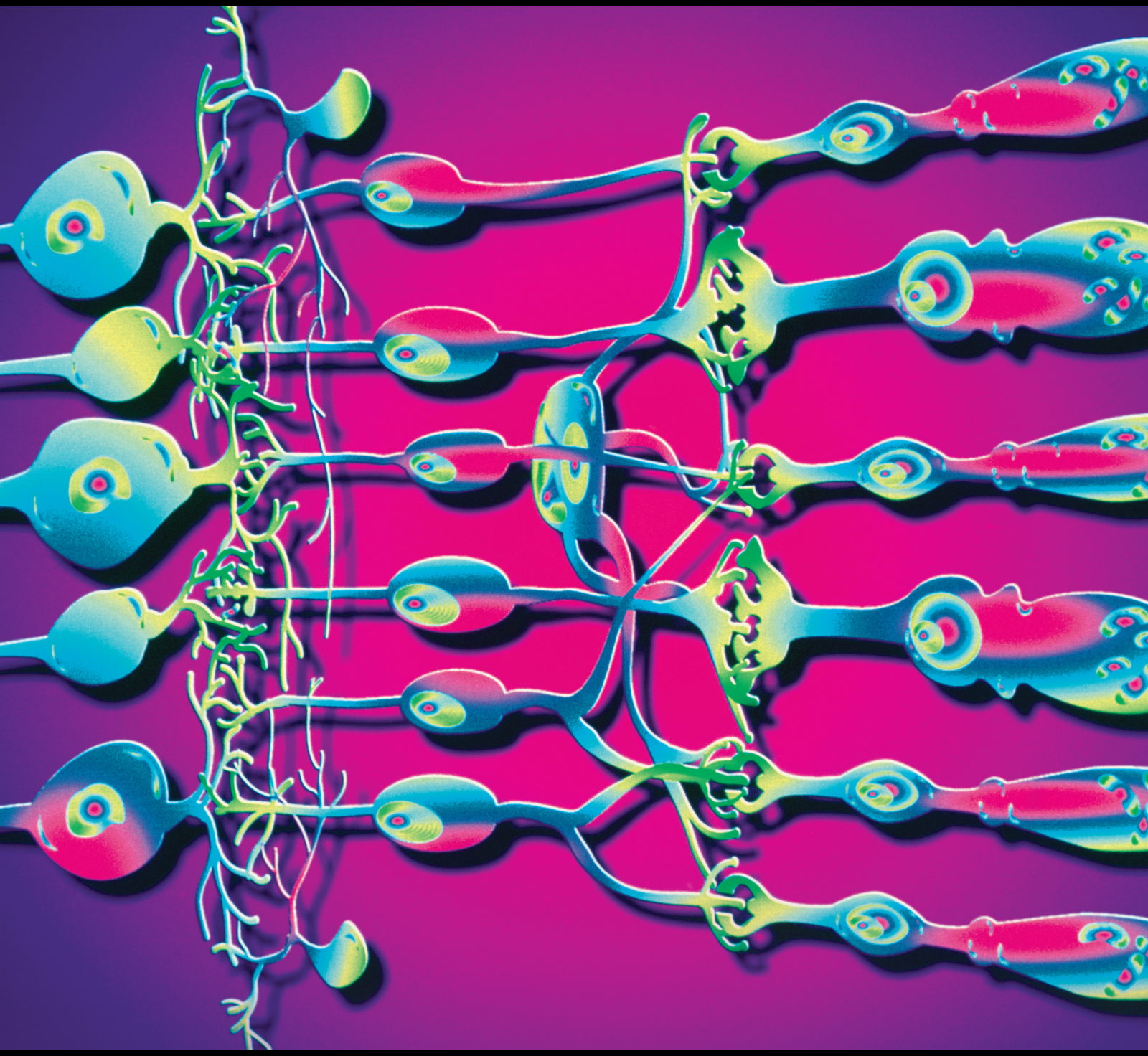
Journal of Ophthalmology

Advances in Retinal Therapeutics

Guest Editors: Petros E. Carvounis, Thomas A. Albini, Andrew J. Barkmeier, and Miltiadis Tsilimbaris



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Editorial Board

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Editorial

Advances in Retinal Therapeutics

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In recent years there have been monumental and exciting advances in the treatment of retinal disease which made a special issue on advances in retinal therapeutics relevant and interesting. Indeed, in this issue there are 11 papers reporting either original basic or clinical research findings or reviewing the literature and reporting recent developments.

The papers in this issue are on a wide range of topics. J. He et al. present original basic research in elucidating the effects of VEGF receptor 1 blockade on diabetic retinopathy while M. L. Perepechaeva et al. present original basic research identifying a potential novel target for the treatment of age-related macular degeneration in a rodent animal model of the disease. O. Chrapek et al. present original clinical research identifying occult CNVM and lesion size less than 5 disc diameters as baseline characteristics predicting inactivity after 3 injections of an intravitreous anti-VEGF agent (ranibizumab) for neovascular age-related macular degeneration (nARMD). S. M. Prea et al. review endogenous inhibitors of nARMD as well as the principles of gene therapy that could use such inhibitors to treat nARMD clinically. There are 2 papers discussing advances in retinal laser treatment—one by Y. G. Park et al. discussing developments on laser photocoagulation for diabetic macular edema and another by K. Inagaki et al. discussing micropulse laser for persistent macular edema from branch retinal vein occlusion in eyes which includes eyes with good visual acuity at baseline. Two additional papers address vitreoretinal surgery topics: one by Dr. C. Pournaras et al. that reports on the outcomes of repair of recurrent retinal detachment, including presenting visual outcomes which are not uncommonly omitted in prior publications and the other paper by A. García-Layana et al. that reviews the current treatment of vitreomacular traction and macular hole. The paper by M. Harrell and P. E. Carvounis is an up-to-date evidence-based review of the treatments of toxoplasma retinochoroiditis which takes into account evidence published within the last 2 years when the previous evidence-based review was published. Finally, the paper by M. Cabrera et al. reviews the 3 sustained-release corticosteroids available for the treatment of retinal disease.

The 54 authors and coauthors should be commended for the quality of their manuscripts; the science as well as the writing is of high caliber. It is our belief that the readership of the Journal of Ophthalmology will enjoy reading the papers in this special issue as much as we did.

Petros E. Carvounis
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Andrew J. Barkmeier
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Research Article

Prognostic Factors of Early Morphological Response to Treatment with Ranibizumab in Patients with Wet Age-Related Macular Degeneration

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Aim. To assess the significance of age, gender, baseline best corrected visual acuity, baseline macula thickness, and type and size of choroidal neovascularization in early morphological therapeutic response to ranibizumab treatment in patients with the wet form of age-related macular degeneration. *Methods*. From 09/2008 to 06/2013 we evaluated 1153 newly diagnosed, treatment-naïve patients treated with ranibizumab. Based on the morphological findings in the macula following the initial 3 injections of ranibizumab, the patients were divided into two groups based on active and inactive choroidal neovascularization. *Results*. After the initial 3 injections of ranibizumab, we examined the sample of 841 eyes with active CNV and 312 eyes with inactive CNV. In the inactive group, we found a statistically higher proportion of occult CNV ($P < 0.001$) and lower incidence of CNV greater than 5DA ($P < 0.001$) compared with the active group. We found no statistically significant difference in age, gender, baseline best corrected visual acuity, or baseline macula thickness between the inactive and active groups. *Conclusion*. Occult CNV and CNV smaller than 5DA are optimistic factors for a better morphological therapeutic response at the beginning of ranibizumab treatment.

1. Introduction

Age-related macular degeneration (AMD) is subdivided into the dry form and the wet form. According to Bressler et al., 90% of legal blindness from AMD is caused by the wet form [1]. The Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA) evaluated the benefit of ranibizumab in a dose of 0.5 mg per month. At 12 months, 95% of patients had lost <15 Early Treatment Diabetic Retinopathy Study (ETDRS) letters from baseline and 34% of patients had a best corrected visual acuity (BCVA) gain of ≥15 ETDRS letters [2]. The study Ranibizumab in Patients with Subfoveal Choroidal Neovascularization (CNV) Secondary to Age-Related Macular Degeneration (SUSTAIN study) was designed to further evaluate the safety, tolerability, and efficacy of optical coherence tomography (OCT)/BCVA-guided, individualized, and flexible pro re nata (PRN, as needed) dosing regimen for ranibizumab. At 12 months, 92.5% of patients had lost <15 ETDRS letters from baseline and 19.3% of patients had a BCVA gain of ≥15 ETDRS letters [3].

Whether the fixed or PRN regimen was used in the treatment of wet AMD, the therapeutic response of individual patients was not always the same. Current observations suggest that the factors which affect the response to the initial treatment with ranibizumab are individual.

The aim of this study was to assess the importance of various factors (age, gender, baseline BCVA, baseline macula thickness, and type and size of CNV) for early morphological therapeutic response to ranibizumab in clinical practice.

2. The Population and Methodology

The treatment of patients with wet AMD is centralized into 9 tertiary referral centres in the Czech Republic (see Appendix). Anonymised data on treatment efficacy and safety have been consecutively entered into the Czech national database AMADEuS (Age-Related Macular Degeneration in patients in the Czech Republic) since September 2008. The main aim of this registry is to collect basic epidemiologic data on patients diagnosed with wet AMD in the Czech Republic, document standard diagnostic and therapeutic patterns, and assess treatment efficacy in standard clinical practice. The data collection is independent of all treatment decisions; it does not affect a patient's access to treatment and fully complies with all ethical as well as legal requirements for noninterventional data collection in the Czech Republic. All patients have given written informed consent to the treatment, as well as data collection. The reported investigations were in accordance with the principles of the current version of the Declaration of Helsinki.

