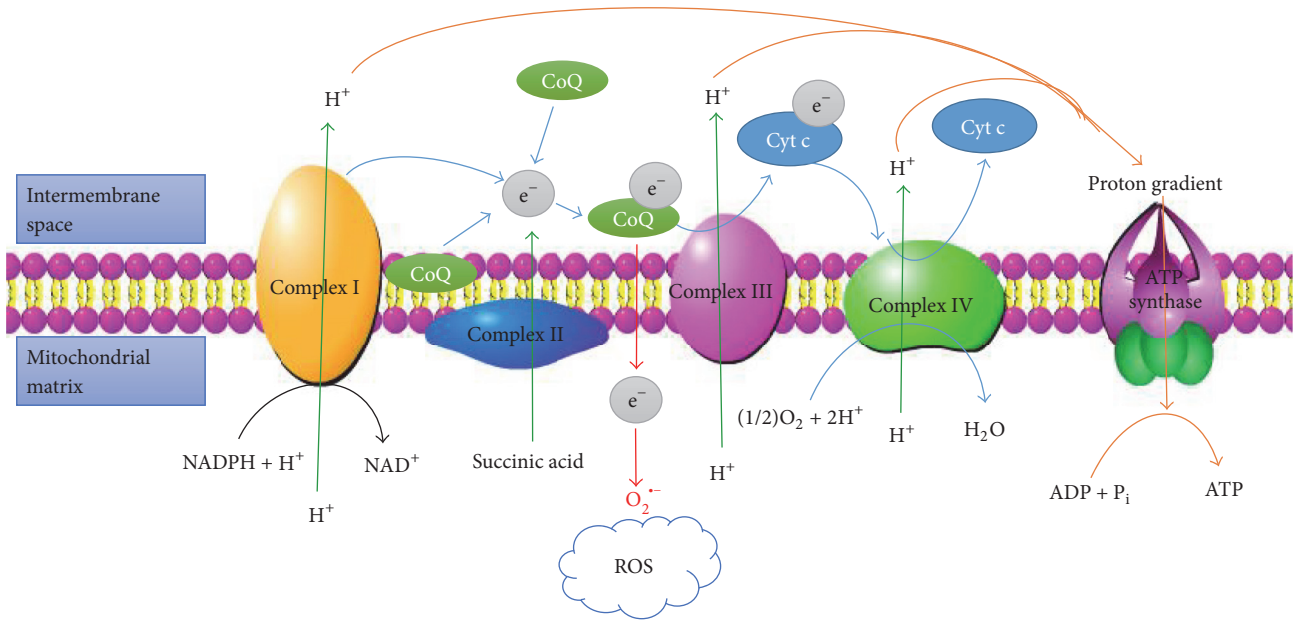

Figure 1: The electron transport in the mitochondrial respiratory chain and the production of ROS. Complexes I–IV are located in the mitochondrial inner membrane. First, complexes I and II can accept electrons from NADPH and succinic acid and then transport them to coenzyme Q (CoQ), while at the same time, they can pump out protons. Then, complex III can transfer electrons from CoQ to cytochrome C (Cyt c). Finally, complex IV sends electrons to O_2, producing H_2O. During this process, a proton gradient is formed, which promotes the synthesis of ATP. If complex III cannot receive electrons from CoQ, the electrons would be accepted by O_2, which could produce ROS and result in oxidative stress.

resulting in DR. It is well known that hyperglycemia can cause vascular damage through four classical mechanisms: increased polyol pathway flux; increased intracellular formation of advanced glycation end-products (AGEs) and expression of the receptor for AGEs; activation of the protein kinase C (PKC) pathway; and activation of the hexosamine pathway [6]. Moreover, the nuclear factor, erythroid 2 like 2- (NFE2L2-) related pathway (NFE2L2 is also known as Nrf2), GTP-binding proteins, and epigenetic modifications have also attracted increasing attention in recent years [7–9]. All these pathways are associated with the overproduction of ROS. In particular, oxidative stress induced by epigenetic modifications can persist for a considerable time, even after the blood glucose concentration returns to normal; this is called "metabolic memory" [10]. Thus, scavenging and/or reducing ROS production by these pathways may provide new therapeutic strategies. At present, multiple antioxidants have been used in clinical trials, but the results are not clear. In this review, we summarize several of the mechanisms induced by oxidative stress that cause DR and focus on the latest research into the treatment of the disease using antioxidants.

2. Diabetes-Induced Production of ROS

Oxidative stress can induce damage to target organs such as the retina [11]. The production of ROS mainly depends on two factors: (a) mitochondrial oxidative phosphorylation [12] and (b) the nicotinamide adenine dinucleotide phosphate-(NADPH-) oxidase (Nox) system [13]. Mitochondria are the major endogenous source of ROS and can utilize 95% of the available oxygen to produce ATP (Figure 1). Normally, ~2% of oxygen enters the electron transport chain and is subsequently oxidized to superoxides such as O^{2-} and hydrogen peroxide. In diabetes, the uncoupling of mitochondrial electron transport leads to excessive superoxide production, which may stimulate several abnormal biochemical metabolic pathways, such as the polyol, PKC, and AGE pathways [14]. It has been reported that the levels of transcription of mitochondrial DNA-encoded NADH dehydrogenase 1 and 6 of complex I and cytochrome b of complex III are subnormal in the retinas of diabetic patients [15, 16], which contributes to the development of DR. Moreover, superoxides can also react with nitric oxide (NO) to form peroxynitrite. Peroxynitrite can oxidize small-molecule antioxidants such as glutathione (GSH), cysteine, and tetrahydrobiopterin [17], resulting in cytotoxicity due to lipid peroxidation, inactivation of enzymes by oxidation of sulfhydryl moieties and nitration of tyrosine, and damage to DNA [14, 18, 19].

The Nox system can generate ROS and catalyze molecular oxygen to produce superoxides and/or hydrogen peroxide by accepting electrons from NADPH and transporting them to molecular oxygen. The Nox system is a major source of oxidative stress in the vascular system and can be divided into different isoforms. Vascular Nox comprises membrane-bound subunits (Nox protein and p22phox) and cytosolic subunits (p47phox, p67phox, and Rac). Isoforms Nox 1, Nox 2, and Nox 4 are highly expressed in the vascular system [20]. The activity of Nox 2 increases in the retinas of diabetic

mice. This increase is associated with increased production of ROS and the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF), which can be inhibited by deletion of the Nox 2 gene [21]. A recent study has shown that Nox 1 expression and activity increase in the microvascular endothelial cells in the brains of diabetic mice [22]. Similar findings have also been reported in kidney vessels, coronary microvessels, and aortic endothelial cells [23, 24].

3. Hyperglycemia-Induced Pathological Changes in DR

The retina is the most metabolically active tissue in the body and is therefore easily affected by diabetes. Sustained hyperglycemia damages the microvasculature of the retina resulting in hemodynamic changes, thickening of the basement membrane, loss of pericytes, and BRB dysfunction [25, 26]. These abnormalities cause retinal ischemia and the release of proinflammatory and proangiogenic factors, which lead to inflammation and angiogenesis. The tight junctions between retinal pigment epithelial (RPE) cells constitute the BRB, which protects the retina from abnormal effusion from the choroid. Hyperglycemia produces elevated levels of methylglyoxal due to increased glycolysis. Methylglyoxal activates matrix metalloproteinases, which may facilitate an increase in vascular permeability by a mechanism involving the proteolytic degradation of tight junction proteins such as occludin. Fluid leakage therefore increases in the surrounding retinal tissue, resulting in macular edema and visual loss [27].

The retina is susceptible to oxidative stress owing to its hypermetabolic state [28], and the levels of oxidative stress markers are related to the severity of DR [29]. Experiments have demonstrated that the degeneration of retinal capillaries in diabetes can be reduced by antioxidant therapy via activation of caspase-3 and nuclear factor-κB (NF-κB), indicating that oxidative stress plays an important role in retinal capillary apoptosis [30, 31]. The dysfunction of rod and cone photoreceptors is thought to contribute to the development of retinal hypoxia and neovascularization [32, 33]. Patients with diabetes and the outer retinal degenerative disorder retinitis pigmentosa have a reduced risk of the development of preproliferative DR [34, 35]. It has been demonstrated that the retinas of rhodopsin knockout mice exhibit vascular attenuation in the capillary bed; however, in the retina of rhodopsin knockout mice with diabetes, it appears to have fewer vascular attenuation than that of nondiabetic counterparts [36]. The reason may be that the loss of rod photoreceptors during retinitis pigmentosa leads to a net reduction in oxygen usage by the retina. Thus, it may offset the exacerbation of hypoxia during DR, thereby protecting the microvasculature from pathogenic change. Furthermore, hyperglycemia-induced endothelial injury can produce reactive nitrogen species through the activation of arginase, leading to disruption of the nitroso-redox balance [37]. Finally, increased levels of nitrogen species, including NO and peroxynitrite, promote leukocyte adhesion to retinal vessels, BRB breakdown, and RPE damage [38, 39].

4. Role of Oxidative Stress in Hyperglycemia-Induced Pathological Changes in DR

4.1. General Mechanisms Underlying DR. As shown in Figure 2, four classical mechanisms are involved in the pathology of DR: increased polyol pathway flux, PKC pathway activity, accumulation of AGEs, and activation of the hexosamine pathway.

Hyperglycemia induces the activation of the polyol pathway and increases intracellular glucose flux [40]. Excess intracellular glucose is converted to sorbitol by aldose reductase, and sorbitol is oxidized to fructose by sorbitol dehydrogenase. During the reaction, these two enzymes can oxidize NADPH to $NADP^+$and convert NAD^+ to NADH, respectively. As a result, NADPH is consumed, the ratio of NADH/NAD^+ increases, and the synthesis of NO and GSH decreases, all of which are important to maintaining the redox balance. Moreover, the accumulation of sorbitol is associated with basement membrane thickening, endothelial cell death, and pericyte apoptosis [41, 42]. Thus, activation of the polyol pathway plays a crucial role in the pathogenesis of DR.

PKC is a serine/threonine-related protein kinase; it mainly has three isoforms that are involved in diabetes: PKC-β, PKC-δ, and PKC-ζ. Hyperglycemia primarily activates PKC-β, which is associated with neovascularization. PKC-β can increase the expression of VEGF [43]. Retinal ischemia induces the overexpression of PKC-β in transgenic mice, whereas a lack of PKC-β reduces angiogenesis [44]. PKC-δ can be activated by aldose reductase [45], and the PKC-δ/p38αMAPK pathway can inhibit platelet-derived growth factor-mediated survival activity, resulting in the apoptosis of pericytes and the formation of acellular capillaries [46]. PKC-ζ can be detected in endothelial cells and is involved in VEGF-mediated proliferation and hyperpermeability induced by tumor necrosis factor-α (TNF-α) and thrombin (Figure 3) [47, 48].

AGEs are the late products of nonenzymatic glycation. Normally, glucose forms a Schiff base through the Maillard reaction; irreversible oxidation and dehydration reactions then convert Amadori products to AGEs [49]. Oxidative stress causes the formation of reactive carbonyl compounds, which react with protein, resulting in the production and accumulation of AGEs [50]. AGEs can cross-link with structural proteins such as elastin, laminin, and collagen type IV, leading to reduced elasticity and sensitivity to proteolytic digestion [51]. In addition, the interaction between AGEs and their cell surface receptor mediates the production of ROS, the activation of NF-κB [52], and the expression of VEGF, monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1. This contributes to hyperpermeability and the breakdown of the BRB, ultimately leading to angiogenesis and thrombosis [53, 54].

Increased levels of ROS under hyperglycemic conditions inhibit the activity of glyceraldehyde-3-phosphate dehydrogenase, which diverts fructose 6-phosphate from the

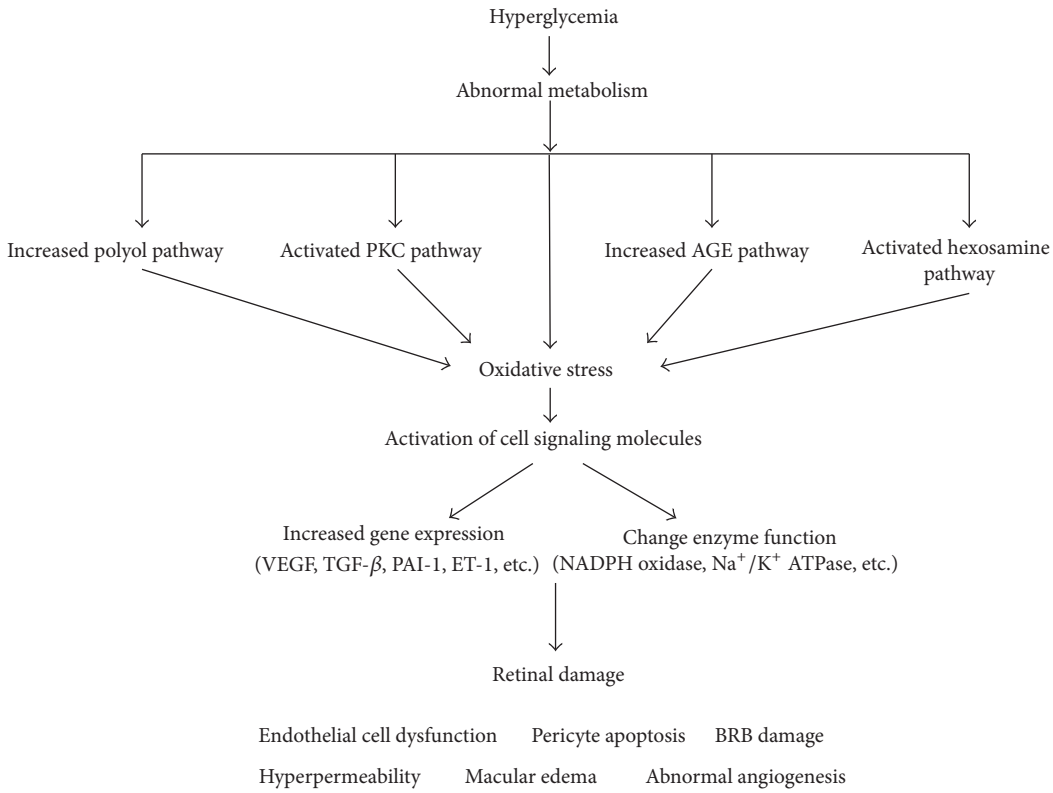

Figure 2: The mechanisms of oxidative stress and diabetic retinopathy. BRB, blood-retinal barrier; ET-1, endothelin-1; NADPH, nicotinamide adenine dinnucleotide phosphate; PAI-1, plasminogen activator inhibitor 1; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

glycolysis pathway to the hexosamine pathway, thereby activating the hexosamine pathway and producing UDP-N-acetylglucosamine [55]. UDP-N-acetylglucosamine is an important substrate in the posttranslational modification of Ser/Thr residues by O-linked β-N-acetylglucosamine. A recent study demonstrated that hyperglycemia promotes the GlcNAcylation of NF-κB, which contributes to the death of retinal gangliocytes [56]. Moreover, elevated angiopoietin-2 (Ang-2) levels cause pericyte loss, which is probably related to the increased GlcNAcylation of stimulating protein-3 (Sp-3) through the promotion of Sp-1 binding to the Ang-2 promoter and activation of the transcription of Ang-2 [57].

4.2. Nrf2-Keap1 Antioxidant Defense System in DR. Nrf2 is a redox-sensitive factor that can regulate the transcription of antioxidant genes and act as a protective factor in inflammation and ischemia/reperfusion injury [58, 59]. Normally, Nrf2 exists in the cytosol and binds to its cytosolic inhibitor Kelch-like ECH-associated protein 1 (Keap1). When oxidative stress occurs, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it binds with the antioxidant-response element (ARE) to regulate the transcription of antioxidant genes (Figure 4) [7]. Thus, the Nrf2-Keap1-ARE pathway is considered one of the major ways by which cells are protected from oxidative stress, and it can regulate the expression of a number of protective factors, such as GSH [7, 60]. GSH biosynthesis can be regulated by glutamate cysteine ligase (GCL), which has a catalytic subunit (GCLC) and a modifier subunit (GCLM). The transcription of GCLC can be regulated by Nrf2 [7]. It has been confirmed that Nrf2 is activated in streptozotocin- (STZ-) induced mouse retinas, and the level of nuclear Nrf2 in diabetic mice is two times higher than in nondiabetic controls. Moreover, in Nrf2-deficient diabetic mice, the levels of ROS, TNF-α, and superoxide increase, and the level of GSH decreases [7, 61]. Under high-glucose conditions, there is a decrease of Nrf2 binding at the antioxidant-response element region 4 (ARE4), leading to the decreased expression of its downstream target gene *GCLC*. Moreover, histone methylation at GCLC-ARE4 can disrupt the binding of Nrf2 with GCLC-ARE4, which is not altered by the termination of high-glucose insult [62]. This suggests an important role of the Nrf2-Keap1-GCLC-GSH signaling pathway in the maintenance of retinal redox status in the development of DR.

Epigenetic modifications of Keap1 also play an important role in the development of DR. Keap1 is a crucial factor during Nrf2 signal transduction because it is a substrate adaptor protein for Cullin3/Rbx1 ubiquitin ligase. The Keap1 promoter has an Sp-1 element site, and the binding of Sp-1 with the Keap1 promoter can activate the transcription of Keap1 [63]. Hyperglycemia can activate methyltransferase enzyme Set7/9 (SetD7), which facilitates the methylation of lysine 4 on histone 3, and increases the binding of Sp-1 to the Keap1 promoter. Moreover, SetD7-siRNA decreases the binding

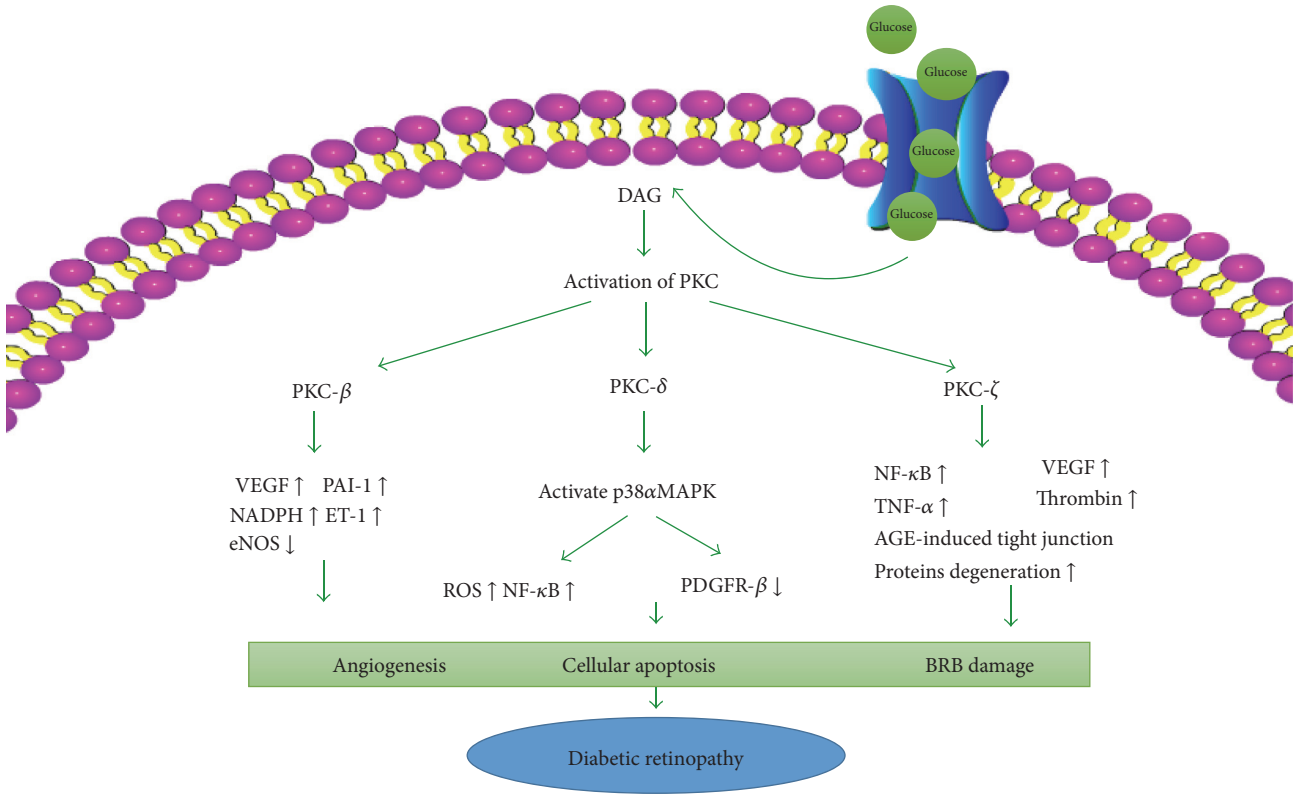

Figure 3: The activation of the three main protein kinase C (PKC) isoforms induced by hyperglycemia. AGE, advanced glycation end-products; BRB, blood-retinal barrier; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; NADPH, nicotinamide adenine dinnucleotide phosphate; NF-κB, nuclear factor-κB; PAI-1, plasminogen activator inhibitor-1; PDGFR, platelet-derived growth factor receptor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

of Sp-1 to the Keap1 promoter and reduces its expression; thus, the functions of Nrf2 are suppressed. Termination of a high-glucose insult fails to reduce the binding of Sp-1 to Keap1, and the methylation of the promoter persists with the increased expression of Keap1, which implies that it contributes to "metabolic memory" [64]. A recent study has shown that NF-κB activity is enhanced by reduction of the deacetylase activity of sirtuin 1 (SIRT1) by high-glucose levels, which leads to downregulation of microRNA-29 (miRNA-29) and inhibition of Keap1/Nrf2 signaling [65]. Accumulating evidence supports the protective role of Keap1 in various cancers via regulation by miRNAs such as miRNA-200a and miRNA-141 [66, 67], although studies on DR are scarce. This suggests that miRNAs may have therapeutic potential for the clinical treatment of DR.

4.3. Novel Small GTP-Binding Proteins in DR. Apart from the classical mechanisms mentioned above, a few studies have recently demonstrated that small GTP-binding proteins play a crucial role in the pathogenesis of DR; these proteins are one of the biological switches of cellular processes. The family of small GTP-binding proteins is one of the regulators of the signaling cascade triggered by oxidative stress. GTP-binding proteins belong to a superfamily consisting of more than 100 members, which range from 20 to 40 kDa in size; there are five major families: Ras, Rho, Rab, Sar1/Arf, and Ran, which regulate processes such as cellular proliferation, survival, and differentiation [68]. These cellular processes cycle between GTP-bound active and GDP-bound inactive states. The Ras and Rho families are regarded as particularly important in the development of DR.