The data are recorded from the moment of diagnosis and start of treatment at regular 3-month intervals for half a year. In the following period, the record is completed every 6 months. Each record presumes biomicroscopic examination of the retina, determination of BCVA using the ETDRS chart, and an OCT examination (OCT 3 Stratus). The first visit involves fluorescein angiography (FA); indocyanine green angiography (ICGA) is used only if it is necessary for determining a diagnosis. Based on the examinations, compulsory and optional data are specified. The compulsory data always include BCVA expressed by the number of ETDRS letters, the central thickness of macula in 1 mm of macula in μm, and volume in 6 mm of macula in mm^3. The first visit also involves recording age and gender and measuring type and size of CNV using FA or ICGA. Patients with diagnosed wet form of AMD who meet the Czech Society of Ophthalmology criteria for initiation of treatment with the ranibizumab are entered into the registry. Ranibizumab therapy in the Czech Republic is indicated in patients with AMD who are older than 50 years, with predominantly classic, minimally classic, or occult CNV in subfoveal localization, a BCVA score between 70 and 35 letters (20/40–20/200 Snellen equivalent), total macular lesion area ≤8 disc area (DA), and submacular haemorrhage ≤25% of the total macular lesion area. Minimally classic and occult CNV must show signs of activity in the form of the presence of hard exudates, subretinal haemorrhages, or decrease in BCVA within the last 3 months by ≥10 letters of the ETDRS chart. In patients treated with ranibizumab in a dose of 0.5 mg, there are two separate phases: the loading phase, followed by a PRN phase. In the loading phase, patients receive 3 consecutive monthly injections of ranibizumab (months 0–2), followed by a PRN phase when further treatment is given between and including months 3 and 11 according to the retreatment criteria.

Retreatment with ranibizumab is performed if the patient's BCVA worsened against BCVA recorded in the previous visit and if there is a demonstrable macular edema on OCT examination. The PRN method of application is also followed in the second year and all succeeding years of patient treatment.

Our study assessed the influence of age, gender, baseline BCVA, baseline macula thickness, and type and size of CNV on early morphological therapeutic response to ranibizumab in clinical practice. We studied these factors in terms of anatomical changes in the macula after 3 consecutive monthly injections of ranibizumab in the loading phase (months 0–2). The monitored factors: age, gender, baseline best corrected visual acuity, baseline macula thickness, and type and size of choroidal neovascularization nor any others parameters (visual acuity, OCT) had no influence on the treatment scheme in the loading phase.

From 01/09/2008 to 24/6/2013, 1153 newly diagnosed, treatment-naïve patients treated with ranibizumab were entered into the registry.

Following the 3 initial injections of ranibizumab, the patients were divided into two groups based on the morphological findings in the macula: a group with active CNV and a group with inactive CNV. In the group with active CNV, OCT screening revealed intraretinal macular edema, subretinal fluid accumulation, retinal pigment epithelium (RPE) detachment, fibrovascular RPE detachment, or the combination of all these findings. No vitreomacular traction was revealed.

In the group with inactive CNV, OCT screening revealed restored foveal depression and a scar at the site of CNV with no signs of exudation was apparent. No intraretinal macular edema, subretinal fluid accumulation, RPE detachment, or fibrovascular RPE detachment was found.

In both groups, we assessed the following parameters: gender and age of patients, type and size of CNV, baseline BCVA on the ETDRS chart, and baseline macular thickness.

Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables and median supplemented with 5th–95th percentiles and mean supplemented by 95% confidence interval for continuous variables. The statistical significance of differences between groups was analyzed using Pearson's chi-square test for categorical variables and Mann-Whitney U test for continuous variables. $\alpha = 0.05$ was adopted as the level of statistical significance in all analyses.

The analysis was computed using the software PASW Statistics 19.0.1. (SPSS, Inc. 2010) and performed by the Institute of Biostatistics and Analyses at Masaryk University, Brno, operating independently of any AMD treating centre in the Czech Republic.

3. Results

The sample included 1092 patients, 38.6% men, average age 73.3 years (SD: 8.4), and 61.4% women, average age 74.2 years (SD: 8.6). The analysis included 1153 treated eyes; the right eye was treated 561 times, the left eye 592 times (both eyes were treated 61 times).

After the initial 3 injections (day 0, month 1, and month 2) of ranibizumab, in month 3 we examined the sample of 1153 eyes. Of these there were 841 eyes with active CNV (the active group) and 312 eyes with inactive CNV (the inactive group).

The sample in the active group included 37.9% of men and 62.1% of women. The sample in the inactive group included 41% of men and 59% of women ($P = 0.338$, Pearson's chi-square test).

The active and the inactive group included 29.3% and 27.9% of patients at the age <70 years, 43.4% and 42.9% of patients aged 70–80 years, and 27.3% and 29.2% of patients at the age >80 years ($P = 0.237$, Mann-Whitney U tests), respectively.

The active and the inactive group included 31% and 20.2% of patients with predominantly classic CNV, 21% and 19.9% with minimally classic CNV, and 47.9% and 59.9% with occult CNV, respectively. The inactive group showed statistically significantly higher presence of occult membranes and statistically significant lower presence of predominantly classic CNVs compared with the active group ($P < 0.001$, Pearson's chi-square test).

The active and the inactive group included 23.8% and 26.9% of patients with CNV < 2 disc areas (DA), 66.8% and 70.2% of patients with CNV 2–5 DA, 9.4% and 2.9% of patients with CNV > 5 DA, respectively. The inactive group showed statistically significantly lower presence of CNV > 5 DA compared with the active group ($P < 0.001$, Pearson's chi-square test).

The baseline BCVA in the range of 15–30 ETDRS letters was shown in 10.2% of patients in the active group and 10.9% of patients in the inactive group. The BCVA in the range of 31–60 ETDRS letters was shown in 62.5% of patients in the active group and 55.1% of patients in the inactive group. The baseline BCVA of >60 ETDRS letters was shown in 27.2% of patients in the active group and 34% of patients in the inactive group. The median of the baseline BCVA was 54 (5–95 percentiles: 22–73) and 55 (5–95 percentiles: 23–75) in the active and inactive group, respectively. We found no statistically significant difference in the value of the baseline BCVA between the groups ($P = 0.066$, Mann-Whitney U test).

19.3% of patients in the active group and 15.6% of patients in the inactive group had a baseline macular thickness of <250 μm. The baseline macular thickness in the range of 250–400 μm was shown in 51.7% of patients and 58.5% of patients, respectively, and baseline macular thickness of >400 μm was shown in 29% and 25.9% of patients, respectively. The median of the baseline macular thickness was 330 μm (5–95 percentiles: 190–600) and 337 μm (5–95 percentiles: 201–535) in the active and inactive group, respectively. There was no statistically significant difference in the value of the baseline macular thickness between groups ($P = 0.663$, Mann-Whitney U test).

4. Discussion

In the literature, we found no publications describing prognostic factors for early morphological therapeutic response to treatment with ranibizumab in patients with wet AMD. We found only articles on prognostic factors for functional therapeutic response. Sarks, Killingsworth, and Gonzales noted that occult CNV may have a good functional treatment response. Sarks et al. [4] and Killingsworth [5] demonstrated histologically that the onset of CNV is characterized by intrachoroidal neovascularization followed by sub-RPE fibrovascular proliferation. Occult CNV which is fibrovascular tissue in the sub-RPE space may in part represent an earlier stage of CNV because it is in the same tissue plane. Up to 50% of occult CNV may then progress to classic CNV. Occult CNV as an earlier stage of the disease is assumed to be associated with less damage to photoreceptors in macula and successful treatment has a better prognosis [6].

In our study, we evaluated the impact of gender, age, baseline BCVA, baseline macular thickness, and type and size of CNV on early morphological therapeutic response following the 3 initial injections of ranibizumab. In the inactive group of 312 patients with complete regression of CNV activity after the initial 3 injections of ranibizumab, we found a statistically significantly higher proportion of occult membranes, statistically significant lower presence of predominantly classic CNV ($P < 0.001$), and statistically significantly lower incidence of CNV > 5 DA ($P < 0.001$) compared with the active group. We observed that smaller and occult CNV lesions have potentially better morphological therapeutic response with the disappearance of the CNV activity and resorption of the macular edema.

We found no significant impact of gender, age, value of baseline BCVA, or baseline macular thickness on early morphological therapeutic response after the initial 3 injections of ranibizumab.

The question remains whether positive morphological therapeutic outcomes are connected to positive functional results. Additional studies are needed to further clarify the relationship of morphological and functional results in ranibizumab treated patients with wet age-related macular degeneration.

5. Conclusion

The results showed positive early morphological therapeutic response with restored foveal depression and no signs of exudation on OCT in patients with higher incidence of occult CNV, lower incidence of predominantly classic CNV, and lower incidence of CNV > 5 DA.

There was no evidence of any effect of age, gender, baseline best corrected visual acuity, or baseline macula thickness on the early anatomical restoration of the macula. We believe that occult CNV and a CNV smaller than 5 DA are optimistic for better morphological therapeutic response at the beginning of ranibizumab therapy. To determine if other factors influence the morphological response to ranibizumab treatment in patients with wet AMD, further clinical studies are needed.

Appendix

Participating AMADEUS Clinical Sites are as follows.

Department of Ophthalmology, Faculty of Medicine in Hradec Králové, Charles University in Prague and University Hospital in Hradec Králové, Czech Republic: Associate Professor Jan Studnička MD., Ph.D., Jaroslava Dusová MD., Ivana Cermanová, Gabriela Blažková; Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic: Zora Dubská, MD., Ph.D., Bohdan Kousal, MD; Department of Ophthalmology, University Hospital, Olomouc, Czech Republic: Professor Jiří Řehák, MD., Ph.D., FEBO, Oldřich Chrapek, MD., Ph.D., Zuzana Prachařová, MD., Martin Šín, MD., Ph.D., FEBO; Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and Central Military Hospital in Prague, Czech Republic: Jan Ernest, MD., Ph.D; Department of Ophthalmology, University Hospital, Brno, Czech Republic: Associate Professor Petr Kolář, MD., Ph.D., Daniela Vysloužilová, MD., Veronika Matušková, MD; Department of Ophthalmology, Masaryk Hospital, Ústí nad Labem, Czech Republic: Martin Hovorka, MD., Martina Závorková, MD; Department of Ophthalmology, University Hospital, Ostrava, Czech Republic: Jan Němčanský, MD., Pavel Šmehlík, MD; Department of Ophthalmology, Faculty of Medicine in Plzeň, Charles University in Prague and University Hospital in Plzeň, Czech Republic: Dagmar Frdlíková, MD., Hana Fidranská, MD., Tomáš Nathanský, MD; Department of Ophthalmology, Teaching Hospital Královské Vinohrady, Prague, Czech Republic: Miroslav Veith, MD., Stanislava Pokorná, MD.