The Ras family is a group of low-molecular weight GTP-binding proteins that are involved in cellular signal transduction; the family includes the three highly homologous proteins H-, K-, and N-Ras [69]. It is well known that diabetes increases oxidative stress and that ROS can influence the interactions between H-Ras and its several effector proteins [70]. The most important effector protein is Raf-1, a threonine/serine kinase. The Ras/Raf complex can, in turn, induce intracellular oxidative stress [71]. It has been demonstrated that the activation of retinal H-Ras in diabetes is mediated by its translocation to the plasma membrane, which can be prevented by simvastatin, an inhibitor that blocks the membrane translocation of H-Ras [72]. Evidence suggests that activated H-Ras produces more ROS, probably through activating Nox [73]. Moreover, H-Ras activation is thought to be involved in cell migration, proliferation, and differentiation, which leads to neovascularization [74]. Previous studies have indicated that the expression levels of H-Ras and its effector protein Raf-1 are increased in the retinas

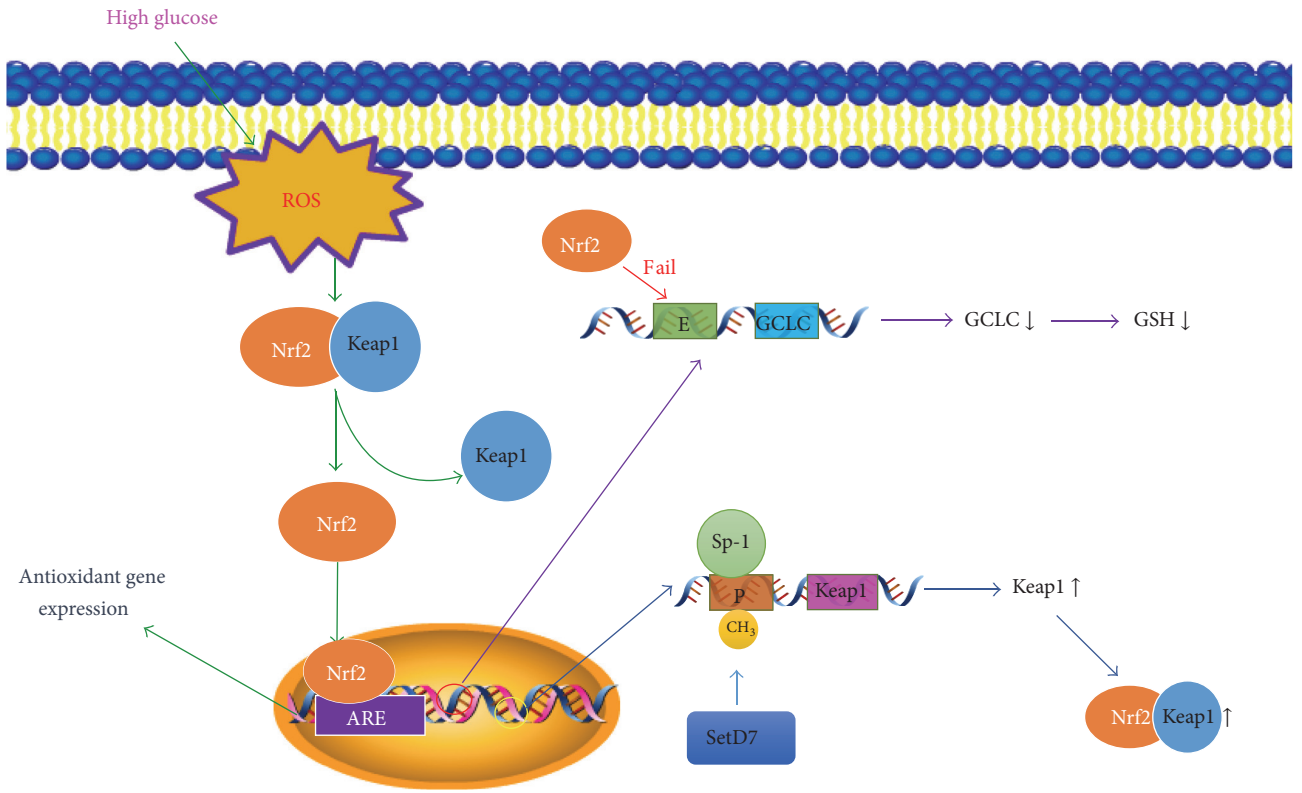

Figure 4: Nrf2-related signal transduction. Oxidative stress activates Nrf2 via dissociating it from its inhibitor Keap1 and translocating to the nucleus, where it binds with the antioxidant-response element (ARE) to regulate transcription of antioxidant genes. At the same time, the expression levels of two other genes are also influenced. On the one hand, Nrf2 fails to bind to the enhancer region of glutamate cysteine ligase catalytic subunit (GCLC), which is an important enzyme in glutathione (GSH) synthesis, thus resulting in the decrease of GSH. On the other hand, hyperglycemia facilities methylation of the Keap1 promoter via methyltransferase enzyme Set7/9 (SetD7), which eases stimulating protein-1 (Sp-1) binding to the promoter, resulting in increased Keap1 expression; therefore, Keap1 combines with Nrf2 and represses Nrf2 in the cytosol. ARE, antioxidant-response element; CH_3, methylation; E, enhancer; GCLC, glutamate cysteine ligase catalytic subunit; GSH, glutathione; P, promoter; SetD7, methyltransferase enzyme Set7/9; Sp-1, stimulating protein-1.

of diabetic animal models, suggesting that H-Ras is involved in retinal capillary apoptosis induced by high-glucose levels; furthermore, inhibitors of retinal H-Ras can prevent the development of DR [75, 76]. Several cell regulatory networks cannot be separated from the Ras/Raf/MEK/ERK pathway, which controls a wide range of downstream targets that regulate gene expression and transcription factors (Figure 5) [69]. There is also evidence that the Raf/MEK/ERK cascade can be activated by ROS, contributing to cellular dysfunction and/or apoptosis [77]. Matrix metalloproteinase-9 (MMP-9), the most complex member of the MMP family, is thought to be involved in vascular permeability and capillary apoptosis in DR owing to its damage to the tight junction complex and activation of caspase-3, respectively [78]. Diabetes causes the activation of MMPs in various tissues, and MMP-9 activity is increased in the retinas of patients and animal models with DR [78, 79]. However, MMP-9 expression is regulated by H-Ras. In rat liver epithelial cells, H-Ras activation can regulate MMP-9 expression [80], and overexpression of H-Ras in human fibroblasts is associated with the upregulation of MMP-9 [81]. Furthermore, MMP-9 activation is decreased following treatment with an H-Ras inhibitor [78]. This suggests that MMP-9 activation is regulated by H-Ras and that MMP-9 appears to be downstream of H-Ras. However, the exact mechanism remains unclear.

The Rho family and its target protein Rho-kinase (ROCK) are involved in cell migration, adhesion, proliferation, and apoptosis [68, 82]. The Rho/ROCK pathway is activated in retinal microvessels during diabetes and affects the expression and function of adhesion molecules, such as ICAM-1, leading to leukocyte adhesion to the microvasculature [82]. In endothelial cells, Rho/ROCK signaling activation is also involved in VEGF-induced migration and angiogenesis [83]. Moreover, it is well known that transforming growth factor-β (TGF-β) is overexpressed in the vitreous of patients with proliferative vitreoretinal disease and that it contributes to the cicatricial contraction of the membrane. Specifically, TGF-β2, the main isoform in the posterior segment of the eye, activates Rho, leading to myosin light chain phosphorylation in hyalocytes [84], which is associated with actin-myosin interaction to form stress fibers and cell contraction [85, 86]. This evidence shows that the activation of ROCK is important in

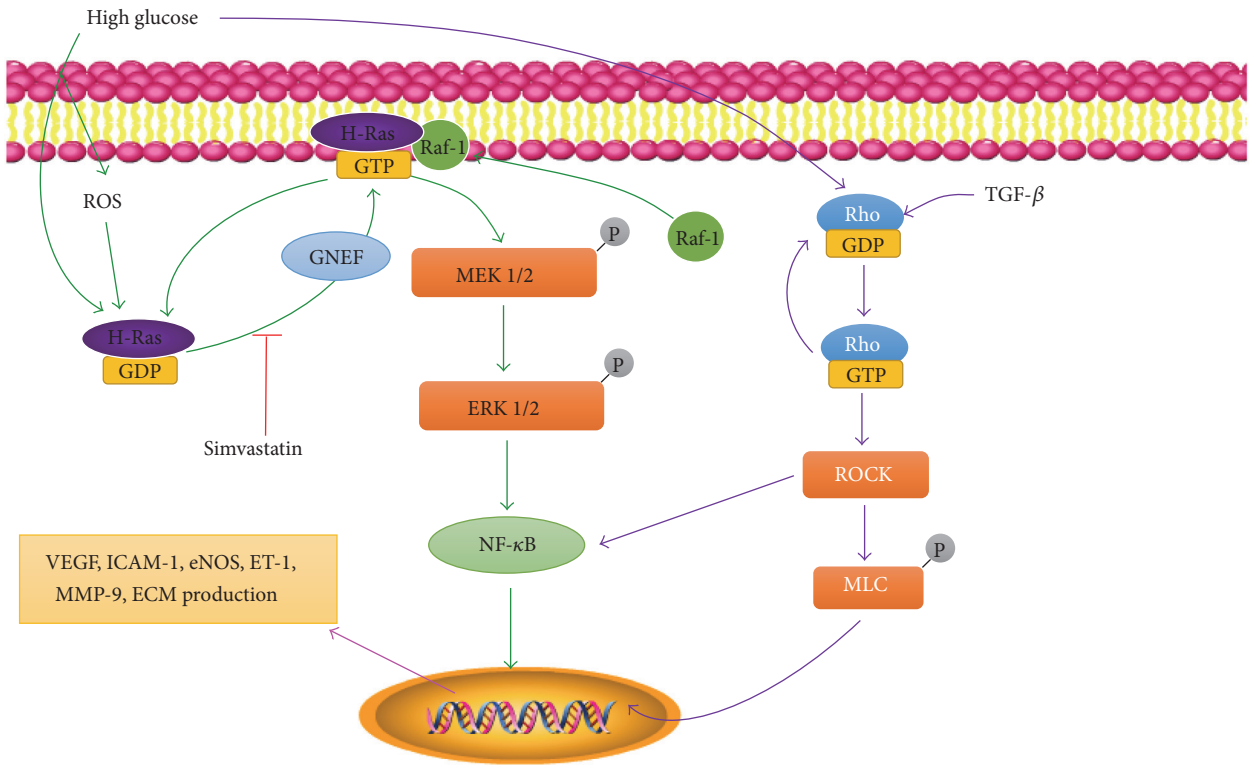

Figure 5: The mechanisms of Ras and Rho proteins in DR. High glucose activates H-Ras via ROS and/or other classical pathways, which contains two steps: H-Ras translocates from the cytosol to the membrane and H-Ras binds to GTP. The former can be inhibited by simvastatin; the latter can be activated by guanine nucleotide exchange factor (GNEF). After that, Raf-1 also can be activated by translocation to the membrane and binding with H-Ras, thus initiating the Ras/Raf/MEK/ERK pathway. On the other hand, Rho can be activated by high glucose and elevate the level of TGF-β, thus promoting the Rho/ROCK pathway. Both of these pathways lead to increased levels of various cytokines involved in DR. ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; ERK 1/2, extracellular signal regulated kinases 1/2; ICAM-1, intercellular adhesion molecule-1; GNEF, guanine nucleotide exchange factor; MEK 1/2, mitogen-activated protein kinase kinase 1/2; MLC, myosin light chain; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-κB; ROCK, Rho-kinase; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

the cicatricial contraction of the proliferative membrane in DR patients.

4.4. Epigenetic Modifications in the Oxidative Stress-Related Pathogenesis of DR. It is well known that the pathogenesis of DR continues after the termination of a high-glucose insult, a phenomenon known as "metabolic memory." It has been suggested that epigenetic modifications are one of the major factors that contribute to the development of DR. Environmental factors, lifestyle, disease progression, and age influence epigenetic changes; DNA methylation, histone modification, and the effects of miRNAs and sirtuin proteins (SIRTs) are considered the major epigenetic modifications.

4.4.1. DNA Methylation. DNA is not a fixed entity; it can modify its properties to adapt to environmental changes. The methylation of cytosine forms 5-methylated cytosine, which alters the chromatin structure by changing protein-DNA interactions; it is considered to be one of the major epigenetic modifications [87]. Normally, CpG islands, which are enriched in the promoter regions, comprise unmethylated cytosines. This guarantees the active expression of genes [88]. In experimental studies, hyperglycemic induction is associated with hypermethylation or hypomethylation of the promoter region in some genes that play critical roles in DR development. For example, research shows that a hyperglycemic milieu can alter the methylation status of the MMP-9 promoter, and the regulation of retinal MMP-9 promoter hypomethylation can prevent mitochondrial damage and the development of DR [89]. Generally, two classes of enzymes are involved in DNA methylation: DNA methyltransferases (DNMTs) and ten-eleven translocation enzymes. The former can transfer a methyl group from S-adenosyl methionine to cytosine [90]; the latter oxidize 5-methylated cytosine to produce 5-hydroxymethyl cytosine, which is easily oxidized to generate 5-formylcytosine and 5-carboxylcytosine [91]. DNMT activity is increased and the expression of DNMT1 is elevated in diabetic retinas and their capillary cells [16]. In the retinas of rats with STZ-induced diabetes, the promoter region of DNA polymerase gamma is hypermethylated, which is sustained even after 3 months of good glycemic control [92]. High levels of glucose also activate ten-eleven translocation enzymes in zebrafish, leading to genome-wide demethylation and aberrant gene

expression [93]. Therefore, DNA methylation is important in the process of DR and metabolic memory.

4.4.2. Histone Modification. Histone modification is another influential factor in diabetes. Chromatin comprises nucleic acids and histones, which are proteins that can regulate the structure of DNA and the expression of various genes. There are five major histone families: H1/H5, H2A, H2B, H3, and H4. Histones constitute the basic unit, the nucleosome, of chromatin and act as a spool around which the DNA is wound. The N-terminal sequences of histones can be modified by acetylation, phosphorylation, methylation, ubiquitination, or sumoylation [87]. Acetylation and methylation are the most common modifications of histones. The acetylation of histones at lysine residues generally occurs with transcriptionally active genes, whereas the methylation of lysine is related to gene activation or repression [94, 95]. Two groups of enzymes are involved in acetylation: histone acetyltransferases and histone deacetylases, which can add or remove acetyl groups, respectively. Histone acetyltransferase overactivity results in hyperacetylation, which can facilitate the binding of transcription factors and RNA polymerases to the DNA template [96]. There is evidence that, in rats with STZ-induced diabetes, the activity and transcripts of histone deacetylases are increased, and histone acetyltransferase activity is reduced, which leads to the reduced acetylation of histone H3 [97]. Another research has shown that modification of histone H3 at lysine 9 (H3K9) at the proximal Cox2 promoter bearing the NF-κB binding site is involved in thioredoxin-interacting protein-mediated inflammation in high-glucose cultured retinal endothelial cells [98]. Moreover, transient hyperglycemia can induce upregulation of NF-κB subunit p65, which acts as an inflammatory mediator in diabetes, via active histone H3 lysine 4 monomethylation at the promoter region [99]. However, hypomethylation of H3K9 is also observed in diabetes, and this frees up the lysine 9 of H3K9 for acetylation, facilitating the recruitment of NF-κB [100]. Furthermore, the promoter and enhancer regions of superoxide dismutase-2 (SOD-2) change in diabetes [101], and histone lysine acylation and methylation in monocytes at inflammation- and diabetes-related gene loci can be induced by hyperglycemia [102, 103]. Both hyperglycemia and hydrogen peroxide treatment increase the expression of coactivator-associated arginine methyltransferase 1 via histone 3 arginine 17 asymmetric demethylation in RPE cells, suggesting that oxidative stress-mediated cell damage is associated with histone modification [104, 105].

4.4.3. MicroRNAs (miRNAs). MicroRNAs (miRNAs) are small, single-stranded, noncoding RNA molecules. They contain 20–24 nucleotides and regulate genes by binding to the target sequences in the 3′-untranslated regions of messenger RNAs [106]. Gene expression can be controlled by miRNA via either degradation or translation repression of messenger RNA, and gene regulation is involved in inflammation, cellular metabolism, angiogenesis, and apoptosis [107]. Circulating miRNA is altered in diabetic patients, and the expression of miRNAs can be a predictive factor for the progression of diabetes [108]. Recent research has shown that miRNA-200b is upregulated in diabetic mouse retinas. This increases the expression of oxidation resistance-1, which prevents oxidative stress [109]; therefore, miRNA-200b has a protective role in DR. Moreover, the expression of miRNA-26b-5p increases after exposure to high levels of glucose [110], and miRNA-26b-5p facilitates cardiomyocyte apoptosis by increasing the production of ROS [111], suggesting its contribution to endothelial apoptosis in DR. Other experiments have provided evidence that oxidative stress regulates SIRT1, which has significant antioxidative and anti-inflammatory effects through miRNA; several miRNAs such as miRNA-217, miRNA-181, miRNA-138, and miRNA-199 downregulate SIRT1 in various cells and tissue types [112]. These studies suggest that miRNAs play an important role in regulating gene expression in DR.

4.4.4. SIRTs. SIRTs are homologs of silent information regulator 2 in higher eukaryotic organisms. They participate in the epigenetic modifications of histone and nonhistone protein deacetylation, which contribute to the mechanisms of vascular dysfunction related to aging, cardiovascular disease, diabetes, and other metabolic diseases [113, 114]. Mammals have seven SIRTs (SIRT1–7) that all depend on NAD^+ cofactor. SIRT1 and SIRT3 are closely correlated with diabetes and its complications. This is particularly true of SIRT1 [115, 116], which regulates oxidative stress response and RPE cell apoptosis via the deacetylation of cytoplasmic p53 [117]. A recent study has shown that SIRT1 activity decreases in diabetic retinas [118]. Moreover, SIRT1 overexpression protects pancreatic β-cells by inhibiting the NF-κB pathway through deacetylation of p65 [119]. In cultured mammalian cells, SIRT1 is activated in response to energy stress and increased oxidative stress. Evidence also shows that SIRT1 is closely related to redox modulation because its activity depends on intracellular NAD^+ [120]. Furthermore, exendin-4 reduces the number of retinal cells and the production of ROS by upregulating the expression of SIRT1 and SIRT3 in the retinas of early-stage diabetic rats [121].

5. Antioxidant Treatments for DR

In the previous sections, we have demonstrated that oxidative stress plays a crucial role in the development of DR. Therefore, it is postulated that antioxidants inhibit abnormal metabolism and slow DR progression by inhibiting the production of ROS, neutralizing free radicals, or augmenting the antioxidant defense system. Therefore, these factors are targets in the treatment of DR.

5.1. Vitamins. Vitamins C and E can protect against the development of DR by scavenging free radicals, reducing the production of ROS, and preventing lipid peroxidation [122, 123]. Therefore, supplementation with vitamins C and E is thought to be helpful in the treatment of DR. Vitamin C can ameliorate endothelial dysfunction in diabetes and decrease leukocyte adhesion in retinal vessels in diabetic rats [123]. Vitamin E inhibits the PKC pathway in diabetes; the PKC pathway induces a decrease of retinal blood flow, resulting in retinopathy [124]. Recently, vitamin D has attracted interest as

being important in the development of DR. Research shows that vitamin D deficiency is very common in type II diabetes, and there is an increased risk of retinopathy in diabetic patients with severe vitamin D deficiency [125]. Further research has demonstrated that vitamin D can inhibit inflammation as well as the expression of VEGF and TGF-β1 in retinal tissues, thereby protecting the retina from neovascularization and cell proliferation [126].

5.2. Green Tea. Green tea, which is rich in polyphenols, is thought to have potent antioxidative, anti-inflammatory, and anticarcinogenic properties [127, 128]. Green tea supplementation in diabetic rats increases the level of GSH and the activities of SOD and catalase, reduces the expression of VEGF and TNF-α, and protects retinal vessels from angiogenesis and retinal endothelial cells from apoptosis [128]. These results indicate the possible mechanisms of the beneficial effects of green tea in the treatment of DR.

5.3. α-Lipoic Acid (ALA). ALA is a powerful antioxidant that is necessary for the function of several enzymes involved in oxidative metabolism [129]. It can reduce the amounts of oxidized forms of other antioxidants such as GSH and vitamins C and E and can modulate several of the signal transduction pathways involved in vascular damage, such as the NF-κB pathway [130]. It has been shown that ALA supplementation in diabetic rats over 30 weeks can prevent damage to retinal vessels, including pericyte dropout, and ALA can also inhibit the expression of Ang-2, VEGF, and AGE receptors, which are related to pericyte dropout, neovascularization, and the activation of NF-κB [131].

5.4. Benfotiamine. Benfotiamine, a lipophilic analogue of thiamine monophosphate, can protect vascular cells from the metabolic damage induced by hyperglycemia. In diabetic animals, benfotiamine has been shown to inhibit three major ROS production pathways (the hexosamine, PKC, and AGE pathways) and can reduce the activation of NF-κB by exciting transketolase [132]. There is also evidence that benfotiamine can protect retinal pericytes from apoptosis via normalizing the Bcl-2/Bax ratio [133]. Therefore, benfotiamine might provide an alternative therapy for DR.

5.5. Poly (ADP-Ribose) Polymerase (PARP) Inhibitors. PARP is one of the downstream oxidant injury mediators. It is involved in retinal cell apoptosis and the regulation of NF-κB activation [134]. PARP can increase the expression of vasoactive factors and the production of extracellular matrix proteins in diabetic animals, and the genetic and chemical blockade of PARP can normalize these changes [135]. Administration of PJ-34, a potent PARP inhibitor, decreases the activation of NF-κB and the transcription of NF-κB-dependent genes such as ICAM-1 in diabetic rats [136]. The evidence supports the value of PARP as a therapeutic target for delaying the development of DR.

5.6. SOD. SOD, the first line of physiological defense against oxidative stress, also contributes to the development of DR. The overexpression of manganese SOD can decrease the hyperglycemia-induced expression of VEGF mRNA and protein in endothelial cells, and the expression of VEGF and fibronectin in STZ-induced DR models can also be reduced [137]. Moreover, overexpression of mitochondrial SOD in hemizygous transgenic mice can prevent diabetic retinal oxidative stress and mitochondrial dysfunction [138]. Tempol, a SOD mimetic, can scavenge $O_2^{\bullet -}$, reduce the amount of NO end-products, and increase GSH biosynthesis, thereby normalizing the oxidative/nitrosative status of DR. Furthermore, decreased copper-zinc SOD expression/activity in treated diabetic rats has been observed [139].

5.7. Other Antioxidants. Other antioxidants also have beneficial effects on the development of DR. For example, zinc, an important nutrient, is involved in oxidative stress, apoptosis, and aging. Zinc can prevent the increase of lipid peroxidation induced by diabetes in the retina and can reduce GSH levels in diabetic rats [140]. Moreover, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and N-acetyl-L-cysteine prevent glycated albumin-induced cell death in pericytes [141]. Nicanartine, an antioxidant with cholesterol-lowering properties, can partially inhibit pericyte loss in diabetic rats [142]. Curcumin, a traditional herb extract, demonstrates significant antioxidant activity by downregulating hypoxia-inducible factor 1 and preventing angiogenesis induced by hypoxia [143]. Curcumin supplementation can inhibit the expression of VEGF in DR rats [144]. Furthermore, β-carotene, taurine, lutein, caffeic acid phenethyl ester, cannabidiol, and 8-hydroxy-N,N-dipropyl-2-aminotetralin also have some antioxidant properties [145].

5.8. Nrf2-Related Treatments. In the previous sections, we have described the role of Nrf2-signaling pathways in DR and inflammation [58]. Sulforaphane, an Nrf2 inducer, can protect the RPE against oxidative injury [146]. Hemin is another potential treatment. In diabetic rats, the levels of heme oxygenase-1, SOD-1, and Bcl-2 increase, whereas the expressions levels of hypoxia-inducible factor 1 and VEGF decrease following treatment with hemin. The activation of Nrf2 nuclear translocation contributes to these results [147]. Mycophenolate mofetil (MMF) is also considered an effective drug. Evidence shows that MMF facilitates Nrf2 nuclear translocation and conserves antioxidant enzymes in diabetic nephropathy [148]. Moreover, zinc, oltipraz, and dimethyl fumarate have different effects in diabetes via the Nrf2 pathway [149–151].

5.9. Epigenetic Modification-Related Treatments. As mentioned above, DNA methylation, histone modification, and miRNAs are three major epigenetic factors that are potential targets for the treatment of diabetes. DNA methylation can be repressed by nucleoside analogues because they inhibit DNMTs. Moreover, DNMT inhibitors, including 5-azacytidine (azacitidine; Vidaza) and 5-aza-20-deoxycytidine (decitabine; Dacogen) for myeloid cancers and cutaneous T cell lymphoma, have been recommended by the US Food and Drug Administration [87]. The regulation of histone modification also has a large effect. For instance, epigallocatechin-3-gallate, a strong histone acetyltransferases inhibitor, can block

the activation of p65 acetylation-dependent NF-κB [152]. Curcumin, genistein, and resveratrol also have an influence on histone modification [153]. Resveratrol has a protective role against inflammation because it controls the expression of several miRNAs such as miRNA-21, miRNA-155, and miRNA-663 [154]. For miRNA, double-stranded miRNA mimics and anti-RNA antisense oligodeoxyribonucleotides are major molecules that have been investigated for targeting specific miRNAs [9]. Metformin, which is involved in the SIRT1/LKB1/AMPK pathway, suppresses hyperglycemia stress "memory" in diabetic rats [155]. Another research has shown that cocoa enriched with polyphenol improves the retinal SIRT1 pathway and protects the retina from diabetic insult [156]. Moreover, resveratrol and fenofibrate also have a protective effect on DR via SIRT1-related pathways [118, 157].

6. Conclusions

DR is a leading cause of blindness in working-age adults, and oxidative stress plays an important role in its development. In this review, we have demonstrated that hyperglycemia induces a series of metabolic abnormalities in the retina by producing ROS and subsequently initiating oxidative stress. This can in turn activate these abnormal metabolic pathways to produce more ROS, thereby creating a vicious cycle. Generally, oxidative stress causes retinal damage by inducing endothelial cell dysfunction, pericyte apoptosis, and angiogenesis. The polyol, AGE, PKC, hexosamine, and Nrf2-related pathways, GTP-binding proteins, and epigenetic modifications are all involved in the pathological process of DR. Therefore, inhibitors of these pathways may protect the retina from damage induced by high levels of glucose. Antioxidants are beneficial for DR because they reduce ROS production, neutralize free peroxynitrite, or augment the antioxidant defense system. Furthermore, herbal extracts have received increasing attention for treating DR in recent years, although the mechanisms underlying their mode of action require verification. At present, most of the evidence has been provided by animal experiments, and the clinical therapeutic effects are still not very clear. Thus, more work is needed in the future.