Conflict of Interests

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

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Review Article

A Review of Current Management of Vitreomacular Traction and Macular Hole

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The paper presents a review of the sequence of events of posterior vitreous detachment (PVD), vitreomacular adhesion (VMA), vitreomacular traction (VMT), and macular hole (MH) from their pathophysiological aspects, clinical features, diagnostic implications, and current management strategies. A treatment algorithm to be used in clinical practice in patients with VMA, VMT, and MH based on the presence of symptoms, visual acuity, associated epiretinal membrane, and width of the vitreous attachment is presented. Observation, pharmacologic vitreolysis with ocriplasmin, and surgical treatment are positioned as treatment options in the different steps of the therapeutic algorithm, with clear indications of the paths to be followed according to the initial presenting manifestations and the patient's clinical course.

1. Introduction

Posterior vitreous detachment (PVD) is a common phenomenon frequently related with aging of ocular structures [1]. The presence of persistent vitreomacular adhesions exerting tractional forces (vitreomacular traction, VMT) may be associated with the development of macular hole (MH) [2, 3]. These alterations in the symptomatic phase may cause visual disturbances, including photopsia, metamorphopsia, blurred vision, and decreased visual acuity, which in addition of causing visual-related problems may affect negatively the patient's health-related quality of life [4]. The introduction of optical coherence tomography (OCT) has allowed a more accurate visualization of the macular anatomy and better knowledge of the pathophysiology of the process, including measurement and assessment of MH characteristics [5–7], facilitating treatment decision-making.

1.1. Anatomy of the Vitreous and the Vitreoretinal Interface. The vitreous gel is responsible for the stabilization of the eyeball through collagen fibers (mainly type II collagen). Collagen fibers are running in an anteroposterior direction through the vitreous center, convering in the anterior vitreous base, and inserting into the posterior vitreous cortex [8]. Spaces between the collagen fibrils are maintained by the protein opticin and the glycosaminoglycan chondroitin sulphate [4]. Spaces between the collagen fibrils are mostly filled with water (98% of the vitreous gel component) and hyaluronic acid, which provides the gel-like consistency of the vitreous.

The vitreoretinal interface is a complex anatomical structure composed by the union between the retina and the vitreous [9]. Densely packed collagen fibrils of the posterior vitreous cortex (100–300 μm in thickness) lie over the macula and are superficially inserted into the internal limiting membrane (ILM) of the retina by means of adhesion molecules, such as laminin, fibronectin, and proteoglycans, which interact with opticin in the vitreous gel [4]. Adherences are more firmly attached to the retina at the vitreous base, optic disc, and fovea, as well as along the major retinal blood vessels. The vitreomacular junction has an annular shape, with a diameter of 3-4 mm.

The set of events that occur as the eye ages are associated with a series of physiological changes in the vitreous gel, with progressive liquefaction (at the age of 80, around 50% of the vitreous gel has been liquefied) and gradual destruction of the collagen-hyaluronic acid network [10]. This occurs as a result of the development of fluid-filled pockets beginning in front of the macula, which over the time coalesce and enlarge, resulting in a weakened adhesion between the vitreous and the retina. This gradually predisposes to PVD, defined as separation of the posterior cortex from the ILM of the retina, which represents the final step of the normal vitreous aging process [11, 12].

PVD is an insidious process that occurs over the course of months or years, being asymptomatic in many cases until complete separation of the vitreous from the macula and optic nerve, which is the final stage. However, the anterior attachment to the vitreous base is very strong and remains for a long time. Acute symptoms of complete PVD include photopsia (by vitreous traction on the peripheral retina) and floaters by condensation of the vitreous collagen, glial tissue, or blood around the optic nerve [4].

Studies in healthy adults have shown that focal perifoveal PVD occurs in 50% of subjects aged between 30 and 39, whereas complete PVD is found in 50% of subjects aged 70 years or older [13, 14]. In addition to advanced age, PVD is more frequent in postmenopausal women by the effects of decreased estrogens on the connective tissue (within the vitreous gel), as well as in the presence of myopia [4].

The normal process of PVD due to vitreous aging may be complicated by the presence of vitreomacular adhesions between the cortex and the macular area, resulting from vitreous syneresis [15]. These adherences may be focal or extensive, affecting the foveola only or a wide region of the macular area and the optic disc. Simple vitreomacular adhesion (VMA) is not associated with distortion of the macular architecture. However, these adherences may exert traction forces on the macula (VMT), increasing secondarily during ocular saccades [16]. This may cause retinal distortion and foveal detachment. On the other hand, continuous anteroposterior traction by vitreous contraction may cause alterations, such as cystoid macular edema.

Full-thickness MH is an anatomic defect in the fovea with interruption of all neural retinal layers [17]. With the use of high-resolution OCT, it has been shown that idiopathic MHs are initiated during perifoveal PVD as a consequence of the dynamic anteroposterior VMT process. This anteroposterior VMT may cause intraretinal cavitation with progression to dehiscence of the outer retinal layers and complete detachment of the cyst roof giving rise to a full-thickness defect. Stages of the development of MH from focal VMT to complete aperture together with accompanying symptoms have been described by Gass [18, 19].

The introduction of enzymatic vitreolysis [20], which can result in the liberation of VMT, opens highly interesting new perspectives in this field.

1.2. Risk Factors and Epidemiology of VMT. A few studies have been specifically addressed to the epidemiology of idiopathic VMT due to the overlapping of this condition with other ophthalmological diseases [4]. A prevalence of isolated idiopathic VMT, without MH, has been estimated as approximately 22.5 cases per 100 000 of the general population, with an incidence of 0.6/100 000 persons-year [21]. In different observational and intervention studies, the mean age of patients with VMT was around 65–70 years (range 48–64), with a predominance of females [4, 15].

Regarding the prevalence of MH, it has been reported around 0.1 to 0.8 in adults aged >40 years [22], with an age-adjusted incidence of 7.8 cases per 100 000 of the general population per year [23]. Also, the risk of development of MH in the fellow eyes, without manifestations of PVD, has been estimated at around 7–12% after 5 years and 17% at 20 years [4].

Approximately two-thirds of patients with MH are women, and the disease is unilateral in 80% of cases. An increase in serum fibrinogen level has been reported as a risk factor for MH [24], whereas the use of estrogen replacement therapy in women decreases the risk [4]. In subjects with myopia, the prevalence of MH may reach 6% [25].

2. Diagnosis, Definition, and Classification of VMT and MH

Now, nearly two decades since the introduction of OCT, it is possible to assess and define the pathologic progression of disorders affecting vitreoretinal interface with a high level of accuracy and reproducibility. On the basis of OCT-derived anatomic findings, a unified classification scheme for disease of the vitreomacular interface has been developed.

With this purpose, a group of experts in diseases of the vitreoretinal interface (*International Vitreomacular Traction Study Group,* IVTS) [26] have proposed a classification system for diseases of the vitreomacular interface. This evidence-based classification is a clinically applicable system that is predictive of therapeutic outcomes and is useful for the execution and comparative analysis of clinical studies.

2.1. VMA. VMA represents a specific stage of partial vitreous detachment in the perifoveal area without retinal abnormalities. In previous classifications, VMA is the equivalent of a stage 1 PVD [11, 15, 27, 28]. VMA is characterized by elevation of the cortical vitreous above the retinal surface, with the vitreous remaining attached within a 3 mm radius of the fovea (as defined arbitrarily). The angle between the vitreous and the inner retinal surface is acute, and the retina displays no

abnormalities in contour or morphological features of OCT. VMA is not accompanied by visual impairment and may be considered a normal finding in the natural course of PVD. Also, VMA may be subclassified by the size of the adhesion into focal (≤1500 μm) or broad (>1500 μm). The cutoff of 1500 μm corresponds to the area of increased vitreous adhesion to the fovea. VMA usually resolves spontaneously as part of the normal process of PVD, although it may progress to VMT and, for this reason, periodic monitoring with OCT is necessary.

2.2. VMT. Macular traction due to progression of PVD causes anatomic changes in contour of the foveal surface, intraretinal pseudocyst formation, and disappearance of foveolar depression, which typically results in reduced or distorted vision. The following anatomic criteria [26] should be present at least in one OCT image to classify an eye as having VMT: (a) evidence of perifoveal vitreous cortex detachment from the retinal surface, (b) attachment of the vitreous cortex to the macula within a 3 mm radius of the fovea, and (c) association of this attachment with distortion of the foveal surface, intraretinal structural changes, foveal detachment from the retinal pigment epithelium (RPE), or a combination of these findings, without full-thickness interruption of all retinal layers. VMT can also be subclassified as focal or broad (using the same cutoff of 1500 μm) depending on the width of the vitreous attachment. Distortion of the foveal profile, formation of intraretinal cysts, intraretinal cavitation, subretinal fluid, and, even, RPE detachment can be observed.

On the other hand, proliferation of residual of vitreous tissue provides the anatomic substrate to form an epiretinal membrane (ERM), which in turn may appear at any stage of vitreous separation. ERM is composed of retinal pigment epithelial cells, fibroblasts, and macrophages. ERM may be associated with peripapillary vitreoretinal traction with blurred disc border.

Although spontaneous resolution of VMT may occur, traction on a large surface or the presence of ERM is poor prognostic factor. In symptomatic patients, enzymatic vitreolysis or vitrectomy may be indicated.

2.3. MH. As stated above, full-thickness MH is an anatomic defect in the fovea featuring interruption of all neural retinal layers. The observation of the anatomic opening on several scans through the fovea is an unequivocal sign. According to the aperture size, MHs are considered small (<250 μm), medium (250 to 400 μm), and large (diameter > 400 μm). Nearly half of full-thickness MHs are large at the time of diagnosis [26]. Also, on the basis of OCT findings, MH can be categorized according to the presence or absence of VMT. Only patients with MH and concomitant VMT are candidates for pharmacologic vitreolysis. The correlations between MH stages commonly used in clinical practice and OCT-based images proposed by the IVTS group are shown in Table 1.

Moreover, MH can be subdivided into idiopathic and secondary. Primary MH results from vitreous traction on the fovea from anomalous PVD (incomplete or inadequate separation of the vitreoretinal interface), whereas secondary MHs are caused by other pathologic conditions and do not have preexisting or concurrent VMT. Secondary MHs have been reported in cases of blunt ocular trauma [29], lightning strike [30], high myopia [25, 31], macular schisis [32], macular telangiectasia type 2 [33], occlusion of the central retinal vein, diabetic macular edema, uveitis, and age-related macular degeneration [26].