Competing Interests

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

Acknowledgments

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Research Article

Nutritional Supplementation Inhibits the Increase in Serum Malondialdehyde in Patients with Wet Age-Related Macular Degeneration

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Purpose. To compare serum levels of malondialdehyde (MDA) in patients with wet age-related macular degeneration (wAMD), patients with dry AMD (dAMD), and patients without AMD and to evaluate the efficacy of nutritional supplementation for treating elevated serum MDA in patients with wAMD. *Methods.* MDA levels were measured in sera from 20 patients with wAMD, 20 with dAMD, and 24 without AMD. Patients with wAMD were randomized to receive or not receive nutritional supplementation (10 patients in each group), and MDA levels were measured after 3 months of treatment. *Results.* MDA levels in patients with wAMD were significantly greater compared with patients without AMD. In eyes with wAMD, there was a significant correlation between MDA levels and choroidal neovascularization lesion area. Serum MDA levels decreased in most patients that received supplementation and significantly increased in those who did not. *Conclusion.* Baseline serum MDA levels were elevated in patients with wAMD, and MDA levels were directly correlated with choroidal neovascularization lesion area. In addition, nutritional supplementation appeared to exert a protective effect against oxidative stress in patients with wAMD.

1. Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness in developed countries [1, 2]. AMD is classified as either wet AMD (wAMD) or dry AMD (dAMD) according to its pathophysiology [3]. wAMD is characterized by choroidal neovascularization (CNV) and an increase in intraretinal/subretinal fluid which is strongly associated with the overexpression of vascular endothelial growth factor (VEGF). dAMD is characterized by the atrophy of retinal pigment epithelium (RPE) and its advanced form is called geographic atrophy (GA) [4, 5]. Multiple risk factors, including obesity [6], hypertension [7], smoking [8], and light exposure [9, 10], have been shown to contribute to the pathogenesis of AMD, presumably by inducing oxidative stress [11–13].

Nutritional supplements, that is, Ocuvite PreserVision with Lutein, have shown to provide therapeutic benefits in both wAMD and dAMD [14–16]. The Age-Related Eye Disease Study 2 (AREDS 2) found that high-dose zinc/antioxidant supplementation inhibited the progression of early stage AMD to late stage AMD compared with placebo [17, 18]. A previous study demonstrated that mean serum levels of cysteine, an oxidative stress marker, decreased in study participants on a regulated diet that received a 5-day course of antioxidant and zinc supplementation [19].

Malondialdehyde (MDA) is a highly reactive three-carbon dialdehyde product of polyunsaturated fatty acid (PUFA) peroxidation by free radicals [20, 21]. Consuming high levels of linoleic acid, a ω-6 fatty acid and the most abundant dietary PUFA, is a risk factor for multiple types of cancers [22] and heart diseases [23]. Photoreceptor outer segments are rich in unsaturated fatty acids. As MDA is generated from the oxidation of unsaturated fatty acids, MDA is present in drusen, the extracellular deposits that accumulate in AMD eyes [24, 25]. MDA is used as a biological marker of oxidative stress [26], suggesting that serum MDA levels are likely to be elevated in patients with AMD compared with healthy subjects [27–29]. Consistent with this hypothesis, MDA exerts cytotoxic effects and upregulates VEGF expression in RPE cells in vitro [30, 31]. We previously demonstrated that not only is MDA a marker of AMD but it also induced autophagy dysregulation and VEGF secretion in AMD eyes. Furthermore, higher levels of dietary linoleic acid intake promoted CNV progression in mice with high MDA levels [32]. In this study, we further analyzed the relationship of clinical characteristics and oxidative stress levels in patients with AMD. In addition, we investigated the efficacy of antioxidant nutritional supplements in reducing serum MDA levels.

2. Materials and Methods

2.1. Patients. We prepared serum samples from patients with wet AMD (wAMD group), patients with dry AMD (dAMD group), and individuals without AMD (control group). All of the study patients were >50 years old and their axial length was >23.0 mm and <26.0 mm. Patients with polypoidal choroidal vasculopathy, retinal angiomatous proliferation, maculopathy with myopic CNV, or CNV based on angioid streaks or patients with axial length >26.0 mm or <23.0 mm were excluded from the study. The diagnosis of wAMD and dAMD was established on the basis of age (over 50 years), clinical examination, fundus photography, optical coherence tomography, and fluorescein fundus angiography as previously described [33, 34]. Patients with wAMD in one eye and dAMD in the other eye were excluded from the study. Control sera were obtained from patients with other ocular diseases, including cataract, glaucoma, retinal detachment, macular hole, and epiretinal membrane. After baseline measurements were obtained, patients in the wAMD group were divided into two groups; patients in the wAMD group were free to choose whether nutritional supplements should be taken. Patients who took the nutritional supplement were referred to as S (+) and those who did not take it were referred to as S (−). The nutritional supplement (Ocuvite PreserVision with Lutein, Bausch & Lomb, Rochester, New York City, The United States of America), which was commercially available at the time of the study, contains vitamin C (408 mg), vitamin E (241 mg), zinc (30 mg), and lutein (9 mg). The participants received the supplement once daily for 3 months. The study was approved by the Nagoya University Hospital Ethics Review Board (#2012-0340-3), and written informed consent was obtained from each patient prior to obtaining the first serum samples.

2.2. Best-Corrected Visual Acuity. The best-corrected visual acuity (BCVA) was measured using a standard Japanese visual acuity chart. The decimal BCVA was converted into the logarithm of the minimum angle of resolution (logMAR) for the statistical analysis.

2.3. Fluorescein Angiography Imaging and Evaluation of Choroidal Neovascular Lesions. Fluorescein angiography (FA) was recorded for all patients in the wAMD group using cSLO (Heidelberg Retina Angiograph, HRA 2, Heidelberg Engineering, Dossenheim, Germany) as previously described [35–37]. To evaluate the area of the CNV lesion, we traced the border of the area of hyperfluorescein in images captured at 5 min and quantified the pixels using NAVIS bundled software (Nidek Co. Ltd., Aichi, Japan). The measurements were conducted by two observers (Toshiyuki Matsuura and Kei Takayama) and both observers were blinded to the patients' clinical features. The area of one pixel in the FA images was defined as 0.0004 mm^2, and the measurements were converted from pixels to the area (mm^2).

2.4. Fundus Autofluorescence Imaging and Evaluation of Geographic Atrophy (GA). Fundus autofluorescence (FAF) was recorded using a cSLO (Heidelberg Retina Angiograph, HRA 2, Heidelberg Engineering, Dossenheim, Germany) as previously described [38, 39]. An optically pumped solid-state laser was used to generate the excitation, and emitted light with a wavelength >500 nm was detected using a barrier filter. The images were immediately digitized. Then, they were processed using a flexible frame processor and displayed on a computer screen. The FAF images were recorded in accordance with a standard operating procedure. The red-free reflection mode was used for focusing, and a series of $30 \times 30^{\circ}$ images were acquired. To evaluate the area of GA from the FAF images, we traced the border of the dark area in the image and measured the pixels using NAVIS bundled software (Nidek Co. Ltd., Aichi, Japan). The measurements were conducted by two observers (Toshiyuki Matsuura and Kei Takayama) who were blinded to the patients' clinical features. The area of one pixel in the FAF images was 0.0004 mm^2, and the measurements were converted from pixels to the area (mm^2).

2.5. MDA Levels. MDA levels in patient sera were measured at baseline. Then, patients in the wAMD group were randomized to the S (+) group or S (−) group, and sera that were obtained were measured 3 months later. Serum levels of MDA were measured using an OxiSelect MDA Adduct ELISA Kit (STA-332; Cell Biolabs, San Diego, CA, USA) as previously described [32, 40]. Duplicate evaluations were performed for each sample.

2.6. Statistical Analysis. All of the data are presented as the mean ± the standard error of the mean. The Mann–Whitney *U* test was used to compare data between the wAMD, dAMD, and control groups and to compare MDA levels at baseline and after 3 months. Spearman's correlation was used to detect the correlation between MDA levels and age, logMAR BCVA,

Table 1: Patient characteristics in wAMD, dAMD, and control groups.

	wAMD	dAMD	Control
Number	20	20	24
Male/female	10/10	13/7	11/13
Mean age (years)	71.8 ± 11.0	70.6 ± 12.5	68.2 ± 10.2
Mean logMAR BCVA	0.29 ± 0.21	0.24 ± 0.24	0.22 ± 0.29
Mean MDA level (pmol/mL)	9.94 ± 1.53	9.30 ± 0.92	9.04 ± 0.96
Mean CNV area (mm^2)	4.65 ± 3.66		
Mean GA area (mm^2)		2.29 ± 1.80	

Table 2: Patient characteristic in the S (+) and S (−) groups.

	S (+)	S (−)
Number	10	10
Male/female	5/5	5/5
Mean age (years)	72.0 ± 9.7	71.6 ± 10.1
Mean logMAR BCVA	0.30 ± 0.25	0.28 ± 0.17
Mean MDA level (pmol/mL)	10.34 ± 2.03	9.54 ± 0.70
Mean CNV area (mm^2)	4.70 ± 4.09	4.62 ± 3.41

CNV area, and GA area. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patient Characteristics. The study included 20 patients in the wAMD group (10 males, mean age 71.8 ± 11.0 years), 20 patients in the dAMD group (13 males, mean age 70.6 ± 12.5 years), and 24 patients in the control group (11 males, mean age 68.2 ± 10.2 years). The patient characteristics were presented in Table 1. The mean logMAR BCVA was 0.29±0.21, 0.24 ± 0.24, and 0.22 ± 0.29 in the wAMD, dAMD, and control groups, respectively. Mean levels of MDA were 9.94 ± 1.53 pmol/mL, 9.30 ± 0.92 pmol/mL, and 9.04 ± 0.96 pmol/mL in the wAMD, dAMD, and control groups, respectively. Mean CNV area in the wAMD group was 4.65 ± 3.66 mm^2, and mean GA area in the dAMD group was 2.29 ± 1.80 mm^2. The patient characteristics of the three groups are presented in Table 2. Individual value of MDA-protein adducts and patient information of S (+) and S (−) group are presented in Table 3. The mean logMAR BCVA was 0.30 ± 0.25 and 0.28 ± 0.17 in the S (+) and S (−) groups, respectively. Mean levels of MDA were 10.34 ± 2.03 pmol/mL in the S (+) group and 9.54 ± 0.70 pmol/mL in the S (−) group. The mean area of CNV lesions was 4.70 ± 4.09 mm^2 in the S (+) group and 4.62 ± 3.41 mm^2 in the S (−) group. There were no significant differences between the S (+) and S (−) groups in any of the parameters evaluated.

3.2. MDA Level. To investigate the relationship between MDA levels and AMD, we evaluated serum MDA levels at baseline and after 3 months of treatment (Figure 1). We observed a 15.0% increase in mean serum MDA in the wAMD group (9.94 ± 1.53 pmol/mL) compared with the control group (9.04 ± 0.96 pmol/mL, $P = 0.031$). An increase in MDA was also observed in the dAMD group (9.30 ± 0.92 pmol/mL) compared with the control group, although the difference was not significant ($P = 0.12$; Figure 1(a)).

Mean levels of MDA in the S (+) group decreased from 10.34 ± 2.03 pmol/mL at baseline to 8.88 ± 1.18 pmol/mL after 3 months of supplementation ($P = 0.064$). Mean levels of MDA in the S (−) group significantly increased from 9.54 ± 0.70 pmol/mL at baseline to 10.41 ± 1.36 pmol/mL after 3 months ($P = 0.012$) (Figure 1(b)). MDA levels decreased in 7 of the 10 patients in the S (+) group and increased in the other 3 patients (Figure 1(c)). In contrast, MDA levels decreased in 2 of the 10 patients in the S (−) group and increased in 8 patients (Figure 1(d)).

3.3. Correlation between Serum MDA Levels and Patient Characteristics. Next, we examined the correlation between baseline MDA levels and age, logMAR BCVA, CNV area, and GA area. Representative images of CNV in eyes with wAMD and GA area in eyes with dAMD are shown in Figure 2. In the representative eye with wAMD, logMAR BCVA was 0.30, baseline serum MDA was 10.28 pmol/mL, and CNV area was 3.27 mm^2 (Figures 2(a) and 2(b)). In the representative eye with dAMD, logMAR BCVA was 0.097, serum MDA level was 8.90 pmol/mL, and CNV area was 3.40 mm^2 (Figures 2(c) and 2(d)).

In the wAMD group, there was a significant correlation between baseline MDA levels and CNV area ($P = 0.038$, $r = 0.39$, Figure 3(c)), although we did not detect significant correlations between MDA levels and age ($P = 0.31$, Figure 3(a)) or logMAR BCVA ($P = 0.13$, Figure 3(b)). In the dAMD group, baseline MDA levels were not significantly correlated with age, logMAR BCVA, or GA area ($P = 0.33$, 0.14, and 0.052, resp.) (Figures 3(d)–3(f)).

4. Discussion

The association between AMD and oxidative stress both in vitro and in vivo has been demonstrated in several studies [12, 41, 42]. We previously demonstrated that MDA is not only a marker of AMD but also a direct contributor to the pathogenesis of AMD [32]. Prospective studies demonstrated that serum markers of oxidative stress are low in patients taking various nutritional supplements, including antioxidants such as lutein, vitamin C, vitamin E, β-carotene, and zinc oxide. However, only one study evaluated a 5-day course of nutritional supplementation and included a relatively small number of patients [19]. In the present study, we measured MDA levels in the sera using ELISA and regarded them as a systemic oxidative stress marker because a previous report describing the complements binds MDA accumulating in the drusen of the eyes with AMD and complement factor H genetically plays an important role in this complement-MDA cleavage [25]. We were able to demonstrate the usefulness of supplementation by measuring MDA-protein adducts in this study. However, free MDA-protein levels measured by high performance liquid chromatography or 4-hydroxy-2-nonenal are considered as more reliable biomarkers of lipid peroxidation in the human plasma [26, 43]. More reliable correlations could be estimated by measuring them. In the

Table 3: Patients of S (+) and S (−) group.

#	Age (years)	Sex	logMAR BCVA	CNV area (mm^2)	Before MDA (pmol/mL)	After MDA (pmol/mL)
				S(+) group		
1	81	M	0.70	9.09	8.94	7.41
2	81	F	0.70	6.70	8.45	10.23
3	80	M	0.30	1.51	8.45	9.46
4	74	M	0.00	11.41	10.74	9.46
5	74	M	0.10	7.37	9.71	7.68
6	73	F	0.40	1.45	9.52	8.90
7	72	F	0.22	0.85	11.07	9.46
8	69	M	0.30	7.27	14.28	8.86
9	68	F	0.30	1.01	8.94	10.23
10	50	F	0.00	0.30	13.29	6.91
				S(−) group		
1	94	F	0.30	0.85	9.71	9.98
2	81	F	0.30	6.61	8.69	8.51
3	80	M	0.40	10.69	8.90	9.54
4	78	M	0.10	1.73	9.71	10.41
5	74	M	0.22	5.68	8.32	9.54
6	74	F	0.70	5.53	9.71	10.74
7	67	F	0.10	1.25	10.41	12.17
8	59	F	0.22	8.53	9.71	13.30
9	55	M	0.22	1.12	9.71	10.23
10	54	M	0.22	4.18	10.48	9.72

present study, we demonstrated that mean serum MDA levels decreased in patients on a regulated diet that received nutritional supplementation, Ocuvite PreserVision with Lutein, for three months, whereas mean levels increased in patients on a regulated diet with no nutritional supplementation. As systemic oxidative stress is a key contributor to the pathogenesis of AMD, these findings suggest that nutritional supplementation might have inhibited systemic oxidative stress in these patients.

In patients with wAMD, MDA levels were significantly correlated with CNV area (Figure 3(c)). CNV is defined as neovascularization resulting from oxidative stress-induced damage to the choroid and RPE [44–46]. We previously demonstrated that MDA levels in the RPE and choroid of AMD patients were significantly elevated compared with control subjects and that MDA administration induced the upregulation of VEGF expression in RPE cells and induced an increase in CNV volume in vivo [32], suggesting that serum MDA levels are correlated with CNV area. To measure CNV lesion area, FA/ICGA were performed in the wAMD group, as previously described. Changes in the CNV area in the S (+) and S (−) groups would be detectable by FA/ICGA after 3-month observation, although FA/ICGA was not performed in the present study. From OCT images, the CNV area was considered to show no remarkable change after 3-month observation. Therefore, the relation between CNV area expansion and MDA change could not be compared. We did not detect a correlation between MDA and GA area in the dAMD group (Figure 3(f)). GA is defined as a loss in RPE area [1, 47]. The RPE maintains the health of the retina by providing protection from oxidation [48, 49]. GA can take more than 6 years to develop, and it arises as a consequence of long-tern exposure to stress [33], rather than current levels of stress. These findings are consistent with the lack of a correlation between GA area and serum levels of MDA in this study. Although we measured only MDA-protein adducts in this study, measuring one more biomarker, for example, 4-HNE, is preferable in the further study.

There are several limitations of the study worth noting. First, the present study included small number of patients and the observational period was only 3 months. More patients and long-term studies are needed to confirm our findings. In addition, the control group should also be treated by the same nutritional supplementation to have a proper control. A better method would be that the patients in the control groups take same supplementation after 3 months and it should be confirmed that their MDA levels will be also reduced after taking supplementation. The present study showed only "tendency ($P = 0.068$)," not "significant difference ($P < 0.05$)," of supplementation for reducing MDA levels after 3 months. It is possible that a longer observation period may result in "significant difference" in MDA levels between S (+) and S (−) groups. However, other factors, that is, food or stress, could affect systemic oxidative stress during the long-time observation. Second, it was not elucidated whether nutritional supplementation directly reduces MDA level in the eye. We previously reported that high levels of MDA were observed in human RPE/choroid obtained from donor eyes from several Eye Banks. The AREDS demonstrated the efficacy of supplementation in the prevention of AMD

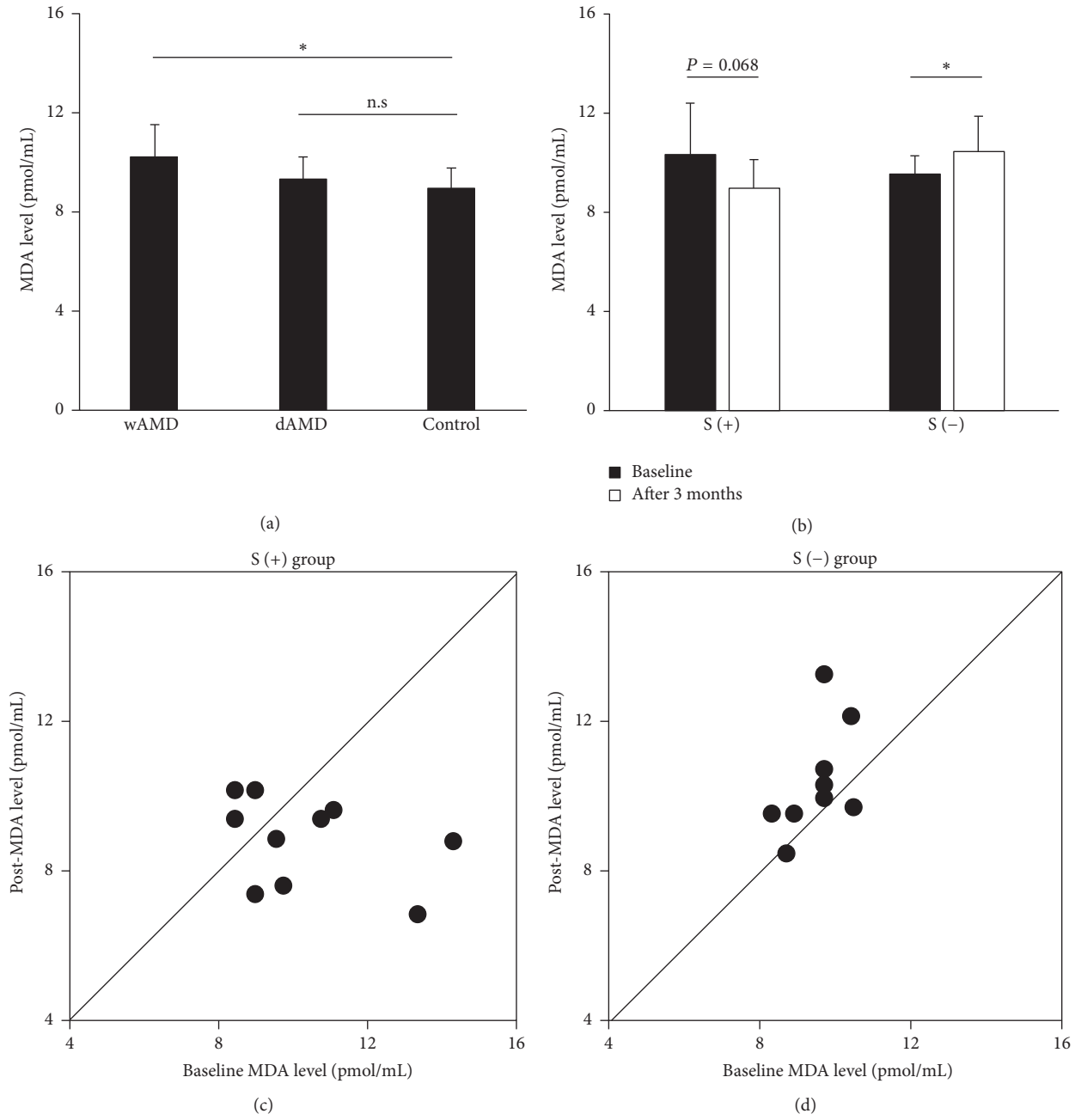

Figure 1: Serum MDA levels in the study population and changes in MDA levels in wAMD patients that received nutritional supplementation. (a) Higher serum MDA levels were observed in the wAMD group. (b) MDA levels tended to decrease in wAMD patients that received supplementation for 3 months. (c) MDA levels decreased in 7 of the 10 patients in the S (+) group and increased in the remaining 3 patients. (d) MDA levels decreased in 2 of the 10 patients in the S (−) and increased in the other 8 patients. $^{*}P < 0.05$.

progression over a period of 5 years [17, 50]. It is difficult to prospectively study the effect of nutritional supplementation on MDA levels in human eyes.

In conclusion, we demonstrated that elevated serum MDA levels were directly associated with the area of CNV lesions in eyes with wAMD and that nutritional supplements appear to protect the eyes from systemic oxidative damage. MDA might be a valuable marker of oxidative stress. The present study is a retrospective short-observation study including small number of patients. To obtain more reliable value of supplementation for AMD and systemic oxidative stress, a double-blinded prospective randomized study of supplementation is necessary.

Competing Interests

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

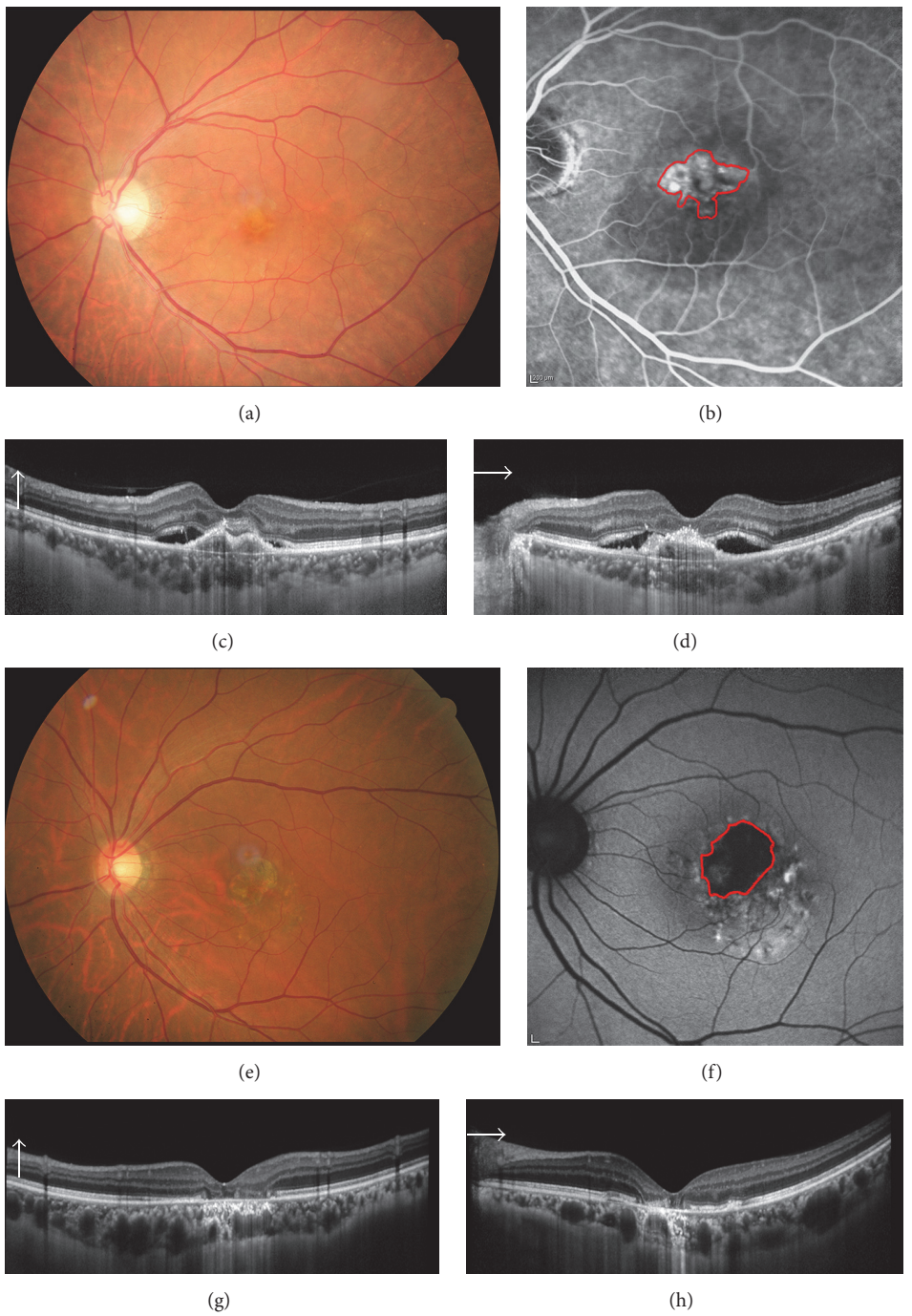

Figure 2: Representative images of wet AMD and dry AMD. (a, b) The color (a) and fluorescein angiography (FA, b) images of a representative case of wet AMD. The CNV lesion area in FA images was measured. (c, d) Representative case of dry AMD. Geographic atrophy area in the autofluorescein angiography image (d) was measured. (e, f) images are the OCT images of wet AMD and (g, h) are of dry AMD.

Authors' Contributions

Toshiyuki Matsuura and Kei Takayama contributed equally.

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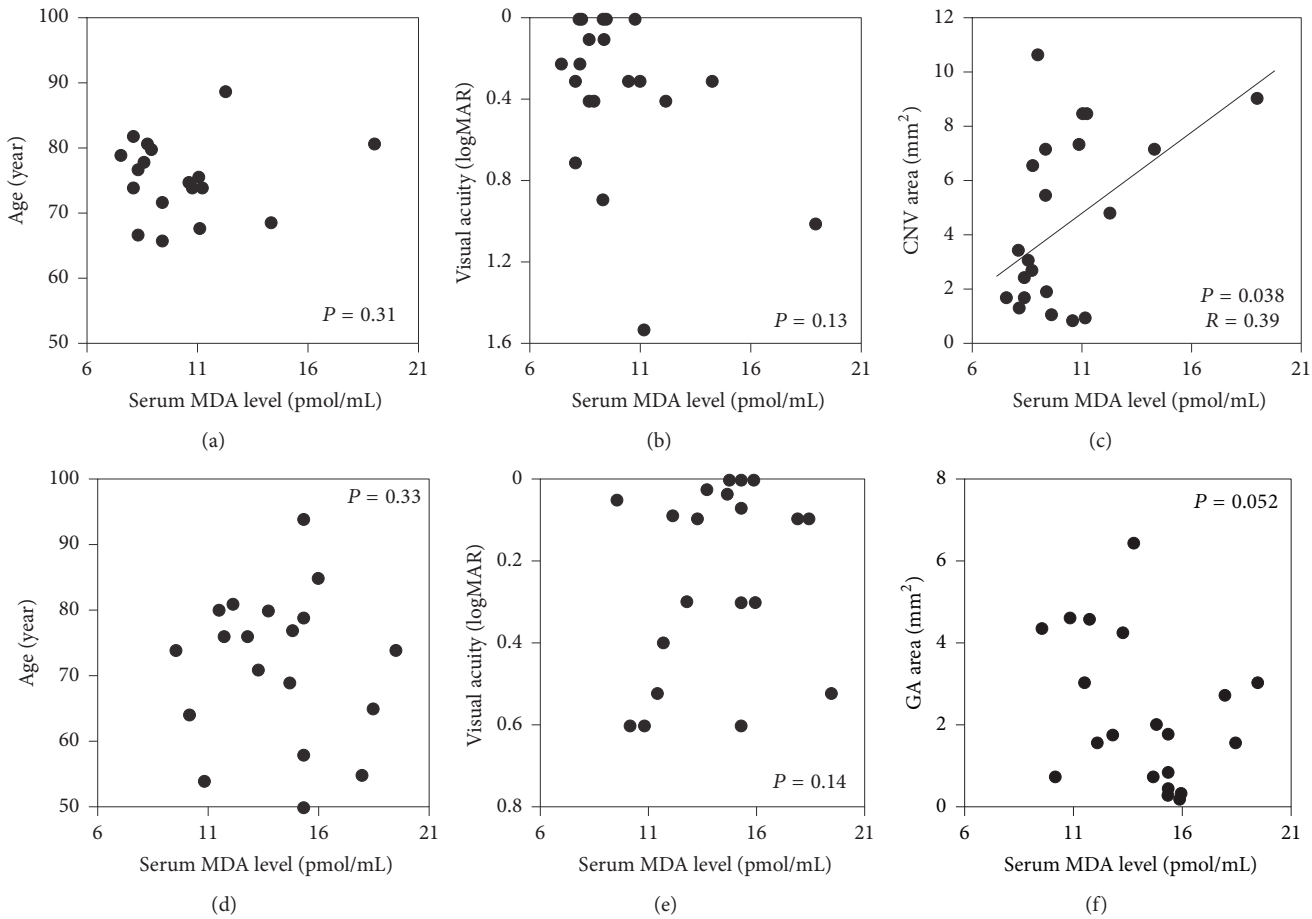

Figure 3: Correlation between MDA levels and patient characteristics in the wet AMD and dry AMD groups. (a–c) In the wet AMD group, serum MDA levels and choroidal neovascularization (CNV) lesion area were significantly correlated, but MDA levels were not correlated with ages or visual acuity. (d–f) In the dry AMD group, there was no significant correlation between serum MDA levels and age, visual acuity, or geographic atrophy (GA) area.

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Review Article

Sirtuins Expression and Their Role in Retinal Diseases

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Sirtuins have received considerable attention since the discovery that silent information regulator 2 (Sir2) extends the lifespan of yeast. Sir2, a nicotinamide adenine dinucleotide- (NAD-) dependent histone deacetylase, serves as both a transcriptional effector and energy sensor. Oxidative stress and apoptosis are implicated in the pathogenesis of neurodegenerative eye diseases. Sirtuins confer protection against oxidative stress and retinal degeneration. In mammals, the sirtuin (SIRT) family consists of seven proteins (SIRT1–SIRT7). These vary in tissue specificity, subcellular localization, and enzymatic activity and targets. In this review, we present the current knowledge of the sirtuin family and discuss their structure, cellular location, and biological function with a primary focus on their role in different neuroophthalmic diseases including glaucoma, optic neuritis, and age-related macular degeneration. The potential role of certain therapeutic targets is also described.

1. Introduction

Neurodegeneration processes are implicated in several eye diseases. These include glaucoma, age-related macular degeneration (AMD), and inherited retinal disorders [1–3]. Recently transcription factors like sirtuins were found to be involved in neurodegeneration. Identification of these cell-based markers and therapeutic modalities of neuroprotection are active areas of research in this field.

Sirtuins (silent information regulator, Sir2) were first identified to prolong lifespan in yeast *(Saccharomyces cerevisiae)*. They are originally categorized as class III histone deacetylase (HDAC) and belong to a conserved family of nicotinamide adenine dinucleotide- (NAD-) dependent protein deacylases [4]. Sirtuins deacetylate both histones and nonhistone proteins. These include transcription factors metabolic enzymes and proteins that have key roles in various cellular processes [5]. In mammals, seven human Sir2 homologues (sirtuins) designated as SIRT1 to SIRT7 have been identified to date. These are associated with calorie restriction, aging, metabolism, cancer, transcriptional silencing, chromosomal stability, stress response, cell differentiation, inflammation, apoptosis, DNA repair, and prevention of age-related ocular diseases. Sirtuins are reported to have key roles in cellular senescence, cell differentiation, and inflammation [6–11].

The aim of this review is to summarize and discuss the cellular location, biological function, and neuroprotective effect of sirtuins as a promising target for the future treatment of related neurodegenerative diseases of the eye.

2. Structure and Biological Function of Sirtuin

High resolution crystal structures of sirtuin family members have provided insight into their substrate, cofactor binding partners, and catalytic mechanisms [12, 13]. All the seven members of the SIRT family share a conserved catalytic core. The central catalytic core is comprised of 245 residues flanked by N- and C-terminal extensions. The core is made up of a large domain consisting of a Rossmann fold. This is typical for NAD-dependent proteins and a small Zn^{2+} ribbon motif containing the consensus sequence Cys-X_{2-4}-Cys-X_{15-40}-Cys-X_{2-4}-Cys and a α-helical region. The two

Table 1: Certain major functions of sirtuins.

Name of the sirtuin (SIRT)	Functions	Subcellular localization
SIRT1	Cellular longevity, tumor promoter, tumor suppressor, inflammation, oxidative stress, glucose homeostasis, cell adhesion, cell metabolism	Nucleus
SIRT2	Mitotic check point, tumor promoter, tumor suppressor	Cytoplasm/nucleus
SIRT3	Tumor suppressor, tumor promoter, mitochondrial oxidation, stress responsive deacetylase	Mitochondria
SIRT4	Glutamine catabolism, TCA cycle, ADP-ribosylation	Mitochondria
SIRT5	Glycolysis, cancer metabolism, fatty acid oxidation, lysine succinylation, malonylation, glutarylation	Mitochondria
SIRT6	Telomere and genome stability, DNA repair, inflammation, glucose homeostasis	Nucleus
SIRT7	Ribosomal production	Nucleus (nucleoli)

domains are separated by a cleft at the interface where the peptide substrate binds. SIRT1 is the most studied human isoform. It is the largest with extended N- and C-terminals and is very flexible and unstructured, which allows it to offer more sites of activity modulation (such as posttranslational modifications, interaction with proteins, and ligands). Unlike other HDACs, where zinc is part of the catalytic mechanism [14], the zinc ion is located in the small domain, far away from the NAD^+ binding domain, excluding the possibility of its participation in the catalysis.

Since sirtuins are protein deacylases the majority of them function as deacetylases (SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7). Their enzymatic activity results in the removal of an acetyl group from *N*-ε-lysine residues and generates *O*-acetyl-ADP-ribose and nicotinamide. In addition SIRT4, SIRT6, and SIRT7 exhibit monoadenosine diphosphate- (ADP-) ribosyltransferase activity. SIRT1, SIRT6, and SIRT7 are predominantly localized in the nucleus; SIRT3, SIRT4, and SIRT5 reside within the mitochondria; and SIRT2 is limited to the cytoplasm. In response to oxidative stress, shuffling of nuclear-to-cytoplasmic localization of SIRT1 has also been reported [15, 16]. Some of the major functions were shown in Table 1.

SIRT1 has been extensively studied due to its deacetylation of transcription factors and apoptotic modulators (including forkhead box O subclass (FOXO), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1 α), nuclear factor kappa-B (NF-κB), Ku70, and p53) [17]. It is associated with inflammation, apoptosis, genome stability, metabolic regulation, senescence, cell differentiation, and oncogenic transformation [18]. SIRT2 regulates mitotic checkpoints, oligodendrocyte, and adipocyte differentiation [15, 19]. It plays a vital role in glucose homeostasis during oxidative stress by deacetylating/activating glucose 6 phosphate dehydrogenase in pentose phosphate pathway [16]. SIRT2 is involved in the regulation of tumor necrosis factor-alpha (TNF-α) induced necroptosis [20]. In contrast, another study revealed that genetic and pharmacological inhibition of SIRT2 did not inhibit TNF-α induced necroptosis [21] suggesting that it may have a minor role.

SIRT3 has been shown to be associated with the human bladder [22] and oral squamous cell carcinoma [23]. It is a stress-responsive deacetylase, whose increased expression protects from obesity induced metabolic deregulation, cancer, and oxidative stress-mediated cell death [15, 24]. It can function either as a tumor promoter or as a tumor suppressor depending on the cell- and tumor-type and the presence of different stress or cell death stimuli [24]. SIRT3 acts as a tumor suppressor, at least in part via its ability to suppress reactive oxygen species (ROS) and regulate hypoxia inducible factor-1-alpha (HIF-1α) [25]. SIRT4 regulates glutamine catabolism and has lipoamidase activity that is induced by high levels of glutamine and negatively regulates pyruvate dehydrogenase complex [26]. Genetic knockdown of SIRT4 has been shown to increase SIRT1 and SIRT3 and enhance the expression of genes associated with fatty acid oxidation and mitochondrial oxidative capacity [15, 27]. SIRT5 regulates lysine succinylation, malonylation, glutarylation, enzymes involved in ketone production, and fatty acid oxidation [15, 28]. It regulates urea cycle through carbamoyl phosphate synthetase 1 via desuccinylase activity instead of deacetylase activity [29, 30]. A recent study revealed that through demalonylation of glycolytic enzymes SIRT5 positively regulates glycolysis and provides a link to cancer metabolism [29].

SIRT6, a chromatin-associated nuclear protein, promotes resistance to DNA damage and suppresses genomic instability in mouse cells, in association with a role in base excision repair [31]. Oxidative stress reduces SIRT6 levels and causes endothelial cell senescence [15, 32]. SIRT6 knockout mice display premature aging symptoms, including excessive loss of subcutaneous fat and a significant reduction in bone density, and die within 4 weeks of birth [31]. However, overexpression of SIRT6 expands lifespan in male mice by regulating insulin-like growth factor 1 (IGF-1) [32].

SIRT7 has a protective effect by deacetylating the transcription factor GA binding protein subunit beta 1 (GABPβ1). SIRT7 deficient mice develop fatty liver and hearing loss and die prematurely from cardiomyopathy [33].

3. Sirtuin Expression in the Eye

Except SIRT5, all sirtuins are expressed in human retina [34, 35]. Retina is a photoreceptive tissue, whose energy

consumption changes depending on light exposure. Retinal cells expend more energy in the dark due to their higher oxygen consumption and lactate production [36–38]. Hence, the retinal expression of sirtuins has been found to be variable, highlighting the regulatory mechanism(s) of sirtuins in the retina. All sirtuins showed significant daily variation under light-dark condition in retina. The mRNA levels of sirtuins except SIRT6 were elevated in dark phase. However, this photosensitive effect is absent in the brain and liver, suggesting that sirtuins may be regulated in a tissue- or organ-specific manner [38].

Jaliffa and colleagues have shown that SIRT1 is expressed in mouse cornea, lens, iris, ciliary body, inner nuclear layer, outer nuclear layer, and retinal ganglion cell layer [39]. SIRT1 deficient mice have been reported to be smaller than normal at birth and usually die during the early postnatal period. They fail to open one or both eyes [40]. In addition, multiple retinal cell layers are significantly thinner than normal mouse eyes, whereas inner and outer nuclear layers are disorganized in these mice. The difficulty in detecting inner and outer photoreceptor cell segments implicates the role of SIRT1 in ocular morphogenesis [11, 41]. In addition, SIRT1 conditional KO mice exhibit p53 hyperacetylation and reduced number of retinal neuronal cells during development [41]. To date, only a few studies reported the role of SIRT1 with the development of cataract [42, 43], retinal degeneration [44, 45], optic neuritis [46], and uveitis [47]. SIRT2 is expressed in human retinoblastoma and other nonaffected normal ocular areas such as nonpigmented ciliary body epithelium, outer and inner plexiform layer, nerve fiber layer, inner and outer nuclear layer, and retinal pigment epithelium [48]. It is also expressed in inflammatory cells at limbus and iris stroma of retinoblastoma cases [49].

SIRT3 is highly expressed in lacrimal gland and neural retina of mice mainly in retinal ganglion and photoreceptor cells [49], but 10-week-old SIRT3 knockout (KO) mice did not show any difference in retinal thickness or electroretinogram. But there is lack of evidence in neural protein expression such as rhodopsin, glial fibrillary acidic protein, and synaptophysin in these SIRT3 KO mice [50]. In humans, the inner nuclear layer (INL) showed weak expression of SIRT3 throughout the retina.

In the human retina, retinal pigment epithelium (RPE) expressed SIRT4, SIRT6, and SIRT7. SIRT4 and SIRT7 were strongly positive in the macula and peripheral retina but not in the outer nuclear layer (ONL) [51].

In the mouse retina, SIRT6 is expressed in all retinal layers and its levels are higher in this tissue compared to brain, heart, liver, or kidney [52]. In humans, SIRT6 is expressed in macula, nonpigmented ciliary body epithelium, ciliary muscle, retinal pigment epithelium, optic nerve fiber, and neurosensory retina except the inner limiting membrane. SIRT6 expression was observed in retinoblastoma [48]. SIRT6 controls the levels of histone H3K9 and H3K56 acetylation. Its deficiency causes major chromatin changes in the retina that are accompanied by marked changes in expression of metabolic genes including GLUT1 and metabotropic glutamate receptor Grm6 and severe functional impairment in the SIRT6-KO retinas. These mice showed profoundly impaired electroretinogram (ERG) [52].

4. Role of Sirtuins in Glaucoma

Glaucoma and optic neuropathy are the leading chronic neurodegenerative disorders over the age of 40 and are associated with increased intraocular pressure, ischemia, oxidative stress, and deprivation of neurotrophic factors [53, 54]. Retinal ganglion cell (RGC) transmits light signals from the neural retina (where the light is captured and converted to electrical impulse) to the visual processing centers of the brain. Ischemic condition in glaucoma leads to hypoxia with increased apoptosis of RGC [55].

SIRT1 was first linked with hypoxia inducible factor (HIF) activity in hepatoma cells, where HIF-2 was acetylated at its C-terminal and consequential decrease in its transcriptional activity. SIRT1 activation reverses the acetylation of HIF-2 and increases its transcription and erythropoietin production [56]. In HEK293 cells, SIRT1 binds to HIF-1, deacetylates at Lys674 and blocks its association with the transcriptional coactivator, p300 [57]. Hypoxia affects NAD^+/NADH ratio that in turn suppresses SIRT1 activity followed by acetylation and activation of HIF-1 [57]. SIRT1 binds to both HIF-1α and HIF-2α. In transfected HEK293 cells, HIF-2α competes with HIF-1α for SIRT1 binding. In support of this, erythropoietin-enhancer and vascular endothelial growth factor promoter reporter analysis showed that SIRT1 facilitated the transcriptional activity of HIF-2α, whereas it repressed HIF-1α activity. This study noted that SIRT1-mediated hypoxic responses appear to be dependent on the α subunit of HIF-1 [57]. Our earlier cell culture study showed that SIRT1 protects the hypoxic RGCs through inhibition of caspase-3 [54].

The Stress-activated Protein Kinase (SAPK)/-c-jun N-terminal kinases (JNK1/2/3) are important signaling kinases that are elevated in neurodegenerative diseases including glaucoma [58]. Differentiated hypoxic RGCs showed that blockade of SIRT1 has higher SAPK/JNK activity whereas inhibition of JNK (SP600125) showed higher SIRT1 activation in [17, 54]. This explains the SIRT1 role in balancing the proapoptotic versus antiapoptotic function. In addition, a study in a rat model of optic nerve axotomy found a direct correlation between SAPK/JNK and induction of apoptosis [59, 60].

In an optic nerve crush injury model, SIRT1 overexpressing (SIRT1-KI) mice had significantly higher RGC numbers compared with severe RGC loss in wild-type [61]. In a similar model, treatment of mice with 250 mg/kg of resveratrol (SIRT1 activator) attenuated the loss of RGC function by preserving pupillary light responses. However, SIRT1-KO mice did not show any effect after resveratrol treatment [61]. Another optic nerve injury study on calorie restricted rats showed decreased SIRT2 mRNA levels compared to the normal diet group but had no effect on survival of RGCs [62]. Retinal ganglion cells in mice pretreated with resveratrol showed protective effect with altered expression of SIRT1 but had minimal alteration in the expression of SIRT2 and SIRT5 followed by optic nerve crush [63].

Glaucomatous human retina showed 2-fold increased expression of SIRT3 compared to normal retina. In addition, human glaucomatous retina showed increased expression of SIRT1, SIRT3, SIRT6, and SIRT7 in the glial fibrillary acidic

protein (GFAP) positive astroglia compared to age-matched nonglaucomatous controls [64].

5. Role of Sirtuins in Age-Related Macular Degeneration (AMD)

AMD, a common cause of blindness in the elderly population, is characterized by either the presence of drusen (dry AMD) or vascular epithelial growth factor- (VEGF-) induced choroidal endothelial cell proliferation with associated leakage (exudative or wet AMD) [65]. Oxidative stress and hypoxia induce several pathological changes in the retina including apoptotic cell death, dysfunction of the retinal pigment epithelial (RPE) cells, accumulation of lipofuscin, formation of drusen, and impairment of Bruch's membrane [66, 67]. SIRT1 deacetylates and activates HIF-2α and regulates VEGF-A promoter [61]. In our earlier study on hypoxic choroidal endothelial cells, we found that SIRT1 regulates vascular endothelial growth factor-A (VEGF-A) through the activation of HIF-2α. Increased VEGF levels in hypoxic cells and the subsequent decrease after the activation of SIRT1 establish a relation between SIRT1 and HIF-2α [68]. Similarly, resveratrol treatment inhibits hypoxic choroidal endothelial cell proliferation through SIRT1-dependent pathway at higher dosage [65].

Three octogenarian patients with AMD who fail to respond to anti-VEGF therapies treated with oral resveratrol showed dramatic short-term anti-VEGF type effect including anatomic restoration of retinal structure with an improvement in choroidal blood flow by near IR multispectral imaging. The improvement of visual function mirrors the effect seen anatomically with added benefit of RPE function and lasted for more than one year when taken daily [69]. In another recent study, Richer et al. reported broad bilateral improvements in ocular structure and function in three patients with AMD over a long-term follow-up of two to three years suggesting its efficacy in AMD [70]. Retinal photo toxicity which is another cofounding factor is associated with AMD. Oral administration of resveratrol showed protective effect against phototoxic degeneration of the mouse retina in vivo via activator protein-1 activation [71].

Cao et al. demonstrated that one of the constituents of drusen is amyloid beta (Aβ) (controlled by activation of SIRT1) induced inflammation in AMD. Amyloid beta induced changes in retinal pigment epithelial cell morphology while barrier integrity was balanced by SIRT1 activation by suppressing nuclear factor kappa-B (p65 subunit). The reduction in the nuclear factor kappa-B activation further decreased the inflammatory cytokine expression of interleukin-8 (IL-8), interleukin-6 (IL-6), and matrix metalloproteinase-9 (MMP-9) [72].

6. Role of Sirtuins in Optic Neuritis

Optic neuritis is an inflammatory optic neuropathy commonly associated with multiple sclerosis [73, 74]. It is a myelin sheath disease with lesions typically located in the optic nerve, brain and spinal cord, or cranial nerves. Normally, myelin helps electrical impulses travel quickly from the eye to the brain, where they are converted into visual information. Optic neuritis disrupts this process and affects vision [75]. Intravitreal injection of SIRT1 agonists inhibits RGC loss in a dose-dependent manner by inducing SIRT1 activity in mice with optic neuritis. This neuroprotective effect is blocked by sirtinol [46, 76]. Resveratrol represents a promising neuroprotective therapy for optic neuritis and traumatic optic neuropathies. Both SIRT1 overexpression and resveratrol treatment reduce the levels of superoxide accumulation in optic nerves following crush injury [61]. Since protective effect of resveratrol on endothelial and cancer cell is dose-dependent caution is necessary in using it as a therapeutic agent [65, 77]. In contrast to SIRT1 overexpression, in an established mouse model of multiple sclerosis, SIRT1 inactivation increased the production of new oligodendrocyte progenitor cells in the adult mouse brain; ameliorated remyelination; and delayed paralysis [78].

7. Role of Sirtuins in Neurodegenerative Diseases

Detailed description of sirtuins protective role in other neuronal diseases has been described in other literature. In a mixed culture of neurons and microglia, SIRT1 deacetylates (at p65 subunit) and reduces NF-kB signaling that protects neurons from amyloid beta induced toxicity in microglia [79]. Ischemic preconditioning and resveratrol treatment reduced neuronal injury of hippocampal CA1 after NMDA challenge in slices and global cerebral ischemic in rats [80, 81].

The inhibition of NF-kB by SIRT1 contributes neuroprotection similar to the effect of glucoside against Alzheimer's disease and ischemia [82, 83]. Resveratrol attenuates neuronal degeneration and death in animal models of Alzheimer's disease and Parkinson's disease associated with the cerebral accumulation of β-amyloid and α-synuclein, respectively [84, 85]. In humans, phase II and phase III clinical trials evaluate the usage of resveratrol in Alzheimer's disease patients. The primary outcome of these trials was evaluation of brain imaging, cerebrospinal fluid marker analysis, and cognitive report [86, 87]. SIRT2 inhibitors are capable of postponing the axon degeneration in Parkinson's disease models due to their presence in cell bodies of neurites and growth cones in axons [62]. Loss of sirtuin 4 (SIRT4) in mice leads to decreased glutamate transporter expression and function in the brain, which can cause increased excitotoxic effects. Loss of glutamate transport function is implicated in epilepsy, traumatic brain injury, and amyotrophic lateral sclerosis [88].