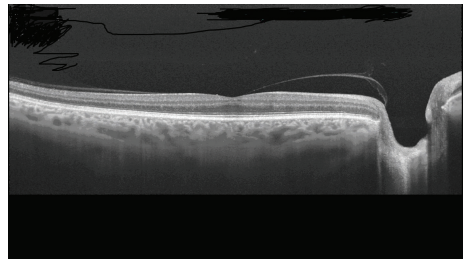


Figure 1: Horizontal image with swept-source OCT at the foveal level showing posterior vitreous detachment, which remains adhered to the fovea and the papillary edge.

3. Treatment Options

3.1. Observation. The availability of OCT, particularly spectral domain OCT (SD-OCT), has allowed a more accurate diagnosis and precise assessment of adhesion of the vitreous to the macula, differentiating VMA from VMT [26]. Before the introduction of OCT, only patients with advanced VMA could have been diagnosed by biomicroscopy and, for this reason, the rates of spontaneous deterioration reported were high (64%) [34].

Studies using SD-OCT have shown that incomplete vitreous detachment with persistent vitreoretinal adhesions is more frequently observed than by clinical diagnosis. During the physiological process of PVD, the vitreous remains attached to the foveal region in the last stages (Figure 1), so that, VMA can be considered a normal stage in the natural history of PVD associated with vitreous aging [13, 26]. Only when symptoms are present or when foveal anatomic changes are observed, VMA can be considered a pathological process [34].

Recently, John et al. [35] investigated the spontaneous clinical course in 106 eyes of 81 patients identified as having VMA by SD-OCT and classified into three grades, with a mean follow-up of 18 months (range 1 to 91). The authors defined three grades to classify adherence: Grade 1 (41%) was incomplete cortical vitreous separation with attachment at the fovea, Grade 2 (52%) was the Grade 1 findings and any intraretinal cysts, and Grade 3 (7%) was the Grade 2 findings and the presence of subretinal fluid. By the last follow-up, spontaneous release of VMA occurred in 32% of cases (34 eyes, in 30%, 30%, and 57% of Grades 1, 2, and 3, resp.). No changes were observed in 23, 31, and 2 eyes (52% of the total), and progression occurred in 7, 8, and 1 eye of Grades 1, 2, and 3, respectively (16% of the total). The authors conclude that the clinical course of patients with VMA managed by

Table 1

Gass classification	OTC findings	Classification IVTS
Stage 0	Minimal changes in the foveal contour with perifoveal detachment of the perifoveal vitreous cortex without traction	VMA
Stage 1A: imminent MH	Foveal cysts and sensory foveolar detachment associated with perifoveal detachment with traction of the posterior vitreous on the foveal internal limiting membrane	VMT
Stage IB	Cyst in the outer retina causing rupture of the cones layer. Perifoveal detachment of posterior vitreous	VMT
Stage 2: small MH	Full-thickness MH of small diameter, with partial rupture of the internal wall of the cyst. Partial detachment of the posterior vitreous, which still remains adhered to the operculum	FTMH small/medium with VMT
Stage 3: large MH	MH of a larger size. Total detachment of the posterior vitreous at the level of the macular area, which persists adhered to the papilla. Occasionally, a free operculum adhered to the posterior vitreous can be seen	FTMH medium/large with VMT
Stage 4: full-thickness MH with PVD	Total detachment of the posterior vitreous. In some cases, the vitreous is not observed on OCT scans. Larger diameter of the hole with halo of outer retinal detachment in many occasions	FTMH small/medium/large without TVM

FTMH: full-thickness macular hole, MH: macular hole, OCT: optical coherence tomography, PVD: posterior vitreous detachment, VMA: vitreomacular adhesion, and VMT: vitreomacular traction.

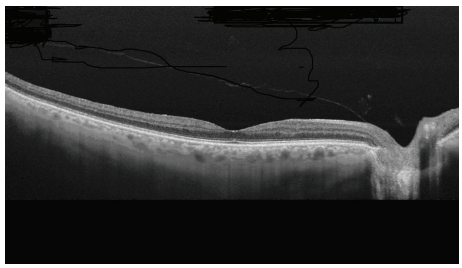


Figure 2: Study of the retinal surface with swept-source OCT showing posterior vitreous detachment, which remains attached at the papillary level.

initial observation was generally favourable in asymptomatic patients or with minimal symptoms of VMT.

Studying the retinal surface with SD-OCT, it has been observed that PVD appears to begin in the perifoveal region, with a slow clinical course taking even years until complete separation of the vitreous from the papilla (Figure 2). In most patients this process is asymptomatic but, in some cases, PVD may be complicated by macular pathology [2]. In a OCT study of eyes with macular edema secondary to VMT, published in 2012, complete and spontaneous resolution of traction was observed in 53% of eyes [36].

The clinical course of VMA, particularly in asymptomatic patients, remains to be fully elucidated. Systematic examination with SD-OCT has been associated with an increase in diagnostic rates and has allowed assessing more accurately the course of this physiological process that may evolve into VMT, remain stable, or resolve spontaneously. Therefore, in the presence of a VMA syndrome, the first approach is to reexamine the patients using OCT at a period of 3 months. Even in cases of evolution to a VMT syndrome, observation still remains an option, given the possibility of spontaneous resolution of VMT.

3.2. Pharmacologic Vitreolysis: Ocriplasmin. Ocriplasmin is a truncated form of human plasmin that induces liquefaction of the vitreous and separation of the vitreous cortex from the retinal surface due to proteolytic activity against main components of the vitreomacular adhesion.

3.2.1. Results of Clinical Trials of Ocriplasmin and Initial Data in Clinical Practice. The efficacy and safety of ocriplasmin have been evaluated in two pivotal, phase 3 clinical trials (TG-MV-006 y TG-MV-007) carried out in the United States and Europe [20]. Both studies were very similar except for the ratio of randomized assignments to ocriplasmin and placebo, which was 2 : 1 in the TG-MV-006 study and 3 : 1 in the TG-MV-007. Overall, 652 patients were randomized, 464 were assigned to treatment with a single intravitreal injection of ocriplasmin (125 μg) and 188 to a placebo intravitreal injection. The primary endpoint was the pharmacologic resolution of VMA at day 28, as determined by OCT. Secondary endpoints included the percentage of patients with complete PVD and nonsurgical closure of full-thickness MH at day 28. Eligible patients had symptomatic focal VMA as seen on OCT and a best-corrected visual acuity of 20/25 or less. Exclusion criteria were high myopia (more than −8 diopters or axial length > 26 mm), prior vitrectomy or prior laser photocoagulation of the macula, and other eye diseases that may affect visual acuity. Patients with a MH > 400 μm in diameter were also excluded. Of note, the presence of an ERM was not a criterion for exclusion.

At day 28, VMA resolved in 26.5% of ocriplasmin-injected eyes and in 10.1% of placebo-injected eyes ($P < 0.001$). The between-group differences did not change substantially at 6 months (26.9% ocriplasmin versus 13.3% placebo, $P = 0.001$). Also, 72% of patients with resolution of VMA showed the release during the first seven days. Results of adhesion release were better in patients without ERM (37.4% in the ocriplasmin group versus 14.3% in the placebo group, $P < 0.001$).

With regard to secondary variables (day 28), 13.4% of patients treated with ocriplasmin showed total PVD as compared to 3.7% of those treated with placebo ($P < 0.001$). Also, nonsurgical closure of full-thickness MH was achieved in 40.6% of ocriplasmin-treated patients and in 10.6% of placebo-treated patients ($P < 0.001$).

According to the investigator's criteria, all patients could be treated with vitrectomy in the framework of the study if macular disease did not resolve. At 6 months, vitrectomy was performed in 17.7% of patients in the ocriplasmin group and in 26.6% of those in the placebo group ($P = 0.02$). At 6 months, there were statistically significant differences in favour of ocriplasmin in the gain of two or more lines (23.7% versus 11.2%, $P < 0.001$) or three or more lines (12.3% versus 6.4%, $P = 0.02$).

Important safety-related problems were not observed. Most adverse events were related to the development of PVD induced by ocriplasmin injection (floaters and photopsia). There was a slightly higher incidence of retinal tears or detachments in the placebo group, which was attributed to the higher proportion of patients treated by means of vitrectomy in this group.

The favourable results obtained in both clinical trials allowed approval of the use of intravitreal injection of ocriplasmin for the treatment of symptomatic VMT and MH by the Food and Drug Administration (FDA) in the United States, in November 2012, and by the European Medicines Agency (EMA) in May 2013.

Outside the context of clinical trials, recent reports have provided data of the use of ocriplasmin in daily practice. In a retrospective study [37], 17 patients with symptomatic VMT were treated with a single intravitreal injection of ocriplasmin 0.125 mg. By day 28, resolution of VMT was verified by SDOCT in eight patients (47.1%), 7 of which (87.5%) had already experienced release by day 7. Those who did not have traction release showed no statistically significant change in VMA diameter. Four of the five patients (80%) with MH at baseline experienced resolution of their MH after injection. Significant differences in visual acuity were not observed (20/49 at baseline and 20/46 at final follow-up). It should be noted that patients meeting the four positive predictor criteria (younger than 65 years, no ERM at baseline, traction <1500 μm, and phakic lens status) showed a response rate of 75% (three of four eyes). Transient outer segment ellipsoid zone loss was documented in 7 cases (41.1%) and subretinal fluid presence following injection was noted in 5 cases (29.4%) [37].

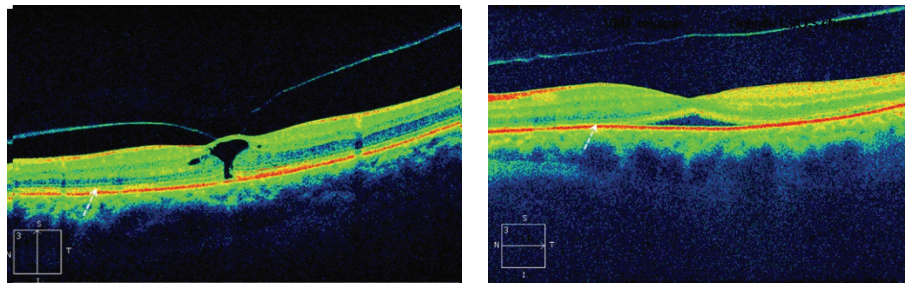
In another study of 19 patients with symptomatic VMA treated with intravitreal ocriplasmin, resolution of VMA was observed in 8 cases (42%) [38]. Results were significantly affected by lens status, with adhesion release in 53% of phakic patients, whereas no case of resolution of adhesions was observed in pseudophakic patients. Also, closure of MHs after treatment was found in 3 of 6 patients (50%). Visual acuity remains stable, with a slight tendency towards improvement in the majority of cases. Only one patient showed an important loss of visual acuity (from 20/70 to 20/200) due to progression of VMT to a full-thickness MH. Significant adverse events were not recorded.