Another common neurodegenerative disease is Parkinson's disease (PD) which affects the elderly population in industrialized countries and is characterized by tremor, postural instability, and rigidity. SIRT1 overexpression protects against Parkinson's disease. SIRT1 activates heat shock factor (HSF1) that impacts the transcription of molecular chaperones including heat shock protein 70 and homeostasis of other cellular proteins [18, 89]. In contrary to this, SIRT2 inhibitors showed dose-dependent effect of α-synuclein mediated toxicity in cell culture models. This effect was mediated by increasing the size of aggregates but reduced the number of synuclein aggregates. SIRT2 showed

protective effect in a mouse model of PD using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Activated SIRT2 in MPTP-induced stress cause Foxo3a deacetylation which lead to increased levels of the proapoptotic factor Bim and trigger neuronal death [90]. In a similar model, absence of SIRT5 increased dopaminergic degeneration whereas presence of SIRT5 maintains the function [91].

Huntington disease occurs due to expansion of CAG repeats codes for glutamine residues which affects conformation and aggregation of huntingtin protein [92]. SIRT1 was shown to be protective against this disease in cell culture models and drosophila systems. In a chemically induced mouse model of HD using 3-nitropropionic acid, treating the animals fed with resveratrol decreased cognitive and motor defects. However, in the N171-82Q transgenic mouse model with overexpressed truncated huntingtin protein, resveratrol treatment did not improve the survival [93, 94]. Similar to SIRT1, SIRT2 inhibition was shown to be protective against HD in cell culture and mouse models. However the mechanism is linked with a decrease in the sterol biosynthesis pathway [95]. Recently, viniferin, another resveratrol derivative, was found to be protective by increasing SIRT3 levels that activate/deacetylate manganese superoxide dismutase (MnSOD) and liver kinase B (LKB) [96].

8. Role of Sirtuins in Metabolic and Health Span

Sirtuins promotes cellular longevity using calorie restriction (CR). It is associated with reduced food consumption of an organism compared to normal consumption. This increases nicotinamide adenine dinucleotide (NAD^+) levels in liver which in turn activates SIRT1 [97]. SIRT1 also activates PGC1α which results in mitochondriogenesis [97]. A decline in mitochondrial activity upon aging is a causative factor in many age-related diseases [98]. SIRT2 has been found to regulate metabolism by deacetylating and stabilizing phosphoenolpyruvate carboxykinase (PEPCK1), which is the rate limiting enzyme for gluconeogenesis, linking SIRT2 with type II diabetes [99]. In response to nutrient deprivation and energy expenditure it promotes lipolysis and inhibits adipocyte differentiation through deacetylation of FoxO [99, 100]. SIRT2 deacetylates FoxO3 and increases its transcription after calorie restriction in mice and after hydrogen peroxide treatment in kidney cells thus reducing cellular levels of ROS [101].

Recently, it has been reported that SIRT3 has been found to regulate many aspects of mitochondrial function, such as metabolism, Adenosine triphosphate (ATP) generation, and modulation of the response to oxidative stress using acetyl-coA synthetase 2 (AceCS2) and glutamate dehydrogenase (GDH). A shift from dependence on liver glycolysis, facilitated by GDH and AceCS2 activity, has been implicated in calorie restriction (CR), suggesting a role of SIRT3 in reprogramming metabolism during CR to allow respiration [102–104].

However, presence of SIRT4 inhibits GDH in pancreatic beta cells and opposes the effects of CR. SIRT4 deficient or CR mice are insensitive to phosphodiesterase (an enzyme that cleaves ADP-ribose and is essential for ADP-ribosylation). This indicates that, in beta cell mitochondria, SIRT4 repress the activity of GDH by ADP-ribosylation, thereby downregulating insulin secretion in response to amino acids, effects that are alleviated during CR [105]. SIRT5 and SIRT6 are sensitive to CR and are induced by low calorie stress. Under CR, upregulated SIRT5 in liver leads to increased physiological needs for nitrogen disposal due to increased amino acid metabolism [106].

SIRT6 is involved in human telomere and genome stabilization, gene expression and DNA repair, glucose homeostasis, and inflammation [107–109]. Aging phenotype was observed in SIRT6 knockout mice where they showed increased transcription of NF-kB that trigger increased apoptotic resistance and cell senescence. This is reversed by the inhibition of RelA subunit of NF-kB. This finding provides the evidence that SIRT6 binds NF-kB subunit of RelA and modulates NF-kB target genes [110]. SIRT6 deacetylate H3K9 and control expression of multiple glycolytic genes lactate dehydrogenase (LDH), triose phosphate isomerase (TPI), aldolase and phosphofructokinase (PFK1). SIRT6 deficient cells showed elevated HIF-1α activity and elevated glucose uptake with an increased glycolysis and reduced mitochondrial respiration. Based on these functions, SIRT6 can serve as a biomarker for metabolic diseases [107].

9. Role of Sirtuins in Cardiovascular Diseases

SIRT1 deficient mice showed developmental defects in the heart and are embryonically lethal. However, heterozygous SIRT1 deficient mice showed absence of fibrosis and decreased cardiomyocyte size [111]. At cellular level, SIRT1 promotes vascular relaxation by activating endothelial nitric oxide synthase (eNOS). SIRT1 interacts with NF-kB by inhibiting its signaling and proinflammatory cytokine release in endothelial cells [109, 112, 113]. SIRT1 promotes angiogenesis by increasing VEGF expression through hypoxia signaling. Pharmacological inhibition of SIRT1 using sirtinol decreases VEGF levels [68]. Inhibition of SIRT1 increases thrombosis by reducing tissue factor activation through the pathway of Peroxisome Proliferated-Activated Receptor δ, cyclooxygenase-2 derived prostacyclin, or NF-kB [114, 115].

Animal studies found that resveratrol (2.5 mg/kg) prevents the cardiac dysfunction in spontaneously hypertensive rats [116]. In a minipig model of heart failure, transplanted stem cell cultures from wild type showed more proliferation compared to transplants from SIRT1 knockout stem cell sheets. They also showed less cytokine release compared to wild-type cells. Similar to this, the Lewis rat model of heart failure also showed lesser cardiac function in a SIRT1 knockout stem cell transplants. These experiments explain that SIRT1 mediates regenerative capability of stem cells in heart failure [117].

SIRT3 knockout mice also showed the signs of cardiac hypertrophy. SIRT3 regulates mitochondrial function and the inhibition of SIRT3 causes mitochondrial dysfunction, which in turn causes reduced oxygen consumption. Overexpression of SIRT3 reduces ROS levels and mitochondrial DNA damage underlying with vascular inflammation in

atherogenesis [109]. SIRT3 deacetylates/activates superoxide dismutase 2 (SOD2) which further increases the deacetylation of transcription factor forkhead box o3a (FOXO 3a) and protects against cardiac hypertrophy [118]. In a recent study, decreased SIRT3 in human pulmonary artery smooth cell was associated with an induction of transcription factors HIF-1alpha, signal transducer and activator of transcription-3 (STAT3), and nuclear factor of activated T-cells cytoplasmic 2 (NFATc2) and was associated with pulmonary arterial hypertension [119].

Similar to SIRT3, SIRT6 knockdown mice showed cardiac hypertrophy and the overexpression of SIRT6 rescues cardiac hypertrophy. SIRT6 interacts with stress reactive kinase c-jun and suppresses the promoter of genes in IGF-signaling pathway. This inhibition further decreases the expression of genes in IGF-akt signaling that participates in the progression of heart failure [120, 121]. Interaction of SIRT6 with a transcription factor NF-kB at Rel A subunit protects against inflammation [110].

In a recent study by Araki et al. [122], myocardial infarction and hindlimb ischemic mouse models showed high levels of SIRT7 expression, whereas SIRT7 knockout mice were more susceptible to cardiac rupture after myocardial infarction and delayed blood flow recovery after hindlimb ischemia. In vitro mechanistic evaluation provided the evidence that cardiac fibroblasts derived from these SIRT7 knockout mice showed reduced transforming growth factor beta (TGF-β) signaling and TGF beta receptor I protein compared to wild-type mice derived cells. They showed low levels of fibrosis related genes [122].

10. Role of Sirtuins in Cancer

SIRT1 role in cancer was widely studied and described in detail in earlier literature. We will briefly describe it in our review. It plays a dual role in promoting angiogenesis and acts as a tumor suppressor. SIRT1 is involved in genome stability, inflammation, DNA repair, and apoptosis processes [123]. SIRT1 activates HIF-2α and Rel A/p65 subunit of NF-kB and promotes angiogenesis through the production of VEGF in choroidal endothelial cells [68]. In hepatocellular carcinoma, SIRT1 promotes accumulation of HIF-α and activates transcription of HIF-1alpha target genes [124]. A recent study found mutations in the SIRT1 gene in several breast cancer cell lines that were related to breast cancer progression [125]. SIRT2 plays a dual role like SIRT1. SIRT2 knockout mice developed tumors after 10 months of age compared to wild-type mice [126]. They developed mammary tumor and hepatocellular carcinoma [127]. In addition to tumor suppression, knockdown of SIRT2 or pharmacological inhibition provides an antiproliferative effect in cancers. A recent study on neuroblastoma cell line found that SIRT2 inhibition downregulates C-MYC and N-MYC oncogenic proteins [128]. Tenovin-D3 another SIRT2 inhibitor was found to increase the tumor suppressor protein p21 [129]. SIRT2 activates lactate dehydrogenase A (LDH-A) and the increased amount of LDH-A was noted in many cancer cells. Thus inhibition of SIRT2 disrupts the cancer metabolism [123, 130].

SIRT3 was reported to be protective in several cancers like oral carcinoma, breast ovarian, and renal cancers [131]. SIRT3 was found to decrease ROS and ROS participates in HIF and akt signaling that plays a critical role in cell proliferation [118]. In vitro studies in human cancer cells revealed that overexpression of SIRT3 decreases the cell proliferation [132]. He et al. [133] found that SIRT3 levels were correlated with clinical features such as metastasis and tumor size in breast cancer. In contrast, recent meta-analysis on 14 studies with 2165 cancer patients assessed the relation between SIRT3 immunohistochemical expression and their respective survival and clinical pathological characters. This study did not find any correlation between SIRT3 expression and clinical pathology. They also concluded that SIRT3 is associated with prognosis and clinical parameters in specific cancers [134].

The inhibitory role of SIRT4 on GDH makes SIRT4 as a tumor suppressor. SIRT4 was found to be downregulated in many cancers. SIRT4 knockout mice developed lung tumors within 18–26 months compared to wild-type SIRT4 mice [135]. SIRT6 also serve as a tumor suppressor by regulating HIFs [107] and NF-kB. SIRT6 plays a role in genomic instability and DNA repair and inflammation explains SIRT6 participation in cancer. A study explains that immortalized mouse embryonic fibroblasts (MEF) cells from $SIRT6^{-/-}$ mice developed more tumorigenicity than MEF from $SIRT6^{+/+}$ mice. They suggest that this is mainly due to reprogramming of metabolism through two transcription factors HIF-1alpha and MYC [136].

So far, various microRNAs (miRNA) have been reported to bind SIRT1 and modulate SIRT1 deacetylation target genes. However, recent in vitro and in vivo analysis found that SIRT7 promotes gastric cancer growth by deacetylating H3K18ac at the promoter of miRNA-34a, whereas reducing/knocking down SIRT7 inhibits the cancer cell growth. They also showed G2/M accumulation in the cell cycle [137]. In addition ovarian and breast cancer cells showed high levels of SIRT7 and reducing SIRT7 downregulated cancerous cell growth and impacts apoptotic related proteins (NF-kB) [138, 139].

11. Conclusion and Future Aspect

Although limited, it is evident from experimental studies that sirtuins promote survival of RGCs, confer protection against cell death, and are important players in eye-related neurodegenerative diseases discussed above. Current data therefore supports the concept that modulation of sirtuin activity to provide neuroprotection in these diseases may have therapeutic implications. Yet, drug delivery still remains a major challenge in ocular treatment, especially for diseases affecting posterior and anterior segment of the eye. Systemic administrations fail to achieve a therapeutic concentration of drug due to the presence of blood-aqueous and blood-retinal barriers. In contrast, intravitreal administration can achieve higher concentration of drug to treat posterior segment diseases, but the process is very painful and suffers from poor patient compliance. Despite these challenges, continued

efforts in this direction have helped to find numerous strategies to improve drug delivery system [140]. These strategies include formulating drugs into implants and use of micro- or nanoparticulate and hydrogel-based systems. Transporter targeted prodrug approach has also been described to deliver drugs to both the anterior and posterior segments of the eye. Noninvasive drug delivery methods utilizing ultrasound, iontophoresis, and microneedle based devices have been promising [140]. In addition, recently, the delivery system of a sirtuin-activating agent, resveratrol, was developed and patented by Allergan Inc. The inventors have shown prolonged retinal ganglion cell survival and neuroprotection by administration of resveratrol embedded in biodegradable polymer like poly-lactic-co-glycolic acid (PLGA) and intend to use this formulation for the treatment of posterior segment disorders like AMD and macular edema.

Future studies are needed to better understand and elucidate the molecular role of sirtuins and identify their substrate partners/cofactors and the intracellular pathways that regulate their activity in different disease models. Nonetheless, it is essential and plausible to develop and test (clinical trials) specific pharmacological activators or inhibitors of sirtuins that may mediate neuroprotection and serve as beneficial strategy for treatment of the neurodegenerative diseases of the eye [141, 142].

Competing Interests

The authors declare they have no competing interests.

Authors' Contributions

The authors contributed as follows: Sankarathi Balaiya reviewed the literature and wrote the manuscript; Khaled K. Abu-Amero and Altaf A. Kondkar reviewed and wrote the conclusion of the manuscript; Kakarla V. Chalam edited and approved the final version. All the authors have approved the final version of the manuscript.

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Review Article

Retinal Diseases Associated with Oxidative Stress and the Effects of a Free Radical Scavenger (Edaravone)

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Oxidative stress plays a pivotal role in developing and accelerating retinal diseases including age-related macular degeneration (AMD), glaucoma, diabetic retinopathy (DR), and retinal vein occlusion (RVO). An excess amount of reactive oxygen species (ROS) can lead to functional and morphological impairments in retinal pigment epithelium (RPE), endothelial cells, and retinal ganglion cells (RGCs). Here we demonstrate that edaravone, a free radical scavenger, decreased apoptotic cell death, oxidative damage to DNA and lipids, and angiogenesis through inhibiting JNK and p38 MAPK pathways in AMD, glaucoma, DR, and RVO animal models. These data suggest that the therapeutic strategy for targeting oxidative stress may be important for the treatment of these ocular diseases, and edaravone may be useful for treating retinal diseases associated with oxidative stress.

1. Introduction

Oxidative stress plays a pivotal role in the development and progression of multiple neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington disease (HD) [1, 2]. Oxidative stress and neurodegeneration are also involved in several eye diseases, for which there are many published reports [3–5]. Aging, gene abnormalities, and excess exposure to exogenous oxidative stressors (e.g., a light exposure) increase oxidative stress in the eye. In this review, we describe the relationship between oxidative stress and retinal diseases, as well as the effects of the free radical scavenger, edaravone.

2. Oxidative Stress

2.1. Reactive Oxygen Species (ROS). Oxidative stress is caused by an imbalance between the antioxidant defense system and the production of reactive oxygen species (ROS), including superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). In particular, the superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$) with an unpaired electron are also known as free radicals. Hydrogen peroxide exhibits a low reactivity, but it can penetrate cell membranes, including the inner and outer membranes of mitochondria. Therefore, hydrogen peroxide (H_2O_2) can react with cellular iron and generate hydroxyl radicals, the most reactive form of oxygen, via the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+}$ [6].

These ROS are produced during the processes of several enzymatic and oxidation reactions. The mitochondrial respiratory chain is the main source of ROS production [7]. In the inner membrane of mitochondria, electrons are transported and oxygen is converted into water. Under hypoxic conditions, this process is not performed to completion, resulting in an increased production of superoxide anions ($O_2^{-\bullet}$). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is the source of ROS, derived primarily from superoxide anions ($O_2^{-\bullet}$), via enzymatic reactions [8, 9]. As part of the NOX family, seven oxidases (NOX1–5 and Duox1-2) are recognized [10]. Of these, NOX4 can produce both superoxide anions ($O_2^{-\bullet}$) as well as hydrogen peroxide (H_2O_2) [11, 12]. Nitric oxide (NO) is produced by the sequential oxidation/reduction of L-arginine to L-citrulline by nitric oxide synthase (NOS), which exists in the form of inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) [13]. NO can react with superoxide anions ($O_2^{-\bullet}$)

and form peroxynitrite ($ONOO^-$) which has a highly potent oxidizing and nitrosating ability [14]. This reaction prompts eNOS uncoupling, resulting in an increase in the formation of superoxide anions ($O_2^{-\bullet}$) [15]. Moreover, peroxynitrite ($ONOO^-$) oxidizes the eNOS cofactor and further promotes eNOS uncoupling [16].

2.2. Cigarette Smoking. Cigarette smoke is known as one of the exogenous sources of ROS [17] and contains multiple ROS producers, such as nicotine and cadmium. Nicotine promotes nitric oxide (NO) production and increases proangiogenic factors [18] and cadmium accumulates preferentially in the RPE and choroid and increases ROS production [19]. Moreover, hydroquinone (HQ) is also included in cigarette smoke. HQ is an abundant oxidant in nature, found in processed foods, plastic containers, and atmospheric pollutants. In addition, cigarette smoke extract (CSE) has been shown to induce alterations to mitochondrial integrity, increase in lipid peroxidation, and significant human RPE cell death [20, 21].

Excess light exposure is also included as a source of ROS. The energy contained in a photon of light changes electron orbitals and can break bonds directly.

2.3. Light Exposure. Light exposure reduces lipofuscin autofluorescence [22]. Autofluorescence photobleaching is an indication of lipofuscin photooxidation [23]. At a higher level of light exposure, such as after prolonged exposure or being subjected to blue light, RPE disruption occurs in a manner which permanently alters the autofluorescence pattern [24]. Usually, autofluorescence photobleaching recovers after several hours; however, the detailed mechanism remains unclear. Excess light exposure induces cell death in a murine retinal cone cell line (661W) and can cause a disruption in the phagocytotic function of a human retinal pigment epithelial cell line (ARPE-19) [25, 26].

Oxidative stress entails an excess amount of reactive oxygen species (ROS) that leads to oxidative damage to DNA, proteins, lipids, and mitochondria. Mitochondria become progressively more incompetent with age. Therefore, oxidative stress is associated with several age-related diseases. For a detailed summary of the factors affected by ROS, please see the review by Davalli et al. [27].

2.4. Endoplasmic Reticulum (ER) Stress. Oxidative stress is closely linked to endoplasmic reticulum (ER) stress [28–31]. During the induction of the unfolded protein response (UPR), ROS are produced by protein disulfide isomerase (PDI), endoplasmic reticulum oxidoreductin (ERO-1), and NADPH oxidase complexes (i.e., NOX4) [32, 33]. ROS are produced during the transfer of electrons from protein thiol to molecular oxygen by ERO-1 and PDI and during protein misfolding due to the depletion of glutathione (GSH) [34]. In addition, after utilizing GSH, thiols interact again with ERO-1/PDI and are reoxidized. These chain reactions then generate further ROS [34]. ROS can also be produced by unfolded proteins independent of disulfide bond formation. Unfolded proteins in the ER can lead to Ca^{2+} release into the cytosol, which then increases ROS formation in mitochondria [35]. ATP depletion caused by protein folding and refolding processes in the ER lumen is also considered to contribute to increased ATP and ROS production by stimulating mitochondrial oxidative phosphorylation.

2.5. Inflammation. Oxidative stress is linked to inflammation [36–39]. It has been reported that oxidative stress-induced RPE cell death primarily due to necrosis induces the expression of an inflammatory gene, high mobility group protein B1 (HMGB1) [40]. Moreover, the inflammatory cytokine, tumor necrosis factor- (TNF-) α, is also induced in macrophages and healthy RPE cells by the medium of dying cells exposed to oxidative stress [41]. Conversely, proinflammatory cytokines, such as TNF-α, interleukin-1β (IL-1β), or interferon-γ (IFN-γ), induce intracellular and extracellular ROS production in human RPE cells [42]. Indeed, these proinflammatory cytokines are upregulated in the eyes of patients with glaucoma, age-related macular degeneration, diabetic retinopathy, or retinal vein occlusion [43–46].

In particular, endothelial cells are affected by inflammation. Inflammation induces shifts in the endothelial cell phenotype, increasing the expression of inflammatory mediators, cytokines, and iNOS activation [47]. These events are observed in both RPE cells and endothelial cells. Moreover, RPE interacts with endothelial cells (ECs) directly and can enhance the proangiogenic potential of the ECs, such as proliferation and migration. For example, TNF-α upregulates the expression of vascular endothelial growth factor (VEGF), a major angiogenic factor, in RPE cells via the ROS-dependent activation of β-catenin [48]. ROS also affects VEGF-stimulated VEGF receptor 2 dimerization and autophosphorylation. Conversely, VEGF further stimulates ROS production through the activation of NOX in endothelial cells [49]. It has been reported that hypoxia-induced microRNA-424 (miR-424), a member of the miR-16 family crucial for the regulation of cell differentiation [50, 51], promotes hypoxia-inducible factor- (HIF-) 1α stability in human umbilical vein endothelial cells (HUVECs). This is achieved by inhibiting the expression of a scaffolding protein, Cullin-2, which is essential for the assembly of the HIF E3 ubiquitin ligase complex [52]. ROS also inhibits the activity of prolyl hydroxylase enzymes (PHD) and factor-inhibiting HIF-1α (FIH) by reducing Fe^{2+} availability [53]. In addition, endothelial cell apoptosis is triggered by high glucose-induced overexpression of iNOS in RPE cells activating the PKR-like endoplasmic reticulum kinase (PERK) pathway [54].

2.6. Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2). Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a nuclear transcription factor regulating antioxidant defense. Nrf2 usually exists in the cytosol and interacts with Kelch-like ECH-associated protein 1 (Keap1), an adaptor for a Cullin-3-(Cul3-) based ubiquitin ligase [55]. Under normal condition, the amount of Nrf2 is maintained at lower levels than that of Keap1 and Cul3 proteins. However, under oxidative stress condition, electrophilic agent increases Nrf2 much more than Keap1 and Cul3 proteins, resulting in the accumulation of Nrf2 in the nucleus. In contrast, Keap1 and Cul3 are not changed in their abundance, subcellular localization,

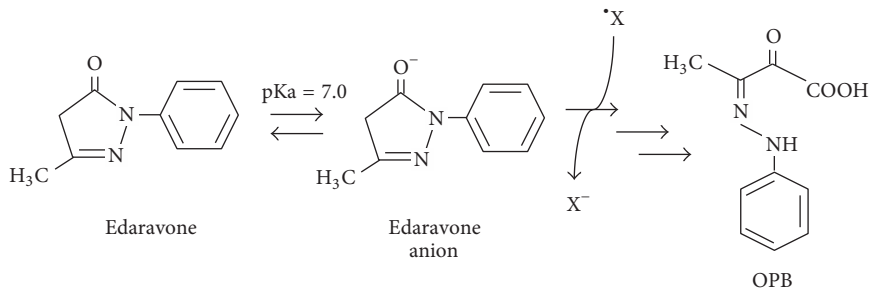

Figure 1: A hypothetical radical-scavenging mechanism of edaravone. Edaravone anion scavenges radicals ($^{\bullet}X$) to produce anion bodies (X^{-}) and edaravone radicals. The final product is 2-oxo-3-(phenylhydrazono)-butanoic acid (OPB), which is without oxidation power.

and interaction in response to electrophilic stimuli [56]. The increased Nrf2 translocates into the nucleus, dimerizes with Maf proteins, and binds to the antioxidant/electrophile response element (ARE/EpRE) in the promoters of its target genes. These genes encode protective proteins against oxidative stress, including superoxide dismutase (SOD), catalase, glutathione S-transferases (GST), NADPH quinine oxidoreductase (NQO-1), peroxiredoxin (PRX), heme oxygenase-1 (HO-1), and thioredoxin reductase-1 (TXNRD1) [57–59]. Catalase and SOD directly neutralize hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{-\bullet}$), respectively [60, 61]. GST and NQO-1 function as a detoxicating enzyme of electrophilic substances and a xenobiotic-metabolizing enzyme, respectively [62, 63]. HO-1 removes toxic heme, producing iron ions (Fe^{2+}), carbon monoxide (CO), and biliverdin [64]. Both biliverdin and its reductive form, bilirubin, are potent antioxidants; bilirubin breaks the oxidation chain reaction of polyunsaturated fatty acids [65].

3. Edaravone

Oxidative stress is highly complex and is linked to other forms of stress and effects on various cells. There are two strategies for reducing oxidative stress: (1) enhancing antioxidant enzymes and (2) reducing ROS directly.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MCI-186, Radicut®) is a free radical scavenger and a drug used to treat acute ischemic stroke [66]. In Japan, edaravone is administered via an intravenous infusion within 24 h of the onset of acute ischemic stroke in patients with lacunae, large-artery atherosclerosis, and cardioembolic stroke.