3.2.2. Safety Profile. The safety profile of ocriplasmin has been evaluated in the two pivotal trials [20]. The proportion of patients who had any ocular adverse event was 68.4% in the ocriplasmin group and 53.5% in the placebo group ($P < 0.001$). This difference was driven primarily by adverse events known to be associated with PVD. The most common complications included vitreous floaters (ocriplasmin 16.8% versus placebo 7.5%, $P = 0.002$), photopsia (11.8% versus 2.7%, $P < 0.001$), blurred vision (8.6% versus 3.2%, $P = 0.01$), and visual impairment (5.4% versus 1.6%, $P = 0.02$). Most of these adverse events were transient and mild in severity. There were no differences between the groups in terms of severe ocular adverse events, including development of MH (5.2% versus 8.6%), retinal detachment (0% versus 1.6%), and reduced visual acuity (0.6% versus 0.5%).

However, since the real-world use of the drug began, there have been some unfavourable reports of visual disturbances after ocriplasmin injection, including transient but profound visual decline, raising concerns regarding its safety. Of 976 patients receiving ocriplasmin injection in clinical trials, 9 patients were reported to have experienced an acute decrease in vision, some to the hand motions level, within 24 hours of injection [39]. In 8 of these 9 patients, vision returned to baseline with a median recovery time of 2 weeks. In the clinical trials of ocriplasmin, dyschromatopsia, and electroretinographic (ERG) changes occurred in a significantly greater number of eyes treated with ocriplasmin than in eyes receiving placebo [20, 40].

Freund et al. [41] recently reported a single case report demonstrating changes seen in the outer photoreceptor segments by SD-OCT. The disruption occurred in the ellipsoid zone and was reversible. Since the clinical trials [20] used only time-domain OCT with inferior resolution to SD-OCT, it is possible that these cases may have been overlooked.

In another study in which 17 patients were included [37], almost all the patients who responded to the treatment (7/8) had ellipsoid zone changes on the SD-OCT (Figure 3). These patients also had transient reduction of visual acuity and demonstrated subretinal fluid during the release process with almost the exact time course as the loss of the OS ellipsoid zone. The loss of the OS ellipsoid zone occurred after an average of 5 days after injection of ocriplasmin and the mean time of resolution on OCT was 29.3 days. The occurrence and resolution of subretinal fluid occurred at an average of 4.8 days and 30 days after injection, respectively. However, in a retrospective review of 62 eyes with symptomatic VMA treated with ocriplasmin, subretinal fluid appeared in 37% of cases, with persistence of fluid in 30% of cases after 5



(a) (b)

Figure 3: (a) Focal VMT. The arrow points to the ellipsoid zone. (b) Release of VMT after injection of ocriplasmin. A severe disruption in the ellipsoid zone is shown (by courtesy of Dr. Peter K. Kaiser, Cleveland Clinic, Cleveland, OH, USA).

months of follow-up [42]. Other studies have also shown resolution of the ellipsoid zone changes in most patients within weeks or months after ocriplasmin injection [43, 44].

Alteration of the ellipsoid zone on SD-OCT and a significant decrease in ERG amplitudes have been also reported in two patients with release of symptomatic VMT after ocriplasmin injection [45, 46]. It is possible that this transient effect of the medication may be due to a diffuse enzymatic effect of the protease on the photoreceptors or the retinal pigment epithelium throughout the retina. The greater reduction in scotopic function compared with photopic function suggests that rod photoreceptors may be more susceptible than cone photoreceptors to the effects of ocriplasmin. If this transient affect occurs for both rods and cones, it may explain the dyschromatopsia, contrast sensitivity changes, dark adaptation issues, and ERG changes reported in the ocriplasmin clinical trials.

An ongoing phase 3b, 24-month randomized clinical trial which will evaluate ERG and microperimetry in ocriplasmin-treated eyes compared to sham, will provide additional clarifications on the observed EGR changes and dyschromatopsia events (*OASIS Study*; NTC01429441) already reported.

3.3. Surgical Treatment

3.3.1. Peeling of the Internal Limiting Membrane. Surgery of idiopathic MH with ILM peeling is a very safe procedure, with good anatomic and functional results and scarce postoperative complications [47]. Data provided by clinical trials have shown that peeling of the ILM significantly increases MH closure rates and is also associated with significantly lower percentages of reoperation and reopening. Therefore, ILM peeling is a cost-effective technique and the procedure of choice for all patients with idiopathic full-thickness MH susceptible to undergo surgical treatment [48–53].

Broad ILM peeling to the vascular arcades is recommended, so that tangential traction forces on the MH edges are removed facilitating approximation and closure [54]. In cases of large MH (>400 μm) with increased risk of failure of primary surgery, alternative techniques have been proposed, such as the inverted ILM flap technique in which instead of completely removing the ILM, a remnant attached to the margins of the MH is left in place. This ILM remnant is then inverted upside down to cover the MH [55]. With the use of this technique closure rates of 98% compared to 88% with the standard technique have been achieved [55]. For refractory MH to the standard technique or for secondary MH after vitrectomy when peeling of the ILM has been already performed, an autologous transplantation of the ILM remnants introduced into the hole with subsequent gas tamponade contributes to the improvement of anatomic and visual outcomes [56].

3.3.2. Vital Dyes for ILM Staining. Vital dyes have become effective and useful tools for identifying ocular tissues during vitrectomy, thereby facilitating ILM peeling and ensuring complete removal of this delicate membrane [57]. The most frequently used vital dyes include triamcinolone acetonide suspension in balanced salt solution (BSS) (Triesence), indocyanine green and infracyanine green, brilliant blue, and trypan blue with brilliant blue (Membrane Blue-Dual).

Triamcinolone suspension in BSS is not a true dye but is very useful for the identification of vitreous remnants and the posterior hyaloid. Deposition of crystals on the ILM surface helps the achievement of complete removal of the membrane, although it is less effective than vital dyes because triamcinolone does not increase the rigidity of ILM.

Indocyanine green and infracyanine green possess a great affinity for the matrix components of the ILM and produce intense staining of the ILM. Besides the ability of indocyanine and infracyanine green to stain the ILM, they cause an increase in the biomechanical stiffness of the ILM, thereby facilitating its peeling. Although in Europe they are no longer used because of potential toxicity, they continue to be used in the United States [58, 59].

Brilliant blue has a remarkable affinity for the ILM and, although ILM staining is less intense than that achieved with indocyanine green, causes adequate staining of the ILM and may be used without fluid-air exchange. In Europe, it is considered the best one for ILM peeling in MH surgery.

The combination of trypan blue and brilliant blue allows staining of the ERM, posterior hyaloid, and ILM simultaneously. This combination has a lower density than water and BSS and circumvents the need for fluid-air exchange. This dual dye is extensively used in Europe [60].

3.3.3. Tamponade and Postoperative Positioning. There is controversy regarding posturing in MH surgery. Although most authors recommend face-down posturing 90% of time for 10 days, different studies have reported successful hole closure in the absence of face-down positioning, given that isolation of the macula by gas tamponade maintaining the macula dried seems to be the most important factor for closure [61–63]. In this respect, OCT studies have shown that hole closure occurs during the first postoperative day independently of the types of gas tamponade and posturing [64], so that after vitrectomy with wide ILM peeling, gas tamponade would be sufficient (preferably short-acting gases, such as SF6) at nonexpansible concentration, without the need of face-down posturing, avoiding the prone position during 3 to 5 days. This approach may be also indicated for phakic patients because it does not seem to increase the incidence of cataracts [54].

3.3.4. Combined Phacovitrectomy. Combined phacovitrectomy or sequential vitrectomy and phacoemulsification are safe and effective for the treatment of MH, with equivalent anatomic and functional results [65]. In most cases, idiopathic MH affects patients older than 50 years in which some degree of lens opacity is frequent. Moreover, cataract develops in 75% to 95% of patients undergoing vitrectomy for MH within 3 years after surgery. For this reason, most authors recommend combined phacovitrectomy in patients over 50 years of age. Both cost and discomfort are lower with a single surgical procedure, and functional recovery is more rapid. Combined phacovitrectomy may also decrease the risk of reopening after cataract extraction in the two-step surgical approach [66, 67]. However, combined vitrectomy, phacoemulsification, and intraocular lens (IOL) implantation may be associated with complications, including a high degree of postoperative anterior chamber inflammation and a higher risk of IOL dislocation or papillary capture, generally as a result of excess gas tamponade and/or poor compliance to positioning [68]. Therefore, the decision of the combined versus the two-step procedure should be individualized according to the characteristics of each case and the patient's and surgeon's preferences.

3.4. Results of Surgery for MH and Complications. In the study of the *Moorfields Macular Hole* (MMHS) Group [69], an overall closure rate of 81% at 2 years was achieved in MHs stages 2, 3, and 4 as well as an improvement in visual acuity of 6/36 to 6/18, which was clearly superior to results obtained in the observation group. In the *Vitrectomy for Treatment of Macular Hole Study* (VMHS), the rate of anatomic closure was 69% and the final visual acuity was higher in the operated than in nonoperated eyes (20/115 versus 20/166) [70].

Once peeling of the ILM has become popular, closure rates of 90% to 100% were reported [71–75]. However, the use of indocyanine green was associated with potential toxicity in some cases [76] and, for this reason, trypan blue and brilliant blue are in widespread use in some countries, with closure rates of 94% to 100%, without apparent severe side effects [77–80]. Despite its clear indication and safety in MH surgery, ILM peeling is a traumatic procedure that has acute effects on the underlying retinal nerve fiber layer. ILM peeling often results in temporary swelling of the arcuate nerve fiber layer (SANFL) which may be the earliest manifestation of dissociated nerve fiber layer (DONFL) which occurs later in the postoperative period. However it is probably a transient feature that does not affect visual recovery [80].