The hypothetical reaction mechanism of edaravone involves the electron donation to free radicals. The final product derived from edaravone is 2-oxo-3-(phenylhydrazono)-butanoic acid which is without oxidation power (Figure 1) [67–70]. The main metabolites consist of sulfoconjugate and glucuronic acid conjugation. Edaravone quenches hydroxy radicals ($^{\bullet}OH$) and inhibits lipid peroxidation dependent and independent of hydroxy radicals ($^{\bullet}OH$) [67, 71, 72]. Indeed, we demonstrated that edaravone scavenged the intracellular not only hydroxy radicals ($^{\bullet}OH$) but also superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) [73]. Moreover, edaravone shows a neuroprotective effect against ischemia/reperfusion brain injury and cardiopulmonary resuscitation through a Bax/Bcl-2 dependent antiapoptotic mechanism [74, 75]. Edaravone also ameliorates photoreceptor cell death after experimental retinal detachment through increasing the level of the antiapoptotic Bcl-2 [76].

Edaravone has not only antiapoptotic effect but also anti-inflammatory effect. In the brain with the treatment of middle cerebral artery occlusion, the expression levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and inducible nitric oxide synthase (iNOS) were effectively suppressed by edaravone [77]. In addition, the expressions of the inflammatory cytokines TNF-α and monocyte chemoattractant protein-1 (MCP-1) in retinal lysates were significantly reduced by edaravone treatment [76].

Edaravone is a low-molecular-weight agent and exerts potency both in water and under lipid soluble conditions [67]. Thus, edaravone is a free radical scavenger with properties of both of vitamins C and E. In addition, edaravone readily crosses the blood-brain barrier, which is unlike other free radical scavengers. These properties of edaravone may be important for its neurovascular protective effects observed in patients with acute ischemic stroke.

Previously, our laboratory demonstrated that combination therapy with normobaric hyperoxia and plus edaravone prevented neuronal damage following focal cerebral ischemia and reperfusion in mice [78]. For a summary of multiple reports on the protective effects of edaravone, please refer to the review by Watanabe et al. [79].

This review describes the relationship between oxidative stress and retinal disease, as well as the effect of edaravone against retinal disease.

4. Age-Related Macular Degeneration (AMD)

4.1. Pathogenesis and Pharmacological Therapy. Age-related macular degeneration (AMD) is the leading cause of blindness in elderly individuals throughout the world, and approximately 50 million people suffer from AMD worldwide [80]. In addition, the number of patients with AMD continues to increase, and it is estimated that approximately 198 million people currently suffer from AMD [81]. AMD is classified into two types: "dry" and "wet." In the dry-type AMD, gradual vision loss and drusen, the yellow deposits located under the retina, are diagnostic features [82]. Lipofuscin is the main constituent of drusen and is produced during the reaction of cell metabolites, such as lipid peroxidation [83, 84].

Lipofuscin is deposited when the production of lipofuscin is beyond the disposal capacity of the photoreceptor pigment in RPE [85]. RPE is particularly susceptible to ROS formation due to its high consumption of oxygen, high proportion of polyunsaturated fatty acids, and constant exposure to light. Drusen causes retinal pigment epithelium (RPE) degeneration and "geographic atrophy" appears as feature in eye fundus. When it spreads to the fovea, rapid and severe vision loss occurs. Some dry-type AMD pathology advances to wet-type AMD pathology. The wet-type AMD accounts for 10–15% of AMD patients, and choroidal neovascularization is characterized. The vessels within Bruch's membrane or the sub-RPE space are very weak; therefore, hemorrhage and/or vascular leakage cause damage to the retina leading to further vision loss.

There are several events that occur during the development of AMD, such as oxidative stress, the formation of drusen and RPE dysfunction, apoptosis, activating immune system, senescent loss of homeostatic control, and Bruch's membrane abnormalities. These events are highly complex and involve crosstalk, as well as interaction with each other. As the name indicates, AMD is major ocular disease in elderly individuals [80]. With aging, antioxidant level declines and ROS level increases, ensuring oxidative stress [86]. By aging, macular carotenoids level [87], glutathione S-transferase-1 expression level [88], and vitamin E level [89] are decreased and lipid peroxidation is increased [90]. In contrast, lipofuscin [91, 92], mitochondrial DNA damage in retina [93, 94], advanced lipid peroxidation, and glycation end products [90, 95] are increased. Aging changes the homeostasis of these factors, which means that the rate of AMD development is high in elderly individuals.

Currently, there is no treatment available for the dry-type AMD. In the Age-Related Eye Disease Study (AREDS), antioxidant micronutrients, including β-carotene, vitamin C, vitamin E, and zinc, showed a suppressive effect on disease progression [96]. As a therapeutic drug for wet-type AMD, the anti-VEGF antibody is commonly used. Anti-VEGF antibody treatment is the current standard therapy that improves the visual function in patients with wet-type AMD [97]. Patients receive the anti-VEGF antibody treatment via an intravitreal injection at regular intervals. An intravitreal injection is the most common and widely recommended route of drug administration to treat posterior ocular diseases [98]. However, this method is highly invasive and is associated with the risk of infection (0.02 to 1.6%) [99–103]. In addition, the anti-VEGF antibody is very expensive, and the financial burden on patients with the wet type of AMD is extremely high. Therefore, the development of novel therapeutic drug is an urgent need.

4.2. The Effects of Edaravone. We demonstrated that edaravone is effective against retinal degeneration both in vivo and in vitro [104–106]. A model of light-induced retinal degeneration in mice is commonly used for the evaluation of retinal damage and photoreceptor cell death induced by excess exposure to light [107–109]. Previously, we demonstrated that oxidative stress was involved in light-induced photoreceptor cell death [110–113]. An electroretinogram (ERG) revealed that the intraperitoneal administration of edaravone at a dose of 3 mg/kg (30 min before and just after light exposure) inhibited visual dysfunction five days after light exposure [104]. Moreover, it decreased the number of apoptotic TUNEL-positive cells and was a marker of oxidative damage to DNA, 8-hydroxy-2-deoxyguanosine- (8-OHdG-) positive cells, and the expression of phosphorylated JNK and phosphorylated p38, but not that of phosphorylated ERK, in the whole retina after light exposure [104]. These data suggest that oxidative stress is involved in light-induced retinal degeneration, and the systemic administration of edaravone may slow the progression of photoreceptor degeneration through antioxidative stress [73] and antiapoptotic effects [74–76] (Figure 2). Moreover, this protective effect of edaravone was also observed in N-methyl-N-nitrosourea- (NMU-) induced retinal photoreceptor degeneration in mice, a model of retinitis pigmentosa [114].

Next, we evaluated the effect of the edaravone eye drop, consisting of edaravone-loaded submicron-sized liposomes (ssLips). Eye drop administration is a noninvasive and simple method of the delivery for patients. The protective effects against visual dysfunction and apoptosis induced by light exposure were shown by edaravone-loaded ssLips at a dose that free edaravone could not prevent [105]. Moreover, the edaravone-loaded ssLips used in the study exhibited a low toxicity in ocular cell lines [105].

Edaravone also demonstrated its effectiveness in the wet-type AMD model. A laser-induced choroidal neovascularization (CNV) model was developed as an animal model of wet-type AMD [115]. Laser photocoagulation ruptures Bruch's membrane and induces CNV, which is the main characteristic feature of the disease [116]. Edaravone administered intraperitoneally or intravenously reduced the CNV area and vascular leakage [106]. Surprisingly, edaravone administered intravenously within 24 h after photocoagulation also demonstrated an inhibitory effect [106]. The mechanism of the effect mediated by edaravone is via the reduction of ROS, lipid peroxidation, and VEGF-induced endothelial cell proliferation. Moreover, edaravone was also found to reduce the laser-induced CNV area in the common marmoset, a small monkey [106]. Edaravone demonstrated effectiveness against experimental laser-induced CNV in both rodents, as well as primates, indicating that it may be effective against wet-type AMD characterized by CNV (Figure 3).

Edaravone is already approved for the treatment of acute ischemic stroke. This means that feasibility of clinical application is high because its effectivity and tolerability are very clear. If a combination therapy of anti-VEGF antibody and edaravone exerts a great inhibitory effect against CNV, edaravone would be a powerful candidate for AMD therapeutic medicine and could extend the period of intravitreal injection.

5. Glaucoma

5.1. Pathogenesis and Pharmacological Therapy. Glaucoma is an optic neuropathy, characterized by retinal ganglion cell (RGC) death, optic nerve head cupping, and visual dysfunction (e.g., scotoma) [117, 118]. Glaucoma is the second

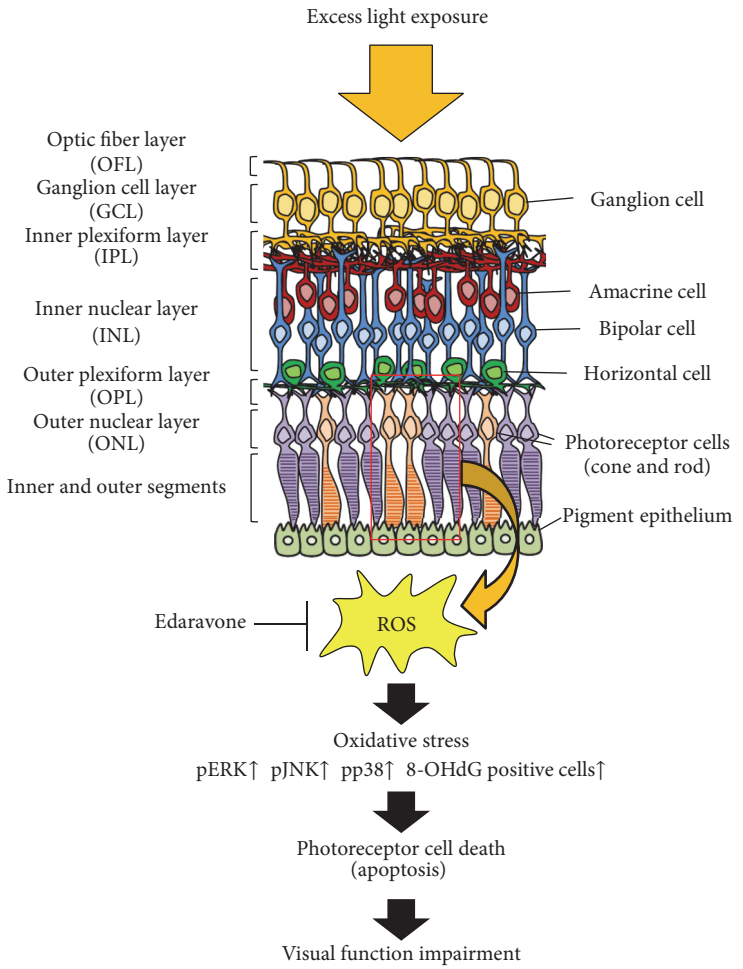

Figure 2: Protective effects of edaravone against light-induced retinal damage. Edaravone scavenges light-induced ROS and rescues light-induced photoreceptor cell death by inhibiting phosphorylated JNK, p38 (but not ERK), and oxidative stress to DNA.

most common cause of blindness worldwide [119], and it is expected that over 80 million people will suffer from glaucoma by 2020 [119]. High intraocular pressure (IOP) was considered as a major cause of developing glaucoma; however, in some cases, RGC loss occurred despite a lower IOP [120]. Therefore, IOP reduction alone may be not sufficient for the treatment of glaucoma.

The axons of the RGCs located within the inner retina constitute the retinal nerve fiber layer (RFNL) and merge to form the optic nerve. Therefore, RGC loss causes a loss of RFNL thickness and optic nerve head cupping [118]. The mechanism of RGC loss remains unknown. Similar to age-related macular degeneration, glaucoma is also associated with oxidative stress [121–123]. Previously, our laboratory demonstrated that antioxidant agents including Coenzyme Q10, Astaxanthin, Zeaxanthin, and Docosahexaenoic acid inhibited RGC death induced by H_2O_2 or the glutamate analog, N-methyl-D-aspartate (NMDA) [124–127]. In a preclinical study, it was revealed that excitatory amino acids (e.g., glutamate and glycine) were increased and that oxidative stress was one of risk factors for RGC death [128–130]. Moreover, oxidative stress leads to the early impairment of trabecular meshwork (TM) cells which are responsible for aqueous humor outflow and further elevation of the IOP [123, 131]. Indeed, multiple reports have shown that, in the aqueous humor of patients with glaucoma, there were lower levels of antioxidants and elevated markers of oxidative stress [132–134].

In preclinical studies, glutamate antagonists, neurotrophic factors, antioxidants, calcium channel blockers, brimonidine, and nitric oxide synthase inhibitors were shown to exhibit the neuroprotective effects [124, 135–143]. A few agents (e.g., brimonidine and memantine) were evaluated in clinical trials. However, these data have not been conclusive [144, 145].

5.2. The Effects of Edaravone. In the model of glaucoma, NMDA-induced retinal damage in mice is commonly used. NMDA induces calcium entry and ROS production, such as NO and superoxide anions ($O_2^{-\bullet}$), and results in RGC death [146, 147].

Edaravone in the form of 5 and 50 nmol intravitreous injections or 1 and 3 mg/kg intravenous injections significantly protected against the NMDA-induced reduction of retinal thickness [73]. Moreover, a 50 nmol intravitreous injection of edaravone decreased the retinal expression of

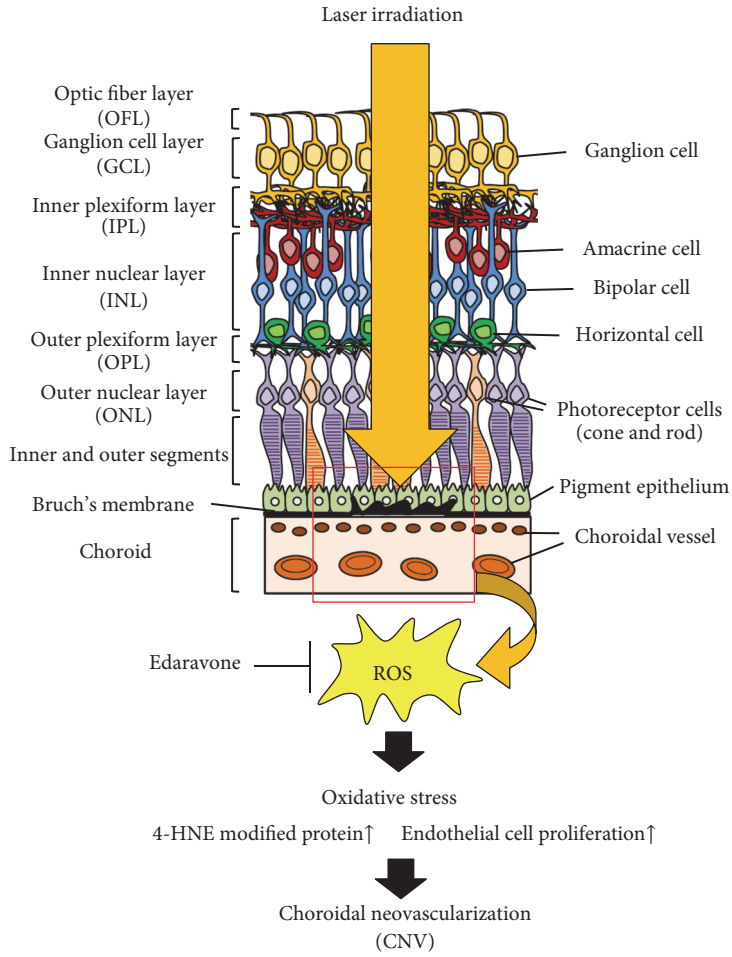

Figure 3: Protective effects of edaravone against laser-induced choroidal neovascularization. Edaravone scavenges laser-induced ROS and rescues laser-induced choroidal neovascularization by inhibiting lipid peroxidation and endothelial cell proliferation.

TUNEL-positive cells, markers of oxidative stress (4-HNE and 8-OHdG), and phosphorylated JNK and p38 but not that of phosphorylated ERK (Figure 4) [73]. Another study reported that an intraperitoneal injection of edaravone at a dose of 3 mg/kg also showed potent neuroprotective activity in a hyaluronic acid-induced glaucoma model [148]. Moreover, edaravone-loaded liposomes suppressed the NMDA-induced reduction of retinal thickness [149]. Elevated IOP induces transient ischemic injury. Edaravone also decreased retinal ganglion cell death induced by oxygen-glucose deprivation (OGD) stress in an ischemia-reperfusion injury model in vitro [73].

6. Diabetic Retinopathy (DR)

6.1. Pathogenesis and Pharmacological Therapy. Oxidative stress is also associated with diabetic retinopathy (DR) [150, 151]. Diabetic retinopathy is one of the most common complications of diabetes mellitus (DM) and the leading cause of blindness and visual dysfunction in working-age populations. Similar to AMD, the number of patients with DM and DR is increasing globally. In the United States alone, 4.1 million people have DR, and the number of patients is expected to double by 2025 [152].

Hyperglycemia induces the excess production of mitochondrial ROS. Increased ROS activates the poly-ADP-ribose polymerase (PARP) pathway and decreases glyceraldehydes 3-phosphate dehydrogenase (GAPDH) activity, which leads to the further activation of the polyol pathway, the protein kinase C (PKC) pathway, advanced glycation end products (AGEs) pathway, and the hexosamine pathway [151, 153, 154]. Under chronic oxidative stress conditions induced by hyperglycemia, Sirt1 and Sirt6 are downregulated and result in endothelial cell senescence [155, 156]. Moreover, increased retinal ROS stabilizes hypoxia-inducible factor-1α (HIF-1α) and leads to the upregulation of angiogenic genes (e.g., VEGF). As a result, pathological angiogenesis is induced [157–160]. Indeed, the concentration of VEGF was found to be upregulated in the vitreous humor of patients with proliferative diabetic retinopathy, compared to the controls with a macular hole [161]. These pathological vessels can result in a hemorrhage or vascular leakage due to its weakness; therefore, such events cause macular edema, retinal ischemia, and retinal detachment. Furthermore, hyperglycemia accelerates premature endothelial cell apoptosis via mitochondrial dysfunction [162].

Increased hyperglycemia-induced ROS affects both endothelial cells, as well as neuronal cells [163]. Increased

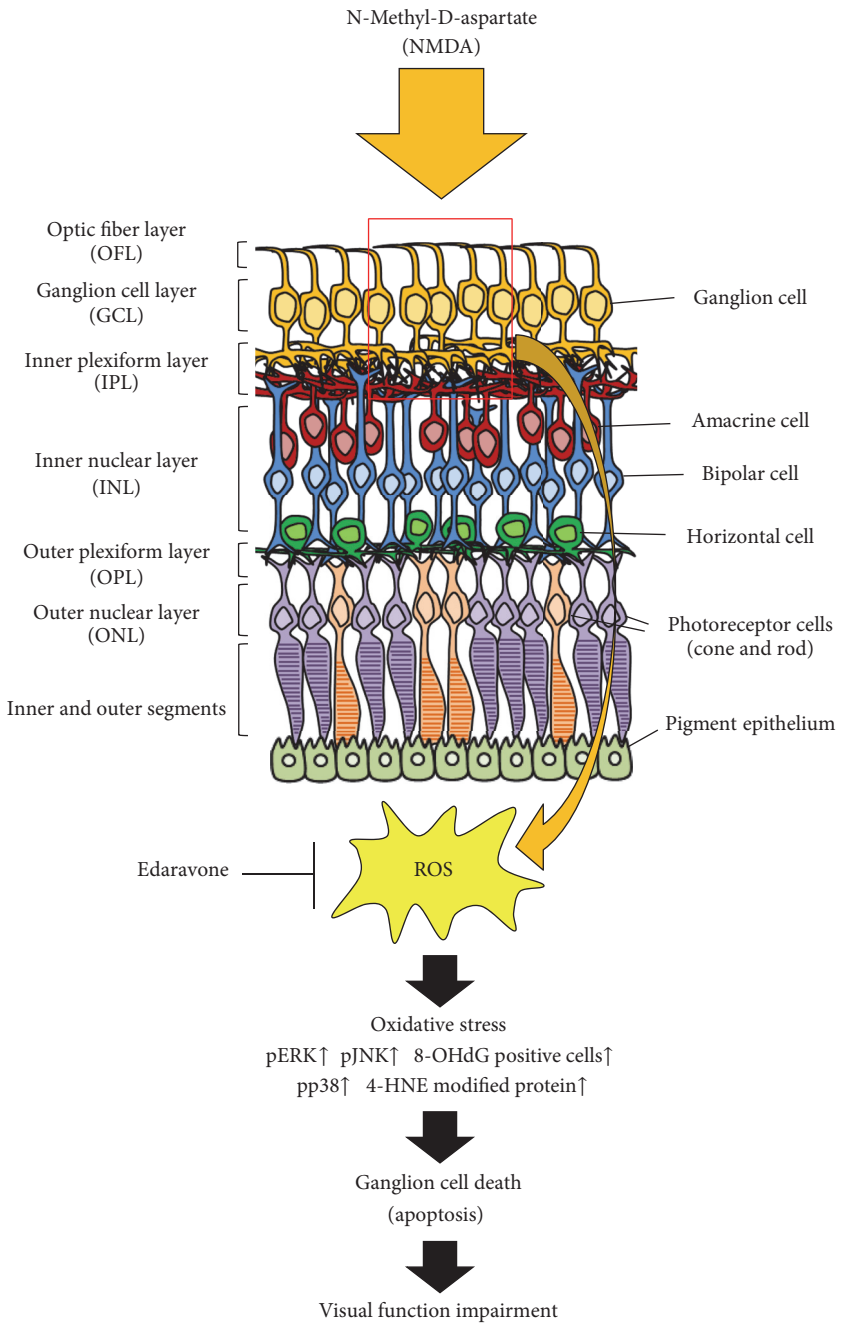

Figure 4: Protective effects of edaravone against NMDA-induced retinal damage. Edaravone scavenges NMDA-induced ROS and rescues NMDA-induced retinal ganglion cell death by inhibiting phosphorylated JNK, p38 (but not ERK), lipid peroxidation, and oxidative stress to DNA.

ROS also decreases brain-derived neurotrophic factor (BDNF) that regulates axonal growth, synaptic activity, and neuronal survival. The damage to the synaptic transmitter and degradation of neurotrophic factors causes neuronal cell apoptosis and visual impairment [164].

Laser panretinal photocoagulation (PRP) is the primary mode of therapy for neovascularization in proliferative diabetic retinopathy (PDR). PRP treatment was proven to decrease the frequency of severe visual loss in PDR with high-risk characteristics (>50% decrease) [165]. Later, Early Treatment Diabetic Retinopathy Study (ETDRS) demonstrated that the frequency of severe visual loss in severe nonproliferative DR (NPDR) and early PDR was decreased by PRP [166]. However, in mild or moderate NPDR, adverse effects of PRP on visual acuity and visual field were also observed [166]. Therefore, for eyes with macular edema, focal photocoagulation is effective in reducing the risk of moderate visual loss. In recent years, anti-VEGF antibody has received a lot of attention. Ranibizumab (Lucentis®) monotherapy provided better visual outcome than standard

focal laser in patients with diabetic macular edema (DME) [167]. Moreover, aflibercept (Eylea®) also provided better visual outcome than standard focal laser in patients with DME [168] and was more effective in improving vision than ranibizumab at worse levels of initial visual acuity [169].

6.2. The Effects of Edaravone. The injection of streptozotocin (STZ) is commonly used for the model of type 1 DM. In this model, retinal damage and visual impairment are observed. An intraperitoneal injection of edaravone at a dose of 3 mg/kg was found to significantly attenuate diabetes-induced RGCs death, the upregulation of ROS, ERK1/2 phosphorylation, cleaved caspase-3, and the downregulation of BDNF [170]. These data suggest that oxidative stress is highly involved in diabetic retinal damage and that the systemic administration of edaravone may slow the progression of retinal neuropathy induced by diabetes.

7. Branch Retinal Vein Occlusion (BRVO)

7.1. Pathogenesis and Pharmacological Therapy. RGC death also occurs under the retinal ischemic conditions during which ROS production is active. Studies have shown that hydroxyl radical ($^{\bullet}OH$) was generated in the retina during ischemic conditions and remained elevated during the reperfusion period [171, 172]. Retinal vein occlusion includes both a branch retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO). In the United States, it is estimated that approximately 100,000 people suffer from RVO.

Similar to DR, the condition including macular edema, retinal ischemia, and fundus hemorrhage is observed. Retinal ischemia impairs the integrity of the blood retinal barrier, and RVO is a common complication of DR. Blood hyperviscosity is also observed in RVO pathology. In determining blood viscosity, erythrocyte deformability plays a critical role. In RVO patients, ROS production and membrane lipid peroxidation, which are indicative of erythrocyte oxidative stress, are observed and positively correlated with erythrocyte membrane viscosity and deformities [173]. A study in young adult CRVO patients revealed that the serum levels of an antioxidant factor, paraoxonase-1 arylesterase (PON1-ARE) activity, were negatively correlated with hyperhomocysteinemia and lipid peroxidation [174]. Moreover, a glucose-6-phosphate dehydrogenase (G6PD) deficiency was associated with increased erythrocyte vulnerability to oxidative stress and developed CRVO [175]. Clinically, antiphospholipid antibodies have been associated with the development of RVO, since it induces oxidative stress in endothelial cells [176].

Anti-VEGF treatment is applied as the therapy for RVO. An intravitreal injection of triamcinolone acetonide is also applied due to the low cost and longer half-life. However, the effects are not permanent, and there are some risks for the development of adverse events, such as cataract formation and elevated IOP [177].

7.2. The Effects of Edaravone. We have reported that the intraperitoneal administration of edaravone at a dose of 1 mg/kg significantly decreased the reduction of retinal thickness and TUNEL-positive cells induced by the ligation of the pterygopalatine artery (PPA) and the external carotid artery (ECA), in a murine retinal ischemic model [178]. Moreover, the intraperitoneal administration of edaravone at a dose of 3 mg/kg lowered a marker of lipid peroxidation, malondialdehyde (MDA), and enhanced superoxide dismutase (SOD) in rodent retinal tissue [179]. MDA is a product of lipid peroxidation and exhibits cytotoxicity. SOD is an antioxidant enzyme that neutralizes superoxide anions ($O_2^{-\bullet}$). In addition, edaravone inhibited the retinal ischemia/reperfusion-induced visual dysfunction and apoptosis of retinal neurons within the inner nuclear, ganglion cell, and outer nuclear layers [179]. These data suggest that edaravone scavenges ROS, thereby reducing lipid oxidation, increasing the activity of antioxidant enzyme, and decreasing the extent of cell death and retinal thickness.

In a clinical trial, edaravone following arteriovenous sheathotomy was effective against macular edema associated with a branch retinal vein occlusion (BRVO) and improved the best-corrected visual acuity [180].

8. Conclusions

Oxidative stress is highly complex and connected to other factors, such as ER stress and inflammation. Moreover, in retinal diseases, including age-related macular degeneration (AMD), glaucoma, diabetic retinopathy (DR), and retinal vein occlusion (RVO), oxidative stress plays pivotal roles in the development and acceleration of these diseases. In the treatment of these ocular diseases, a therapeutic strategy which targets oxidative stress may be effective.

Edaravone demonstrates protective effects against AMD, glaucoma, DR, and RVO models, suggesting that edaravone may be promising as a novel therapeutic drug candidate.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Integrated Approaches to Drug Discovery for Oxidative Stress-Related Retinal Diseases

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Excessive oxidative stress induces dysregulation of functional networks in the retina, resulting in retinal diseases such as glaucoma, age-related macular degeneration, and diabetic retinopathy. Although various therapies have been developed to reduce oxidative stress in retinal diseases, most have failed to show efficacy in clinical trials. This may be due to oversimplification of target selection for such a complex network as oxidative stress. Recent advances in high-throughput technologies have facilitated the collection of multilevel omics data, which has driven growth in public databases and in the development of bioinformatics tools. Integration of the knowledge gained from omics databases can be used to generate disease-related biological networks and to identify potential therapeutic targets within the networks. Here, we provide an overview of integrative approaches in the drug discovery process and provide simple examples of how the approaches can be exploited to identify oxidative stress-related targets for retinal diseases.

1. Introduction

The retina is exposed to chronic oxidative stress (OS) through several mechanisms, including constant exposure to light and reactive oxygen species generated by visual signal transduction pathways [1]. In the healthy state, all cell types in the retina are able to maintain homeostasis under conditions of OS [2]. However, when the balance between pro- and antioxidative signaling is compromised, excessive OS induces dysregulation of functional networks and deleterious changes that result in various retinal diseases, including glaucoma [3–5], age-related macular degeneration (AMD) [6, 7], diabetic retinopathy [8], and retinitis pigmentosa [9]. Due to a combination of lifestyle changes and extended life expectancy, an increasing number of people are at risk for these retinal diseases, and the resulting economic burden imposed on health care systems is increasing accordingly. Various therapies have been developed for retinal diseases [10] with some success, most notably the prostaglandin analogs for glaucoma [11] and antivascular endothelial growth factor (anti-VEGF) agents for AMD [12] and diabetic retinopathy [13]. However, there has been a chronic lack of innovation in drug discovery for retinal diseases [14], as there has been for other diseases [15]. For example, therapies targeting OS in retinal diseases have failed to show efficacy in clinical trials; examples are an antioxidant supplement mixture [16] and a hydroxylamine with antioxidant properties [17]. Thus, there is a clear need for novel approaches to the drug discovery process [18–25]. Here, we first provide an overview of some emerging integrative approaches to therapeutic target discovery and then provide some examples as they relate to OS in retinal diseases.

2. Integrative Approaches in the Search for Therapeutic Targets for OS in Retinal Diseases

2.1. Overview of the Integrative Approaches. Improving our understanding of disease pathogenesis is an important step in the identification of therapeutic targets [14]. Recent technological advances have enabled us to obtain large amounts of

multilevel omics data [20, 21]. For example, DNA microarray and next-generation sequencing technologies have made it relatively easy to obtain genomics data, including profiling of single nucleotide polymorphisms, copy number variations (CNVs), and mutations, which can be used for genome-wide association studies (GWAS) to identify diseases associated with these changes. The same technologies have facilitated the collection of epigenomics data, such as profiles of DNA methylation and DNA-binding sites, and of transcriptomics data, such as messenger and noncoding RNA profiles. In addition, advanced mass spectrometry-based technologies have been indispensable for profiling of protein expression and protein-protein interactions (proteomics), intermediary metabolites (metabolomics), and lipids (lipidomics) [26, 27]. The more recently developed clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) technology has revolutionized the approach to large-scale loss-of-gene-function experiments and phenotypic analysis (phenomics) at both the in vitro and the in vivo levels [28, 29]. In turn, the need to distill these multilevel omics datasets into biological knowledge has led to equally important advances in data curation supported by public databases and novel bioinformatics tools. Some examples are ENCODE for gene expression regulation [30], STRING for protein-protein interactions [31], gene ontology for gene function [32], KEGG for signaling pathways [33], OMIM for human diseases [34], and DrugBank for chemical structures and drug targets [35]. The biological research literature, in combination with text-mining tools such as Agilent Literature Search [36], represents a rich and ever-expanding source of biological knowledge [37]. Integration of multiomics data with existing biological knowledge is essential for generating accurate disease-related networks and for identifying potential therapeutic targets (Figure 1). For example, a recent meta-analysis of omics data identified a number of approved drugs that could potentially be repurposed for the treatment of rheumatoid arthritis (RA) [36]. In that study, RA-associated genes identified by GWAS were prioritized based on eight criteria, including expression quantitative trait loci, protein-protein interactions, pathway analysis, and text mining. This analysis revealed that targets of approved therapies for RA and other indications were significantly enriched in the prioritized genes [38]. Such network-based frameworks generated by integration of multilevel omics data and biological knowledge can be extended to address numerous problems, including interpretation of GWAS data, identification of disease modules located near the drug target, and discovery of disease-disease relationships [39].

Nevertheless, the potential therapeutic targets identified by such integrated approaches will still need to be validated using in vitro and in vivo disease models. Three-dimensional retinal cultures derived from human or mouse embryonic stem cells and induced pluripotent stem cells have been developed and can be used to validate the therapeutic targets for retinal diseases [40–42]. Advances in genome-editing technologies such as transcription activator-like effector nucleases (TALEN) and CRISPR-Cas9 have made it possible to knock out any gene of interest in various species, including teleosts such as zebrafish [43–47]. Indeed, a number of retinal disease models have been developed in zebrafish [48–55], rodents such as mouse and rat, lagomorphs such as rabbit [56–59], and primates such as the common marmoset and cynomolgus monkey [60–62]. Such models have been used successfully to validate the efficacy of therapeutic drugs for retinal diseases, allowing them to be moved forward into clinical trials (Figure 1).

2.2. An Integrative Approach towards Drug Discovery for Glaucoma-Related OS. To illustrate how this integrative approach can be used for OS in retinal diseases, we examined (i) whether glaucoma-associated genes identified by GWAS might be connected through genes related to OS and, if so, (ii) whether the network could identify therapeutic targets to reduce OS in glaucoma. To this end, we used Agilent Literature Search [36], a literature mining tool that can extract biological associations related to a target entity (e.g., gene, mRNA, protein, molecule, chemical, drug, and disease) in a particular context from biomedical literature databases such as OMIM [34] and PubMed [63]. The relationships identified through this analysis (e.g., in our case, glaucoma-associated gene X induces the expression of gene A through interaction with gene B under conditions of OS) can be represented as a network(s) using Cytoscape [64]. If the network of glaucoma-associated gene X and that of glaucoma-associated gene Y share a node(s), these networks can be connected through the node(s), resulting in an integrated network. We used 39 glaucoma-associated genes identified by GWAS [65–67] as the target entities and "oxidative stress" as the designated context in an Agilent Literature Search. The resulting network, shown in Figure 2, contains two glaucoma-associated genes: ATP-binding cassette subfamily A member 1 (ABCA1) and thioredoxin reductase 2 (TXNRD2) (shown in yellow in Figure 2). Gene ontology analysis using DAVID [68] revealed that genes related to "response to oxidative stress" are significantly ($p = 2.5 \times 10^{-10}$) enriched in the network, suggesting that ABCA1 and TXNRD2 are connected through genes related to OS. Superoxide dismutase 2 (SOD2, underlined in red in Figure 2) is another gene related to "response to oxidative stress" and connects to both ABCA1 and TXNRD2.

Probucol, an approved drug for hyperlipidemia, has been reported to inhibit ABCA1 activity [69] and increase SOD2 activity [70]. Both SOD2 and TXNRD2 are mitochondrial antioxidant stress enzymes [71], and mitochondrial dysfunction has been causally related to glaucoma [3]. Because probucol can ameliorate mitochondrial dysfunction [71], this observation raises the possibility that probucol could be used therapeutically as an OS suppressor for glaucoma. In fact, probucol protects against glutamate-induced cytotoxicity in a neuronal cell line [72] and, intriguingly, glutamate toxicity is one of the main pathogenic mechanisms of normal-tension glaucoma [73–76].

Other genes in the network could also be potential therapeutic targets for glaucoma-associated OS. For example, amyloid β (1–42), which is a proteolytic processing product

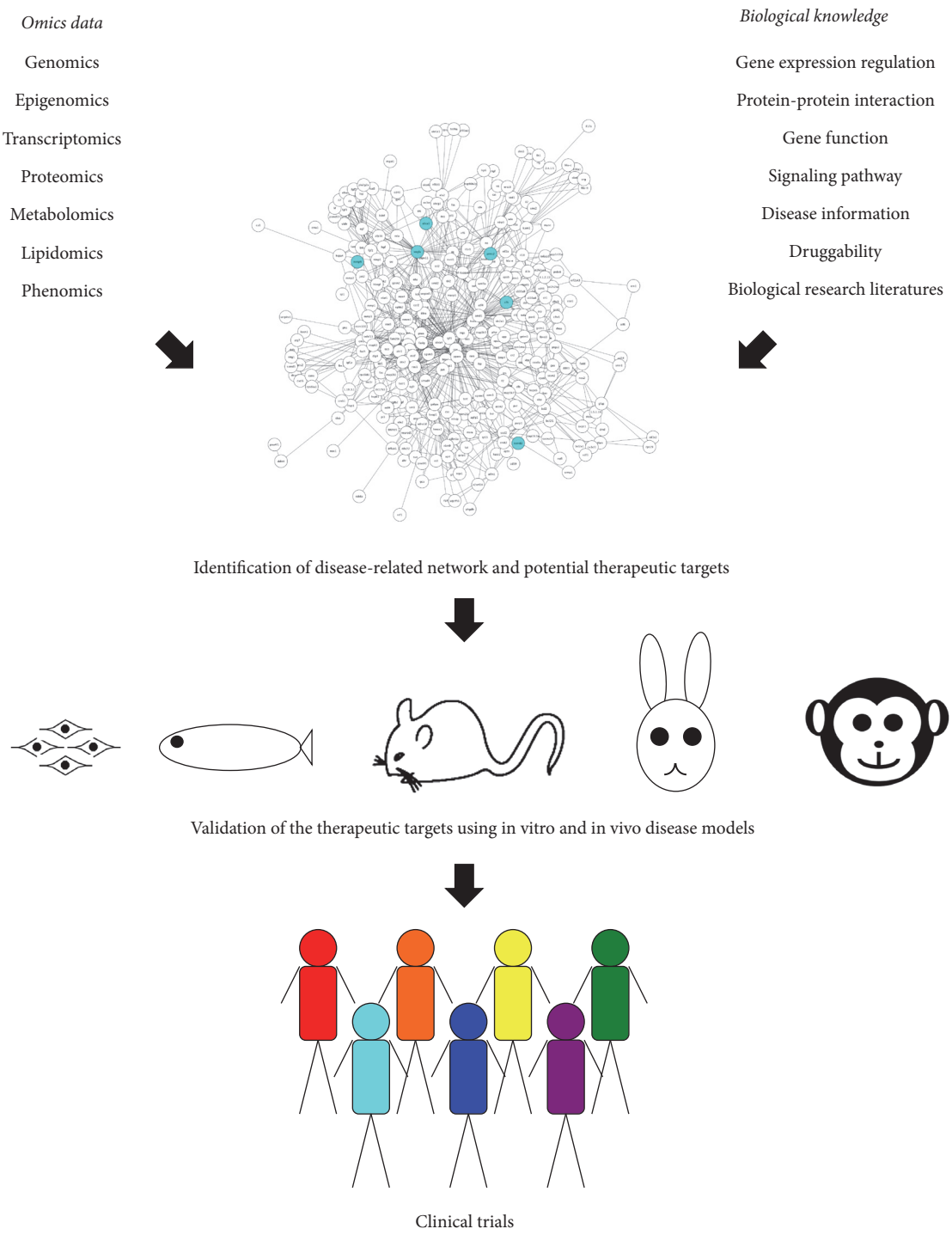

Figure 1: Integrative approaches to identify therapeutic targets. Integration of multilevel omics data and biological knowledge allows construction of disease-related networks and the discovery of potential therapeutic targets (represented as cyan circles in the network). In vitro and in vivo disease models can be used to validate the therapeutic targets and drugs, and drugs displaying efficacy in preclinical models can then be moved into clinical trials.

of amyloid precursor protein (APP, underlined in green in Figure 2), is increased in the optic nerve head of monkeys and humans with glaucoma [77, 78]. Modulation of amyloid β aggregation can reduce apoptosis of retinal ganglion cells in a rat model of glaucoma [79]. OS and amyloid β aggregation exhibit reciprocal stimulation and induce neurodegeneration in both the brain and the retina [80]. In humans, an antibody against amyloid β reduces plaque formation and attenuates

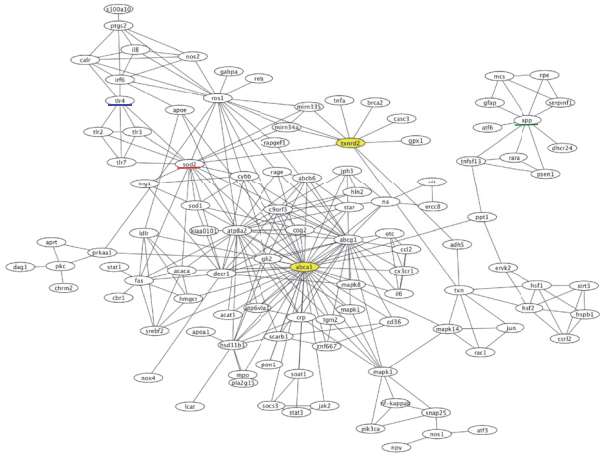

Figure 2: A network related to oxidative stress in glaucoma. An Agilent Literature Search was used as the basis for the network. We used 39 glaucoma-associated genes identified by GWAS as the target molecules and "oxidative stress" as the designated context in the Agilent Literature Search. The glaucoma-associated genes in the network are shown in yellow and other potential therapeutic targets are underlined.

clinical decline in Alzheimer's disease [81]. Collectively, these findings suggest that amyloid β is a potential therapeutic target for glaucoma-associated OS.

Toll-like receptor 4 (TLR4, underlined in blue in Figure 2) is another potential target. Oxidized lipoprotein activates macrophages/microglia through TLR4 and promotes inflammation [82, 83]. Expressions of TLR4 and angiotensin II type 1 receptor (AGTR1) are increased in a mouse model of glaucoma, and an AGTR1 antagonist suppresses neurodegeneration in the mouse retina by inhibiting the TLR4-apoptosis signal-regulating kinase 1 pathway [84]. Modulators of TLR4 signaling have already been developed [85, 86], and the studies described here suggest that such modulators could be used to target OS in glaucoma.

2.3. An Integrative Approach towards Drug Discovery for AMD-Related OS. We applied the same approach to identify potential therapeutic targets for AMD-related OS. We used 25 AMD-associated genes identified by GWAS [87, 88] as the target molecules and "oxidative stress" as the designated context in an Agilent Literature Search. The resulting network (shown in Figure 3) contains ten AMD-associated genes (shown in yellow), including vascular endothelial growth factor A (VEGFA, Figure 3(a)) and matrix metallopeptidase 9 (MMP9, Figure 3(b)). Gene ontology analysis using DAVID [68] revealed that genes related to "response to oxidative stress" are significantly ($p = 5.2 \times 10^{-16}$) enriched in the network, suggesting that these ten AMD-associated genes are connected through genes related to OS.

OS is a major stimulator of VEGFA production and secretion by retinal pigment epithelial cells [89]. Notably, high VEGFA levels also increase oxidative damage, resulting in early degenerative changes in retinal pigment epithelial cells followed by neovascular AMD [90]. Intravitreal injection of anti-VEGF agents can slow the progression of neovascular AMD [12, 89], suggesting that VEGFA is one of the most important therapeutic targets for AMD-related OS.

OS also increases the expression of MMP9 [91] at an early stage of choroidal neovascularization (CNV) [92, 93]. The MMP inhibitors batimastat and marimastat reduce CNV when applied early in the process [93, 94], suggesting a potential therapeutic role for MMP9 inhibitors in AMD.

Other genes in the AMD-OS network may also be therapeutic targets. For example, peroxisome proliferator-activated receptor α (PPARA, Figure 3(c)) is the pharmacological target of fibrates such as fenofibrate, clofibrate, and bezafibrate [35]. PPARA is associated with both antioxidant and anti-inflammatory activities and has previously been suggested as a therapeutic target in AMD [95–97]. In fact, several clinical trials have revealed that fenofibrate can improve diabetic retinopathy [98, 99], which shares some pathophysiological mechanisms with AMD, including OS [100, 101]. These findings suggest that PPARA-activating drugs might have therapeutic utility for AMD.

Several components of the renin-angiotensin system, including angiotensin (ANG), angiotensin I converting enzyme (ACE), ACE2, and angiotensin II receptor type 1 and type 2 (AGTR1 and AGTR2), are shown in the subnetwork (Figure 3(d)). The renin-angiotensin system regulates various biological functions, including OS [102], and is hyperactivated in both AMD and diabetic retinopathy [103, 104]. Several clinical trials have demonstrated some efficacy of renin-angiotensin system inhibitors in slowing the progression of diabetic retinopathy [102, 105]. Thus, the renin-angiotensin system may also be a source of therapeutic targets for AMD-related OS.

3. Conclusion

Here, we provided an overview of some integrative approaches to drug discovery for OS in retinal diseases. We used two simple examples of glaucoma and AMD to illustrate how the approach can identify network hubs as potential therapeutic targets for these retinal diseases. The rapid advances in technology and increasing volume of multilevel omics data continue to create larger and more complex datasets to understand disease-associated biological networks and to build more extensive drug-target networks. Further progress in computational methodology combined with improved in vitro and in vivo disease models will facilitate the prioritization of therapeutic targets in the networks. The integration of multilevel omics data, computational approaches, and validated disease models will thus provide a strong foundation for deciphering the complex mechanisms of OS in retinal diseases and for discovering novel therapies with the greatest potential for efficacy in clinical trials.

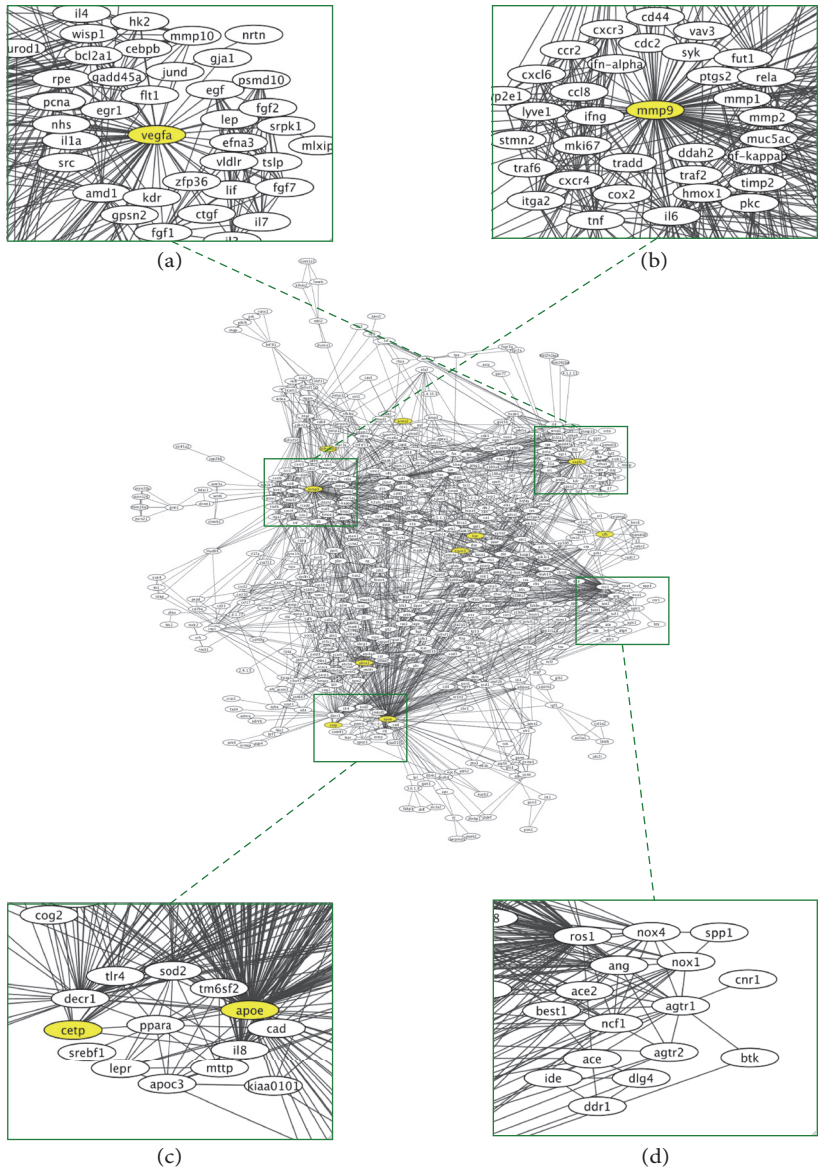

Figure 3: A network related to oxidative stress in age-related macular degeneration (AMD). An Agilent Literature Search was used as the basis for the network. We used 25 AMD-associated genes identified by GWAS as the target molecules and "oxidative stress" as the designated context in the Agilent Literature Search. The AMD-associated genes in the network are shown in yellow. The subnetworks containing potential therapeutic targets for AMD-related OS are enlarged in (a)–(d).

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Nrf2 Is an Attractive Therapeutic Target for Retinal Diseases

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that binds to antioxidant response elements located in the promoter region of genes encoding many antioxidant enzymes and phase II detoxifying enzymes. Activation of Nrf2 functions is one of the critical defensive mechanisms against oxidative stress in many species. The retina is constantly exposed to reactive oxygen species, and oxidative stress is a major contributor to age-related macular diseases. Moreover, the resulting inflammation and neuronal degeneration are also related to other retinal diseases. The well-known Nrf2 activators, bardoxolone methyl and its derivatives, have been the subject of a number of clinical trials, including those aimed at treating chronic kidney disease, pulmonary arterial hypertension, and mitochondrial myopathies. Recent studies suggest that Nrf2 activation protects the retina from retinal diseases. In particular, this is supported by the finding that Nrf2 knockout mice display age-related retinal degeneration. Moreover, the concept has been validated by the efficacy of Nrf2 activators in a number of retinal pathological models. We have also recently succeeded in generating a novel Nrf2 activator, RS9, using a biotransformation technique. This review discusses current links between retinal diseases and Nrf2 and the possibility of treating retinal diseases by activating the Nrf2 signaling pathway.