Although peeling of the ILM has been widely adopted in MH surgery, the high percentages of hole closure obtained in the years prior to systematic ILM peeling add uncertainty about whether to use it in all cases. Recently, Spiteri Cornish et al. [81, 82] carried out a systematic review and meta-analysis to assess the success of HM surgery with ILM peeling compared with the nonpeeling technique. Four randomized clinical trials comparing both techniques were identified [48, 49, 51, 81, 82]. There was no evidence of a difference in the primary outcome (distance visual acuity at six months), nor in distance visual acuity at 12 months between randomized groups. Overall, 66.2% achieved a visual acuity equal or greater than 69 letters on ETDRS charts (corresponding Snellen visual acuity 20/40) and 77.9% gained more than three ETDRS lines. Improvement of visual acuity was higher in patients in which primary anatomic closure was achieved (final visual acuity 72.8 ± 7.6 letters and a mean improvement of 21.6 ± 7.1 letters) than in eyes in which further surgery was required (66.4 ± 8.6 and 17.4 ± 7.7 letters, resp.) [49].

However, visual improvement was obtained somewhat earlier in the ILM peeling group and, at 3 months, improvement was greater if ILM peeling was performed. In addition, the percentage of primary closure was higher in the ILM peeling as compared with no peeling (89.9% versus 50.3%, with an odds ratio (OR) of 9.27 and 95% confidence interval [CI] of 4.98–17.24). When reoperations were excluded from the analysis, the ILM peeling group continued to have more favourable results (OR 3.99, 95% CI 1.63–9.75). Also, in MH stage 2, the efficacy rate was better for ILM peeling than no peeling (91.6% versus 61.3%, with an OR of 6.19; 95% CI 1.65–23.20) [48, 49, 83].

This higher success rate was not accompanied by an increase of perioperative complications, neither in the reports in which the ILM was stained with indocyanine green. In the meta-analysis [81], the rate of intraoperative complications was 19.32% for the ILM peeling group as compared with 21.1% for the nonpeeling group (OR 0.94, 95% CI 0.47–1.87). The most frequent intraoperative complications were small retinal hemorrhage (6–19%), retinal tears (5.4–32%), retinal detachment (2–6%), and choroidal hemorrhage (0–3%).

According to these data, the authors conclude that ILM peeling offers more favourable cost-effectiveness compared with no peeling in MH surgery [81, 82].

3.5. Surgery-Related Prognostic Factors and Management of Reopening. Although anatomic closure in MH surgery is achieved in more than 90% of cases, sometimes it does not correlate well with improvement in visual acuity. Multiple studies using OCT have assessed hole configuration in an attempt to establish a correlation with postoperative visual acuity [84–91], emphasizing the importance of changes in the outer retina. Kusuhara et al. [84] defined a macular hole

index (MHI) as a ratio of hole height to base diameter of hole, calculated from OCT transverse images of the macular area, establishing that a MHI ≥0.5 was correlated with better postoperative visual acuity than MIH <0.5. Ruiz-Moreno et al. [85] described the diameter hole index (DHI) as a ratio between minimum hole diameter and base diameter, showing the minimum diameter was the best preoperative predictive prognostic factor.

Different studies have shown a direct correlation between integrity of the hyperreflective line as IS/OS junction of photoreceptors and postoperative improvement of visual acuity. In the study of Kitaya et al. [86], postoperative vision ≥0.7 was correlated with good reconstitution of the IS/OS junction. However, Sano et al. [87] showed that a continuous IS/OS line was not a reliable prognostic factor in the early postoperative period given that abnormalities of the IS/OS line seen on SD-OCT can be gradually repaired, with achievement of a continuous IS/OS line at 6 months. Spaide and Curcio [88] assessed the correlation of the outer retina analyzed by means of SD-OCT and histopathological findings, showing that the hyperreflective line identified as IS/OS junction of photoreceptors corresponded to the ellipsoid portion of the photoreceptor inner segment, containing mitochondria. Wakabayashi et al. [89] using SD-OCT described that reconstitution of the external limiting membrane (ELM) was more important to predict subsequent restoration of the foveal photoreceptor layer than the ellipsoid zone restoration. Restoration of ELM seems to be a necessary factor for reconstitution of the ellipsoid band, with subsequent migration of photoreceptors and complete closure of the full-thickness MH. Ruiz-Moreno et al. [90] have analyzed 164 eyes with MH treated by vitrectomy and ILM peeling showing that restoration of the ellipsoid portion of the photoreceptor inner segment is an important prognostic factor for visual rehabilitation after MH surgery.

Reopening of the hole (Figure 4) is one of the best known complications after initially successful MH treatment with vitreous surgery [67, 91–101]. Peeling of the ILM during primary MH surgery is one of the factors that has been mostly related to the incidence of reopening, varying between 0% and 8% in eyes with ILM peeling [67, 95–97] and between 2% and 16% in eyes with no peeling [67, 93–95, 97]. The variable percentages reported in the studies are due in part to differences in the length of follow-up, with higher rates associated with prolonged follow-up periods. Paques et al. [93] reported a 9.5% incidence with a mean follow-up of 2 years, whereas Scott et al. [94] found a 12% incidence with a mean follow-up of 7 years. Kumagai et al. [95] analyzed the results of surgery in a series of 877 cases of MH, increasing the reopening percentage to 28.1% with no ILM peeling. The incidence of recurrence was 0.39% in eyes with peeling of the ILM, increasing to 7.2% with no peeling. Besides no peeling, statistically significant risk factors for reopening were myopia of more than 6 diopters and intraoperative retinal tears. Retinal tears treated with laser may be one of the factors that increase the development of ERM, with subsequent tangential traction and reopening of the MH.

No peeling of the ILM may be associated with a higher risk of ERM formation [92, 93, 97, 100]. Yoshida and Kishi [97] observed the presence of ERM in all cases of reopening of the hole. However, Kumagai et al. [95] did not report ERM in none of the cases with hole reopening assessed by SD-OCT.

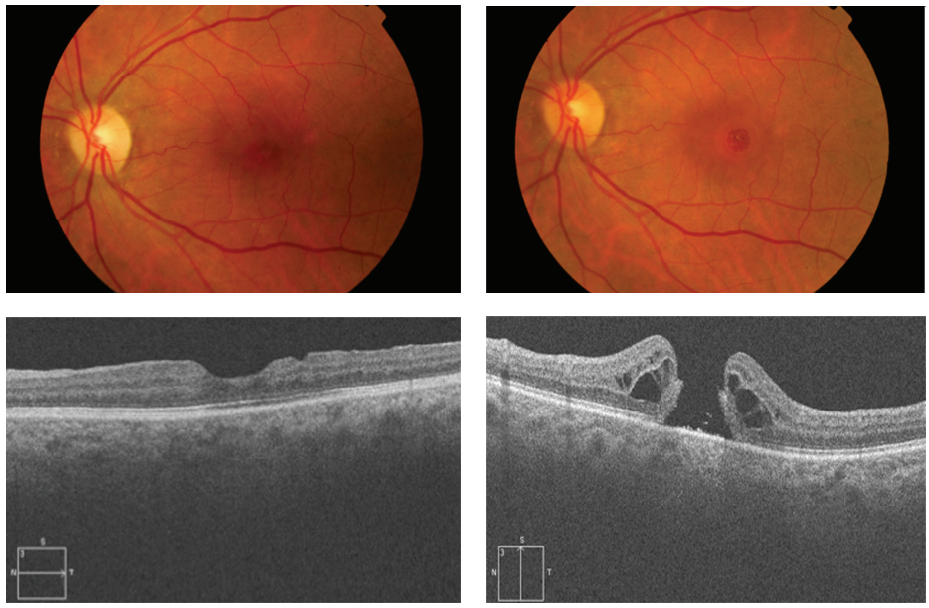
In relation to the incidence of reopening with bilateral MH, Duker et al. [92] reported bilateral reopening in 38% of cases, Christmas et al. [99] in 59%, Scott et al. [94] in 38%, and Kumagai et al. in 14.9% [95].

Cataract surgery in the postoperative period of MH surgery has been involved in the reopening of MH. Paques et al. [93] observed that 73% of cases of hole reopening occurred after a secondary cataract surgery. Bhatnagar et al. [67] reported that in the presence of cystic macular edema after secondary cataract surgery, there was a sevenfold increase in the risk of reopened holes, and García-Arumí et al. [101] reported recurrence of MH reopening after posterior capsulotomy. However, other authors, including Kumagai et al. [95] and Sheidow and Gonder [102] reported cystoid edema in combined surgical procedures and that the incidence of hole reopening did not increase in secondary cataracts.

With regard to treatment of persisting MH, ILM peeling and ERM removal should be performed in those cases in which these procedures were not performed at the initial macular surgery, together with long-acting gas tamponade (C3F8) and strict face-down positioning during the first postoperative days. In these patients, the anatomic and functional success is high. When ILM peeling and removal of the ERM have been performed in the first surgical procedure, the success of reoperation decreases. In a series of 30 patients reported by D'Souza et al. [103] with initial ILM peel who underwent repeat surgery involving vitrectomy, enlargement of ILM rhexis, and gas tamponade with C3F8, the anatomic closure rate was 88% for primary surgery and 46.7% for reoperation. More extensive ILM peeling causing tangential traction due to fibrosis of dissection margin may contribute to the anatomic closure. The use of growth factors, such as platelet-derived growth factors as a stimulus of glial progenitor cells, may be useful if the ILM has been adequately peeled (Figure 5), as well as the use of heavy silicone oil in patients with positioning difficulties [104].

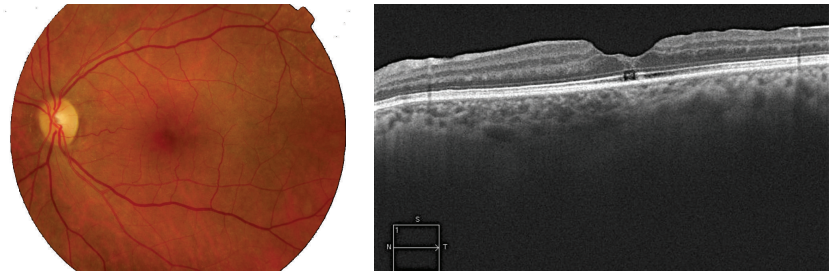
4. Practical Considerations: Therapeutic Algorithm

Based on the aforementioned data and as shown in the schematic representation in Figure 6, patients with VMA can be observed without the need of any intervention. In cases of VMT it is crucial to take into account the patient's symptoms. If the patient is asymptomatic, a follow-up control at 3 months may be sufficient. During this interval, the patient should be advised to perform periodic self-examinations with the Amsler grid or monocular reading tests. In case of symptoms, intensity and disability should be assessed. There is no consensus criterion regarding the degree of vision loss that should be considered significant and amenable to treatment. However, in the TG-MV-006 y TG-MV-007 clinical trials, patients with visual acuity equal or lower than 20/25 were eligible [20], so that this level of visual impairment can be already considered to be susceptible of treatment. Also, other causes that may justify decreased visual acuity should



(a) (b)

Figure 4: (a) Fundus photography and OCT of a patient who underwent macular hole surgery with ILM peeling and adequate reconstitution of the outer retina (ELM and ellipsoid bands) and visual acuity 20/30. (b) Reopening of the MH after 3 years with cystoid edema surrounding the hole and decreased visual acuity to 20/200. The ERM is not observed.



(a) (b)

Figure 5: Fundus photography and OCT after reoperation using platelet-derived growth factors. Successful anatomic hole closure is observed but glial type scar in the inner retina and the absence of a continuous ellipsoid band determined a final visual acuity of 20/60.

be excluded. Metamorphopsia clinically significant for the patient and visual loss progression are also key factors at the time of adopting a more interventional therapeutic attitude. Despite these considerations, a period of observation may be an option for these patients, because spontaneous resolution is still possible. In case of deciding an active treatment, the presence of other associated macular diseases, such as ERM, should be excluded [20]. When traction is ≤1500 μm, enzymatic vitreolysis with ocriplasmin is the treatment of choice. In the presence of >1500 μm traction or ERM, surgical treatment with vitrectomy is associated with better outcomes [105].

In cases with a full-thickness MH, it is necessary to assess the diameter size. In cases of holes ≤400 μm in size with MVT and in the absence of ERM, enzymatic vitreolysis with ocriplasmin is again the most recommendable option [20]. In cases of holes >400 μm, or in the absence of evident VMT, or in the presence of ERM, vitrectomy is the first option [105].

Patients undergoing enzymatic vitreolysis with intravitreal injection of ocriplasmin should be evaluated at 7 and 30 days. Most cases of VMT or MH resolve within the first week of treatment [20] and also at this time the occurrence of potential treatment-related complications should be excluded. If resolution of VMT and/or hole closure had not occurred after a month of treatment, the likelihood of success is highly improbable and vitrectomy can be performed. Patients with lamellar MH or pseudomacular holes in which traction is usually absent are also candidates for enzymatic vitreolysis. In cases of VMT associated with other retinal diseases, such as age-related macular degeneration, diabetic macular edema, or vitreomacular interface pathology in the myope, it is still too early to make a

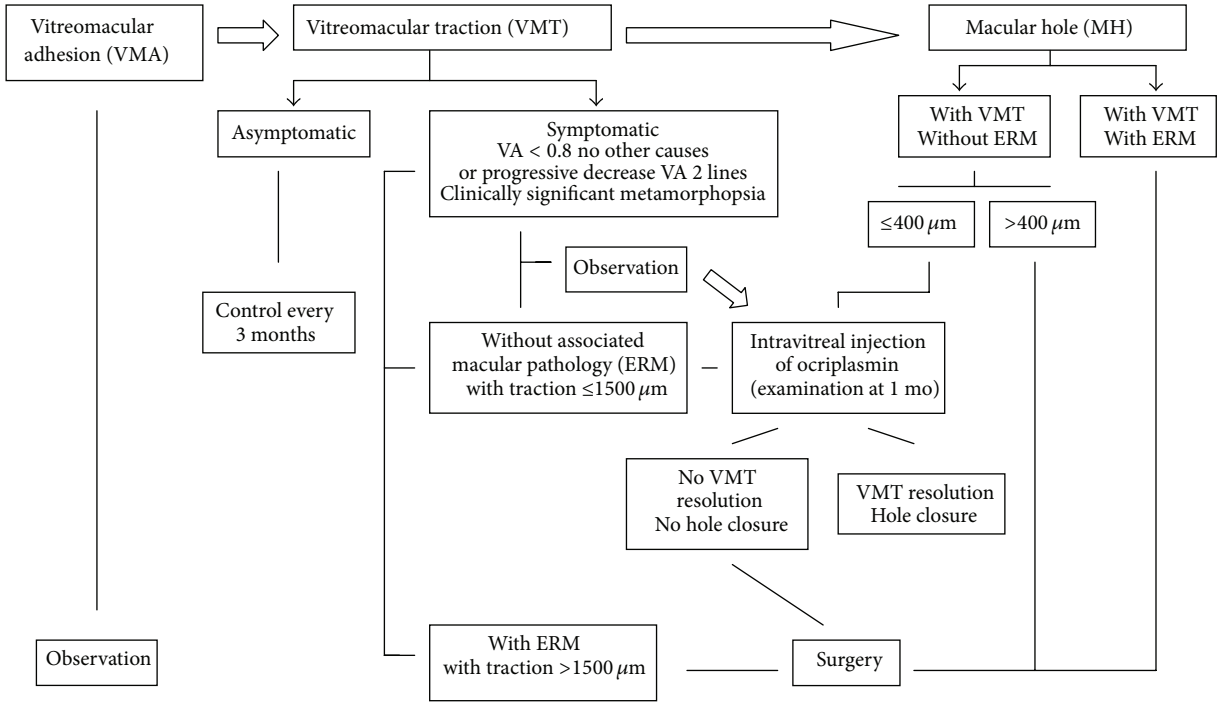


Figure 6: Treatment algorithm for VMA, VMT, and MH (VA: visual acuity, ERM: epiretinal membrane).

recommendation on the impact of enzymatic vitreolysis with ocriplasmin in the treatment of these conditions, and we should await for results of ongoing clinical trials on this topic [106].

Finally, in all cases, the final decision regarding treatment with enzymatic vitreolysis with ocriplasmin or vitrectomy should be consensuated with the patient. All cases in which the use of ocriplasmin is considered a first treatment option can be successfully treated by means of vitrectomy. Also, it may be possible that patients who initially are not ideal candidates for enzymatic vitreolysis may have their pathologic condition solved by treatment with ocriplasmin [20]. Favourable prognostic factors for the choice of vitreolysis have been identified including young age and phakic status, but difficulties to maintain postoperative face-down posture or the waiting lists for vitrectomy are variables that should also be considered.

5. Conclusions

Enzymatic vitreolysis based on the intravitreal injection of ocriplasmin is a treatment option with proven efficacy and adequate safety profile in selected patients with VMT and MH. In cases of VMT, treatment with ocriplasmin is indicated when traction is ≤1500 μm and in the absence of concurrent macular diseases (ERM). In the case of MH, the hole diameter should be ≤400, traction has to be present, and ERM should be absent. When resolution of the process after one month of the procedure is not achieved, vitrectomy with ILM peeling would be the surgical treatment of choice.

Conflict of Interests

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

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Research Article

Blockade of Vascular Endothelial Growth Factor Receptor 1 Prevents Inflammation and Vascular Leakage in Diabetic Retinopathy

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Diabetic retinopathy (DR) is a leading cause of blindness in working age adults. The objective of this study is to investigate the effects of vascular endothelial growth factor receptor 1 (VEGFR1) blockade on the complications of DR. Experimental models of diabetes were induced with streptozotocin (STZ) treatment or Insulin2 gene mutation (Akita) in mice. Protein expression and localization were examined by western blots (WB) and immunofluorescence (IF). mRNA expression was quantified by PCR array and real-time PCR. The activity of VEGFR1 signaling was blocked by a neutralizing antibody called MF1. Vascular leakage was evaluated by measuring the leakage of [^{3}H]-mannitol tracer into the retina and the IF staining of albumin. VEGFR1 blockade significantly inhibited diabetes-related vascular leakage, leukocytes-endothelial cell (EC) adhesion (or retinal leukostasis), expression of intercellular adhesion molecule- (ICAM-) 1 protein, abnormal localization and degeneration of the tight junction protein zonula occludens- (ZO-) 1, and the cell adhesion protein vascular endothelial (VE) cadherin. In addition, VEGFR1 blockade interfered with the gene expression of 10 new cytokines and chemokines: cxcl10, il10, ccl8, il1f6, cxcl15, ccl4, il13, ccl6, casp1, and ccr5. These results suggest that VEGFR1 mediates complications of DR and targeting this signaling pathway represents a potential therapeutic strategy for the prevention and treatment of DR.

1. Introduction

Diabetes mellitus (DM) is a widespread disorder with a prevalence of about 285 million in 2010 and predicated increase to 439 million by 2030 [1]. DR is one of the most common complications of DM and affects about 93 million people worldwide [2]. Clinically, DR is divided into two forms: nonproliferative DR (NPDR) and proliferative DR (PDR). Diabetic macular edema (DME) and retinal neovascularization are the two main causes of visual impairment and blindness in patients with DR [3]. Its pathological features include increased vascular permeability or breakdown of BRB, neovascularization (NV), capillary nonperfusion, endothelial cell damage, and apoptotic cell death of retinal neurons, endothelial cells, and pericytes. The early events, such as endothelial cell-leukocyte adhesion (or retinal leukostasis) and oxidative stress, contribute to these clinical and pathological characteristics in DR.

VEGFR1 has been reported to play various roles in the vascular development, angiogenesis, cell survival, and inflammation. First of all, as a VEGF-A trap or sink, VEGFR1 (mainly soluble VEGFR1 or FLT1), has been characterized as a negative regulator in both embryonic and postnatal vascular development [4, 5]. Secondly, VEGFR1 has been shown to be a positive mediator of pathological angiogenesis in the experimental models of some primary tumors and

wet age-related macular degeneration (AMD) [6]. Thirdly, VEGFR1 has been reported to promote cell survival under some stress conditions. For instance, in the oxygen-induced retinopathy (OIR) model, VEGFR1 activation by placental growth factor (PlGF) could prevent vessel obliteration or degeneration during the hyperoxia phase, thereby preventing the subsequent vessel proliferation during the hypoxia phase [7]. In addition, VEGFR1 signaling plays a role in regulating the chemotaxis of inflammatory cells [8–10].