1. Retinal Diseases and Oxidative Stress

The free radical theory of aging was advocated in 1954 [1] and had a dramatic effect on contemporary medicine, including molecular biology. It was hypothesized that oxidative stress, especially within mitochondria, led to a vicious cycle in which cells are directly damaged. Although many researchers were initially reluctant to accept this concept, accumulating evidence has now consolidated a role for oxidative stress, which is exerted by the intracellular accumulation of reactive oxygen species (ROS) [2], in aging and many diseases. Retinal diseases are no exception to this concept. More than 99% of ultraviolet radiation is absorbed by the anterior segment of the eye and the crystalline lens, but the remaining 1% reaches the light-sensitive retina [3, 4]. Studies have linked the early development of age-related macular degeneration (AMD) with exposure to intense ultraviolet radiation or bright sunlight. Drusen deposits, which are initially formed by oxidized lipids, have been shown to be associated with AMD, along with laminar deposits in Bruch's membrane [5, 6]. It is also well known that the major lipofuscin fluorophore A2E mediates age-related pathophysiological processes in the retinal pigment epithelium (RPE) [7]. Light-induced A2E derivatives such as oxiranes and epoxides were shown to cause DNA damage and induce cellular death [8, 9]. Light emitting diode light was shown to result in cellular damage, which is wavelength-dependent in that short-wavelength light is not easily absorbed into the cornea and lens [10]. Hyperglycemia is also a critical factor in retinal degeneration, especially in diabetic macular edema (DME) [11, 12]. High glucose levels activate the polyol pathway and cause accumulation of advanced glycation end products (AGEs) and overactivation of hexosamine and protein kinase C (PKC) pathways that exaggerate inflammation. In many cases of DME, abnormal retinal neovascularization is observed, as is also seen in wet AMD, and the generation of ROS is indirectly involved in the pathogenesis. The final common pathway in a variety of retinal diseases, including glaucoma and retinitis pigmentosa, is linked to retinal cellular death, and it is likely that ROS triggers cellular damage in these cases. Factors such as an abundance of polyunsaturated lipids, high oxygen consumption ratio, hypoxia, psychological stress, radiation,

air pollution including ozone, smoking, and oxidized foods are other typical risk factors that can induce the generation of ROS in the retina [13–16].

Based on the relationship between retinal diseases and oxidative stress, vitamins and minerals have been regarded as a promising preventive remedy, especially for dry AMD. The Age-Related Eye Disease Study (AREDS) is a major clinical trial sponsored by the National Eye Institute to investigate the effects of vitamin C, vitamin E, beta-carotene, and zinc. AREDS2 is a multicenter, randomized trial designed to investigate the effects of adding macular xanthophylls (lutein and zeaxanthin) and/or long-chain omega-3 fatty acids (docosahexaenoic acid [DHA] and eicosapentaenoic acid [EPA]) to the original AREDS formula on the progression to advanced AMD [17]. These supplements did appear to reduce the risk of progression towards advanced AMD, but the effects were not significant. Results using other antioxidants such as alpha-tocopherol, nicanartine [11], N-acetyl-L-cysteine [18], vitamin A [19], and OT-551 [20] were disappointing or remain controversial, questioning the validity of antioxidants as a therapeutic target for retinal diseases. However, the extremely short half-life and weak activity of standard radical acceptors remain critical issues that should be considered in future clinical trials.

2. Oxidative Stress and Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2), which belongs to the basic leucine zipper (bZIP) transcription factor and heterodimerizes with small Maf proteins, functions as a key player in the redox homeostatic gene regulatory network. The expression of Nrf2 is observed in many tissues, particularly those exposed to the external environment (skin, lungs, and gastrointestinal tract) and those associated with detoxification (liver and kidneys) [21]. Under resting conditions, Nrf2 is trapped within the cytosol by an adaptor protein, Kelch-like erythroid cell-derived protein with CNC homology- (ECH-) associated protein 1 (Keap1) (Figure 1). This protein is an adaptor component of Cullin 3- (Cul3-) based ubiquitin E3 ligase; therefore Nrf2 is rapidly degraded via the proteasomal pathway. Several cysteine residues within Keap1 serve as primary sensors of stress signals, and their modification leads to conformational changes in Keap1, thereby inhibiting ubiquitination of Nrf2 [22]. As a result, stabilized Nrf2 is translocated into the nucleus. Nrf2 binds to a specific consensus cis-element, named the antioxidant-response element (ARE; also called the electrophile-responsive element [EpRE]), present in the promoter region of genes encoding antioxidant and phase II detoxifying enzymes. There are over 250 Nrf2-targeted genes, including NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutamate cysteine ligase, glutathione S-transferase, glutathione peroxidase, catalase, superoxide dismutase, and thioredoxin UDP-glucuronosyltransferase [23]. Therefore, it is predicted that driving robust expression of these genes would be activated by Keap1-Nrf2 signaling, which would rescue cells from a variety of stimuli such as reactive toxicants, proinflammatory factors, apoptosis, and carcinogenesis [22]. Among them, the potential crosstalk between the nuclear factor-κB (NF-κB) and Nrf2 pathways remains still unclear [24].

3. Nrf2 and Retinal Diseases

Oxidative stress is one of the main causes of the pathogenesis of chronic obstructive pulmonary disease (COPD) [25]. Cigarette smoke directly damages the alveolar epithelium and lung structures, leading to deterioration of lung function. As a result, targeting oxidative stress has been intensively studied in the pulmonary field, and accumulating evidence from patients with COPD suggests a role for Nrf2 in this process. The level of Nrf2 mRNA in lavaged macrophages of young patients is not dependent on smoking status; however, levels were reduced in samples from old current smokers compared to old nonsmokers [26]. These observations strengthen the idea that the aging process weakens Nrf2 activity. In the ocular field, a decrease in Nrf2 DNA-binding activity was reported in the retina of patients with diabetic retinopathy [27], and a role for Nrf2 in the onset of AMD has been well documented [15, 28]. These data identify either inhibiting the decrease or activating Nrf2 as potential strategies for treating retinal diseases. This approach could be suitable for scavenging ROS and suppressing retinal degeneration. In addition, it seems Nrf2 is highly expressed in the ganglion cell layer and the inner nuclear layer with the strong expression of Muller cells [29]. Further investigations to scrutinize the expressional change of Nrf2 in pathological conditions are important to establish appropriate remedies.

Data from Nrf2 knockout mice represent the rationale for the concept of Nrf2-mediated cellular protection. Although Nrf2 is considered one of the master genes, Nrf2 knockout mice develop without critical deficits and reach adulthood [30, 31]. This indicates that Nrf2 is dispensable for development and/or that its absence may be compensated for by Nrf2-like genes. However, the ocular phenotypes seen in these mice are exacerbated under oxidative circumstances. Age-dependent deterioration in the RPE, including accumulation of lipofuscin and drusen-like deposits and spontaneous choroidal neovascularization (CNV), was observed in Nrf2 knockout mice compared to wild-type controls [32]. Cigarette smoke induced more profound damage to the RPE-Bruch membrane in Nrf2 knockout mice, where large cytoplasmic vacuoles were observed [33]. Retinal degeneration in these mice is exacerbated under many pathological conditions including retinopathy of prematurity (oxygen-induced retinopathy) [34], glaucoma (axonal injury by nerve crush) [35], diabetic retinopathy [29], posterior uveitis [36], and central retinal artery occlusion (retinal ischemia-reperfusion) [37]. In addition to oxidative stress and inflammation, visual dysfunction, retinal thickness, leukocyte adherence, and cellular survival are employed as surrogate markers to evaluate the retinal deterioration. Such preclinical data sets are motivating researchers to work towards identifying novel Nrf2 activators.

Keap1 knockout mice were generated with the expectation that Nrf2-targeted genes would be increased and the mice would become resistant to oxidative stress [38]. However, these mice died before weaning due to

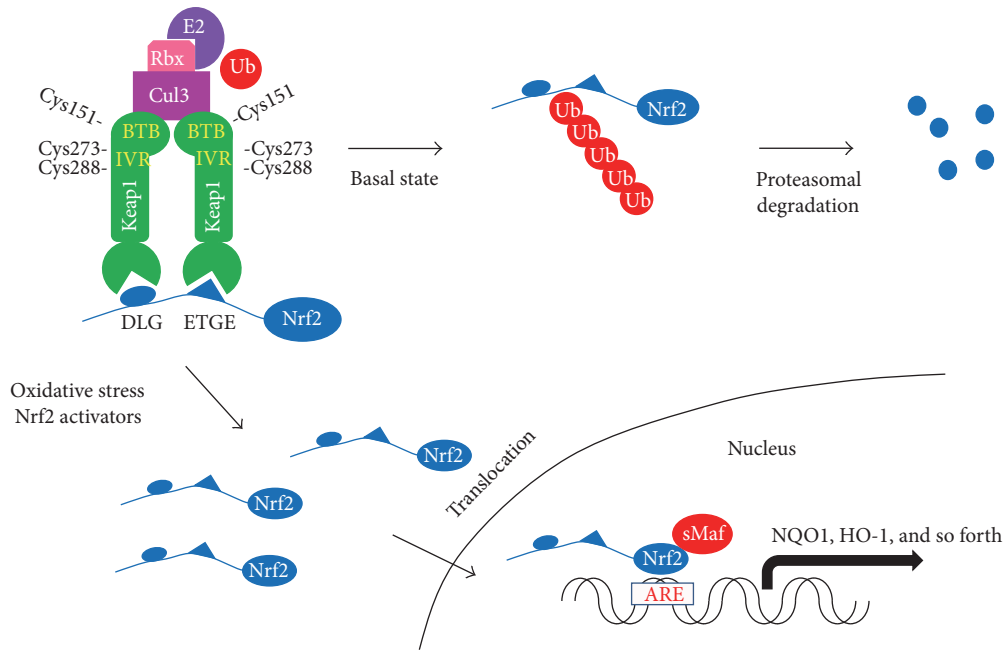

FIGURE 1: Keap1-Nrf2 signaling. The Keap1 homodimer binds to a single Nrf2 molecule via DLG and ETGE motifs; the latter has a high affinity for Nrf2. Under basal conditions, the Keap1-Nrf2 complex is degraded by Cul3-dependent E3 ubiquitin ligase. However, Keap1 also works as a stress sensor, and oxidative stress induces conformational changes of Keap1-Nrf2 complex. As a result, ubiquitination of Nrf2 is inhibited, and then stabilized Nrf2 is translocated into the nucleus. Binding of Nrf2 with small Maf proteins to the antioxidant response element (ARE) induces many antioxidant and phase II detoxifying enzymes.

hyperproliferation of keratinocytes in the esophagus and forestomach, resulting in an abnormal upper digestive tract. In a subsequent study, Keap1 was disrupted in a hepatocyte-specific manner, and these homozygous mice showed no deficits in the development or physiological integrity of the liver. Moreover, the mice showed chronic activation of Nrf2 and resistance to acetaminophen-induced hepatotoxicity [39]. These results strongly support the concept that tissue-specific activation of Nrf2 would be an ideal therapy for treating retinal diseases, without causing severe adverse effects.

4. Nrf2 Activators Ameliorate Oxidative Stress-Related Retinal Diseases

Sulforaphane, which is found in broccoli sprouts and other cruciferous vegetables, has long been employed as a benchmark Nrf2 activator [40] (Figure 2). This compound is a weak electrophile that has the ability to react with cysteine thiols of Keap1 [41]. The *in vitro* activity of sulforaphane is generally determined by the concentration required to double NQO1 activity (CD) in murine Hepa1c1c7 hepatoma cells. From this, the CD value of sulforaphane is known to be approximately 200 nM. Similar well-known electrophiles include curcumin, resveratrol (a polyphenolic compound found in mulberries, grapes, and red wine), oltipraz, ebselen, tertiary-butylhydroquinone (tBHQ), and dimethyl fumarate (also called BG-12 or Tecfidera), some of which have undergone clinical trials [42]. Despite the fact that not all of them show low CD values [43], it is worth noting that dimethyl fumarate has been approved by the FDA as a new oral drug treatment for patients with relapsing-remitting multiple sclerosis [44]. Because the CD value of dimethyl fumarate is over 10 μM, Nrf2-independent modes of action also seem to be involved in its efficacy, including the activation of hydroxycarboxylic acid receptor 2 (HCAR2) and immunomodulatory properties.

A breakthrough in the study of Nrf2 activators was the finding that some synthetic oleanane triterpenoids have CD values in the subnanomolar range [45, 46]. Within this context, a variety of triterpenoids have been chemically synthesized and intensively tested over a long period. The best-known triterpenoid is bardoxolone methyl [2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) methyl ester/CDDO-Me/RTA 402/bard], which has a CD value of approximately 1 nM, and is classified as an oral "antioxidant inflammation modulator." Clinical data from a phase 2b trial were partially reported in 2011 [47]. In the BEAM study ("Bardoxolone Methyl Treatment: Renal Function in Chronic Kidney Disease/Type 2 Diabetes"), the safety and efficacy of RTA 402 in patients with moderate to severe chronic kidney disease associated with type 2 diabetes (227 patients, 52 weeks) were investigated. RTA 402 improved the estimated glomerular filtration rate at 24 weeks and this efficacy continued to 52 weeks. But the phase 3 trial, called the BEACON study ("Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes: The Occurrence of Renal Events") (1600 patients with Stage 4 chronic kidney disease), was to be terminated in 2012 because of a higher rate of cardiovascular mortality [48]. It seems

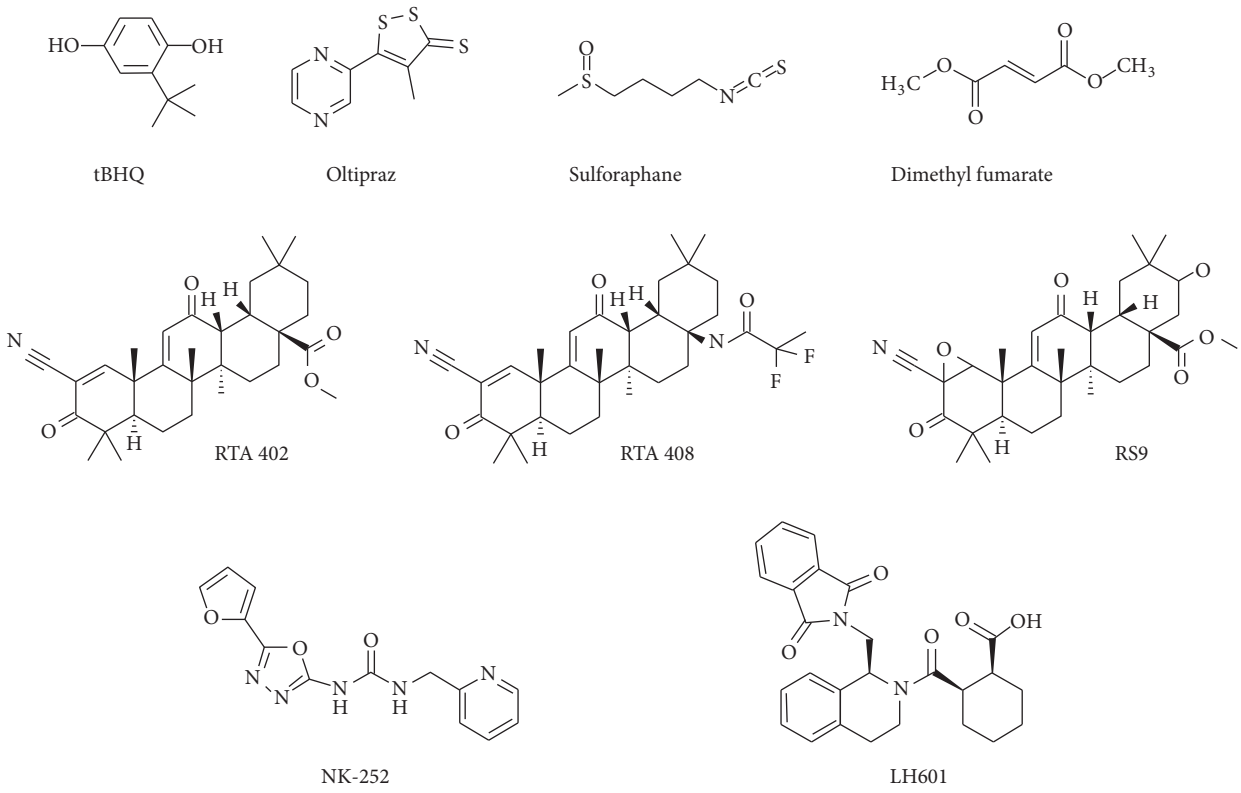

Figure 2: Chemical structure of typical Nrf2 activators.

that volume retention and acute sodium were the cause of these cardiovascular events [49]; however, the controversy has not been fully cleared as to whether both efficacy and adverse events were the result of Nrf2 activation [50]. RTA 402 is thus undergoing further evaluation regarding its use in the treatment of chronic kidney disease and pulmonary hypertension [42].

Oleanolic acid is commonly used as the starting material for synthetic oleanane triterpenoids, and modification at position C17 is often used to synthesize CDDO scaffolds such as imidazole (CDDO-Im), methyl amide (CDDO-MA), ethyl amide (CDDO-EA), trifluoroethyl amide (CDDO-TFEA), nitrile (di-CDDO), and difluoro-propanamide (RTA 408/omaveloxolone). In addition, RTA 408 is currently being tested in clinical trials into postsurgical corneal endothelial cell loss (https://clinicaltrials.gov/; Identifier: NCT02128113) [42].

The triterpenoids described above have been also employed in preclinical experiments and have been shown to be active in several ocular models. Light damage causes an increase in retinal oxidative stress immediately following exposure, and this has been widely used to study the mechanisms of age-related retinal cellular death. When mice were fed CDDO-TFEA, the thinning of the outer nucleus layer was suppressed [51]. The ratio of CDDO-TFEA in their diet was 100–200 mg/kg, which is approximately equal to oral administration of 0.1–0.2 mg/kg. Optical nerve crush injury is commonly used as an experimental disease model for glaucoma and traumatic optic neuropathy. Oral administration of 16 mg/kg CDDO-Im attenuated the death of retinal ganglion cells in mice [35]. Endotoxin-induced uveitis is one of several animal models of ocular inflammation that is triggered by lipopolysaccharide. Ocular inflammatory responses, including leukocyte adherence to retinal vasculature, were decreased by intraperitoneal pretreatment with CDDO-Im at a dose of 1.6 mg/kg [36]. Retinal capillary degeneration following ischemia-reperfusion was also markedly attenuated when mice were intraperitoneally pretreated with 0.5 mg/kg RTA 402 [37]. In the latter three experiments, neither CDDO-Im nor RTA 402 was effective in Nrf2 knockout mice, indicating that the mechanism of triterpenoid function is dependent on Nrf2 activation. It is likely that the effective oral dose range is approximately 1–10 mg/kg, which seems rational considering the dose of 20 mg/kg used in the BEACON study [48]. However, because a preventive protocol and acute injury were employed in these experiments, their effects should be also investigated using therapeutic protocols and/or in chronic disease models. Exposure to cigarette smoke for 6 months induced alveolar apoptosis, alveolar destruction, and pulmonary hypertension, and this damage, including lung oxidative stress, was inhibited when mice were fed a diet containing CDDO-Im (60 or 90 mg/kg) [52]. These results imply that triterpenoids could also be effective in chronic retinal disease models.

We recently identified a novel potent Nrf2 activator, RS9 (CD value: 0.2 nM), using a biotransformation technique. Using *in vivo* studies we demonstrated that oral

administration of RS9 inhibits neovascularization in oxygen-induced retinopathy in rats, and an intravitreal injection of RS9 suppressed blood-retinal barrier permeability in glycated albumin-injected rabbits [53]. The chemical structure of RS9 is unique because it contains epoxidation in the A-ring and hydroxylation in the E-ring. From our structure-activity relationship study, it is likely that the epoxidation contributes to a reduction in cytotoxicity and the hydroxylation is involved in the improvement of activity. The efficacy of RS9 was also confirmed in rhodopsin Pro347Leu transgenic rabbits, which were previously generated as a model of retinitis pigmentosa [54]. Intravitreal injections of RS9 microspheres were employed in this study, but small implants that release more compounds could be a more realistic method for future clinical trials.

Although both preclinical and clinical data have suggested the therapeutic potential of Nrf2 activators for treating retinal diseases, selection of the best diseases remains to be discussed. Because continuing oxidative stress damages the retina over time and activation of Nrf2 is not expected to reverse cellular death, preventing the progress of dry AMD might be a more promising target, as in the case of the AREDS study. Systemic adverse events are still a big concern of triterpenoids; therefore, administering them via eye drops or intraocular implants would be a promising approach for lowering these risks [55, 56].

5. Novel Strategies for Activating Nrf2

Human Keap1 is a 70 kDa cysteine-rich protein (624 amino acids and 27 cysteines), of which residues Cys 151, 273, and 288 have been shown to be essential for regulating Nrf2 in biological studies. Most of the currently used Nrf2 activators are electrophiles and are thought to form covalent adducts with the sulfhydryl groups by Michael addition reactions on the specific cysteine residues of Keap1. Higher concentrations of these compounds interact with other cysteine-rich proteins with lower binding affinities, leading to activation of caspases and induction of apoptosis [46]. To overcome this issue, peptide and small molecule inhibitors of the Keap1-Nrf2 protein-protein interaction (PPI) have been investigated [57, 58]. The Keap1 homodimer binds to Nrf2 through a "hinge and latch" mechanism, in which the ETGE and DLG motifs of Nrf2 interact with Keap1-DC domains (the double glycine repeat domain [DGR] plus the C-terminal region of Keap1) [59]. X-ray crystallography data suggest that the druggability of the interface is not low. The peptide mimetics of the ETGE and DLG motifs are therefore expected to be effective, although it is still important to solve peptide-related issues, such as cellular permeability and plasma stability. Several chemical compounds have been identified from high-throughput screening, and some of them, such as LH601A, have been shown to accelerate the translocation of Nrf2 to the nucleus [60]. Few studies into PPI inhibitors of the Keap-Nrf2 interaction have demonstrated *in vivo* efficacy, but NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea) has been shown to interact directly with Nrf2-containing Keap1-DC domains. Furthermore, oral administration of NK-252 inhibits the progression of nonalcoholic steatohepatitis-related fibrosis in rats at a dose of 20 or 60 mg/kg [61]. This study used a weak electrophile, oltipraz, for comparison, which has since been tested for liver fat reduction in patients with nonalcoholic fatty liver disease, excluding liver cirrhosis (https://clinicaltrials.gov/; Identifier: NCT02068339). It will be intriguing to determine whether the effect of NK-252 is diminished in Nrf2 knockout mice, whether efficacy is observed in retinal disease models, and whether NK-252 shows acceptable tolerability.

Keap1 functions as an adaptor protein for the regulation of proteasomal degradation of Nrf2 by Cul3-dependent E3 ligase. Therefore inhibiting the interface between Keap1 and Cul3 represents another approach for activating Nrf2 [62]. In this context, the intervening-region (IVR) domain [63] and the Broad complex, Tramtrack and Bric-à-Brac (BTB) domains [64] of Keap1 have been studied using experimental deletion. Because many protein-protein interfaces are flat and large, confirming the existence of suitable drug target sites in the ubiquitin-proteasome system will be essential before drug candidates can be identified. However, the X-ray structure of the Keap1-Cul3 interaction is still unknown. Such an analysis could identify a number of promising compounds that act to inhibit the Keap1-Cul3 interface, such as the proteasome inhibitor bortezomib (marketed as Velcade for the treatment of multiple myeloma).

Small interfering RNA (siRNA) is a breakthrough technology used to silence genes by causing destruction of specific mRNA molecules. Considering the mechanisms of Keap1-Nrf2 signaling, Keap1 siRNA is predicted to be a highly successful strategy to specifically potentiate Nrf2 signaling. The half-life of Nrf2 in peritoneal macrophages is estimated to be 18.5 minutes [65], indicating that Nrf2 is constantly produced and rapidly degraded. This profile is suitable to the application of an siRNA-based method. Although there have been no reports to date that Keap1 siRNA is effective in *in vivo* retinal disease models, other siRNA-based drugs are being tested in clinical ophthalmological trials. QPI-1007 is new synthetic siRNA designed to inhibit caspase 2 expression and has been tested in patients with nonarteritic anterior ischemic optic neuropathy and other optic neuropathies [66]. PF-655 (REDD14NP/RTP801i) is an siRNA that targets the RTP801 (DDIT4/Redd1) gene, which is induced under hypoxia and cellular stress and inhibits the mTOR pathway. This siRNA has been evaluated for the treatment of wet AMD and diabetic macular edema [67]. It is possible that the results from these trials will encourage researchers to investigate the use of Keap1 siRNA to activate Nrf2 signaling in retinal disease models.

6. Concluding Remarks

Many questions remain unanswered regarding the mechanisms by which oxidative stress Nrf2 is involved in the onset of retinal diseases. However, activation of Nrf2 would be worth testing further using pathological models. With respect to clinical applications, the reversibility of Nrf2 activation should be considered. In this regard, RTA 402 and RTA 408 have been tested in clinical trials; however, the mode of action of Keap1 is Michael addition, which is known

to be an irreversible process. Reducing these irreversible properties, using reversible compounds, or further improving selectivity might also be useful in order to avoid such adverse events as those observed in the BEACON study [48]. In addition, because cancer cells harness the aberrant activation of Nrf2 for proliferation and survival under stress conditions, carcinogenesis should be also studied in some detail [68]. To date, the efficacy of Nrf2 activators has been shown mostly by oral administration. Therefore, focal administration such as via eye drops or intravitreal injection could be one strategy that would minimize adverse systemic events during the treatment of ocular diseases. In terms of drug delivery to the retia, nanoparticle techniques may be appropriate for long-term treatment. Although *in vivo* pharmacological and toxicological studies will certainly be needed in the future, data are continuing to accumulate that are shedding light on the use of Nrf2 activation to treat oxidative stress-related retinal/retinovascular diseases. The success of these methods will depend on careful optimization of the compounds, appropriate selection of diseases, and use of a proper drug-delivery system to the retina.

Competing Interests

The author declares that there are no competing interests regarding the publication of this paper.

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