The functions of VEGFR1 vary depending on the pathophysiological microenvironment, the type of ligand that binds (PlGF, VEGF-A, or VEGF-B), and the formation of VEGFR1-VEGFR2 heterodimers. Whether the VEGFR1 plays a role in the pathogenesis of DR remains unknown. In the present study, we address this question by blocking the VEGFR1 activity with an antibody called MF1. This VEGFR1-specific antibody has been previously reported by us and other investigators [8, 10, 11]. We found that VEGFR1 blockade prevented vascular leakage and retinal leukostasis, degeneration, and disorganization of the tight junction protein zonula occludens- (ZO-) 1 and the adhesion molecule vascular endothelial (VE) cadherin in DR.

2. Methods

2.1. Mouse Models of Diabetes. All animals were used in accordance with the approved protocols by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two mouse models of diabetes were used: one was streptozotocin- (STZ-) induced method and the other was an Akita diabetic mouse, both of which were described by our previous paper [12].

2.2. Administration of Anti-VEGFR1 Antibody. The monoclonal antibody MF1 was used to block VEGFR1 activity. 50 mg antibody per 1 kg mouse body mass was intraperitoneally (IP) injected three times per week as we performed previously [6]; rat IgG was used as the treatment control. This dose was used because it showed high efficacy in inhibiting pathological angiogenesis and infiltration of inflammatory cells in the mouse models of laser-induced CNV and oxygen-induced retinopathy (OIR) [6, 11]. In the present study, the preventative approach was implemented: the treatments started shortly after the onset of hyperglycemia and long before the occurrence of diabetic complications.

2.3. Western Blots (WB) and Quantification Analysis. Retinas were homogenized in an ice-cold lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and complete mini EDTA-free protease inhibitor] by sonication for 3–5 seconds, were incubated for 30 minutes, and were centrifuged at 14,000 g at 4°C for 10 minutes. Supernatant was collected and protein concentrations were measured by the bicinchoninic acid (BCA) method. Twenty-to-thirty micrograms of protein were electrophoresed on 4%–15% gradient SDS PAGE gels and then transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA) or 5% nonfat milk for 1 hr, protein blots were incubated with primary antibodies in the cold room overnight, followed by horseradish peroxidase- (HRP-) conjugated secondary antibodies (1 : 2,000–5,000) and developed with chemiluminescence reagents (Pierce Technology Co., Holmdel, NJ). The optical density (OD) of each protein band was determined with Image J software according to the user's instructions. The OD of VEGFR1 protein was normalized by dividing with that of β-actin of the same protein sample. The normalized OD ratio of the diabetic sample to the nondiabetic sample was designated as fold changes.

2.4. Immunofluorescence (IF) Staining and Quantification. 10 μm cryosections were blocked/permeabilized with PBS buffer containing 0.25% Triton X-100 and 10% goat serum for one hour and incubated at 37°C for two hours or overnight at 4°C with primary antibodies: rat anti-VEGFR1 (ImClone, Somerville, NJ), mouse anti-ICAM-1 (DSHB, Iowa city, IA), rabbit anti-albumin (Nordic, Capistrano Beach, CA), rat anti-CD31 (Novus Biologics, Littleton, CO), mouse anti-VE cadherin (DSHB), and rat anti-ZO-1 (DSHB). After a rinse was repeated 3 times with PBS buffer, specimens were incubated with appropriate secondary antibodies at 1000 dilution. For double-labeling IF staining of albumin and CD31/PECAM1, the cocktail of two primary antibodies from distinct species was applied, and the appropriate secondary antibodies conjugated with Alex fluo 594 or 488 were used to visualize the staining with fluorescence microscopy. DAPI was used for counter-staining. In the case of mouse primary antibodies, the anti-mouse secondary antibody was preincubated with 0.03 mg/ml normal mouse IgG (Invitrogen, Cat no: 10400C) to prevent its association with the endogenous mouse IgG, thus minimizing background staining.

For the quantitative comparison of IF images, the specimens and images were prepared to eliminate the errors caused by variations with particular care, as described previously [13]. Briefly, the eye samples of control and experimental groups (n = 4 each group) were cryopreserved in the same module with the superior quarter up. The cryosections including optical nerves were collected for immunostaining. The procedures were therefore parallel with the treatment and control groups. A Zeiss Axioplan2 fluorescent microscopy was used to acquire IF Images with Axion 4 software. The IF-stained specimens were used for quantification within 1 week. The fluorescence intensity and area of IF images were quantified by Image J software. The IF-positive areas were first identified as a region of interest (ROI) by running Image > Adjust > Threshold. The mean intensity and area of ROI were then determined by running Analyze/Analyze particles. The results were averaged from the 4 cryosections and then expressed as mean ± SD per section (10 μm).

2.5. PCR Array and Real-Time Quantitative (Q) PCR. Total RNA was prepared from the retinas of 5~6-month-old Akita diabetic male mice (with 4~5 months' diabetic duration) and the nondiabetic littermate male mice by using Trizol agents based on the manufacturer's manual. cDNA was synthesized with SuperScript III First-Strand Synthesis System

(Invitrogen). The mouse inflammatory cytokine PCR array (Cat no: 330231 PAMM-011A, SABiosciences), containing 84 important cytokine, chemokine, or receptor genes, was used to screen the novel genes whose expression was affected by diabetes. Real-time QPCR was used to quantify the gene expression alterations of cytokine and chemokine due to VEGFR1 blockade. Primers were designed as follows: the mRNA nucleotide sequences were obtained from the Unigene database by searching their gene symbols. The achieved sequences were aligned with the mouse genome by using an online Blat program to identify the locations of exons. The primers were designed with the primer3 software. To circumvent the contaminations from genomic DNA with intron sequences, the forward and reverse primers were located in the two or more distinct exons (see Supplementary Table 1 for sequence information available online at http://dx.doi.org/10.1155/2015/605946). Quantification of real-time PCR was performed as we previously described [6].

2.6. The Quantitative BRB Assay. The quantitative BRB assay was performed according to a previously described technique [12] with some modifications. Mice were sedated as above and given an IP injection of 1 μCi/gram body weight of [^{3}H]-mannitol. One hour after injection, the mice were sedated and retinas from the experimental and control eyes were rapidly removed. The posterior portion of the globe was firmly grasped with forceps and a razor blade was used to cut across the cornea and extrude the lens, vitreous, and retina. Retinas were dissected free from the lens, vitreous, and any RPE that was extruded and were placed within preweighed scintillation vials within 30 seconds of sacrifice. The thoracic cavity was opened and the left superior lobe of the lung was removed, blotted free of excess blood, and placed in another preweighed scintillation vial. A left dorsal incision was made and the retroperitoneal space was entered without entering the peritoneal cavity. The renal vessels were clamped with forceps and the left kidney was removed, cleaned of fat, blotted, and placed into a preweighed scintillation vial. Superficial liquid was allowed to evaporate over 20 min from the open vials. The vials containing the tissue were weighed and tissue weights were calculated and recorded. 1 ml of NCSII solubilizing solution was added to each vial and the vials were incubated overnight in a 50°C water bath. Solubilized tissue was brought to room temperature (RT) and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. The vials were brought to RT and 5 ml of Cytoscint ES and 30 μl of glacial acetic acid were added. The vials were stored for several hours in darkness at 4°C to eliminate chemiluminescence. Radioactivity was counted with a LS 6500 Liquid Scintillation Counter (Beckman, Brea, CA). The CPM/mg tissue was measured for the lung, kidney, and experimental and control retinas. Retina/lung and retina/kidney ratios were calculated and compared.

2.7. Retinal Leukostasis. Mice were first anesthetized with excess carbon dioxide, the descending aorta was clamped, and the right atrium was cut. The mice were perfused with 5 ml PBS to remove erythrocytes and nonadherent leukocytes, followed by perfusion of fluorescein-conjugated Con-A to label the adherent leukocytes. Another PBS perfusion was used to flush out unbound fluorescein. Retinal flat mounts were prepared to assess leukostasis. The eyes were harvested and fixed for more than 1 hour with phosphate-buffered formalin. The cornea and lens were removed and, under a stereomicroscope (Stemi 2000C; Carl Zeiss Meditec, Inc., Thornwood, NY), the entire retina was carefully dissected from the eye cup and rapidly cut from the edge to the equator in all four quadrants and was flat-mounted with the photoreceptors facing upward. Leukocytes adherent to the vessel walls were labeled with fluorescein, and leukocytes within the vessels of each retina were counted under an epifluorescence microscope (Axio-pan2; Carl Zeiss Meditec, Inc.) by an investigator masked to the nature of the specimen. The counting began at the optic disc. The vessel at the 12-o'clock position was first examined from the optical disk to the edge of vasculature, and the focus was adjusted as necessary to include all the arteries, veins, and capillaries in the field. This process was repeated in a clockwise direction for each vessel radiating from the optic disc, so the total number of adherent leukocytes in all of the vessels of the retina was counted.

2.8. Statistical Analysis. The Mann-Whitney test was used for the statistical analyses of comparisons between groups: diabetic retina versus nondiabetic retina and antibody treatment versus rat IgG treatment. $P < 0.05$ was designated as being statistically significant.

3. Results

3.1. Increased Expression of VEGFR1 Protein in Diabetic Mouse Retina. Increased expression of VEGFR1 has been previously shown in the retinal vasculatures of diabetic rat and human [14–16], but whether its expression is also increased in diabetic mice remains unknown. We therefore examined VEGFR1 protein expression in diabetic mouse retina. The WB results showed that VEGFR1 expression was increased in STZ-induced diabetic mice (3-month diabetes duration) compared with nondiabetic controls; and IF staining further showed its vascular localization (Figure 1). In Akita diabetic mice (both 6- and 8-month diabetes duration), a robust and specific protein band of approximately 180 KDa was detected by anti-VEGFR1 antibody, but no or weak protein band was detected in nondiabetic mice (Supplementary Figures 1(a) and 1(b)). Similarly, IF staining showed that there was no or barely detectable immunoreactivity for VEGFR1 in nondiabetic mice (Supplementary Figure 1(c)); whereas IF intensity was evidently elevated in diabetic mice with an apparent vascular localization (Supplementary Figure 1(d)). Quantification showed that VEGFR1 protein expression was significantly upregulated by diabetes (Supplementary Figures 1(e) and 1(f)).

3.2. VEGFR1 Blockade Inhibits Retinal Leukostasis in DR. Six-week-old STZ-induced diabetic mice (about 2-week diabetes duration) and nondiabetic mice of the same age were used for this analysis. Similar to our previous results [12], 2-week diabetes duration caused a significant increase of leukocytes in comparison with the nondiabetic mice (Figure 2(a)). We

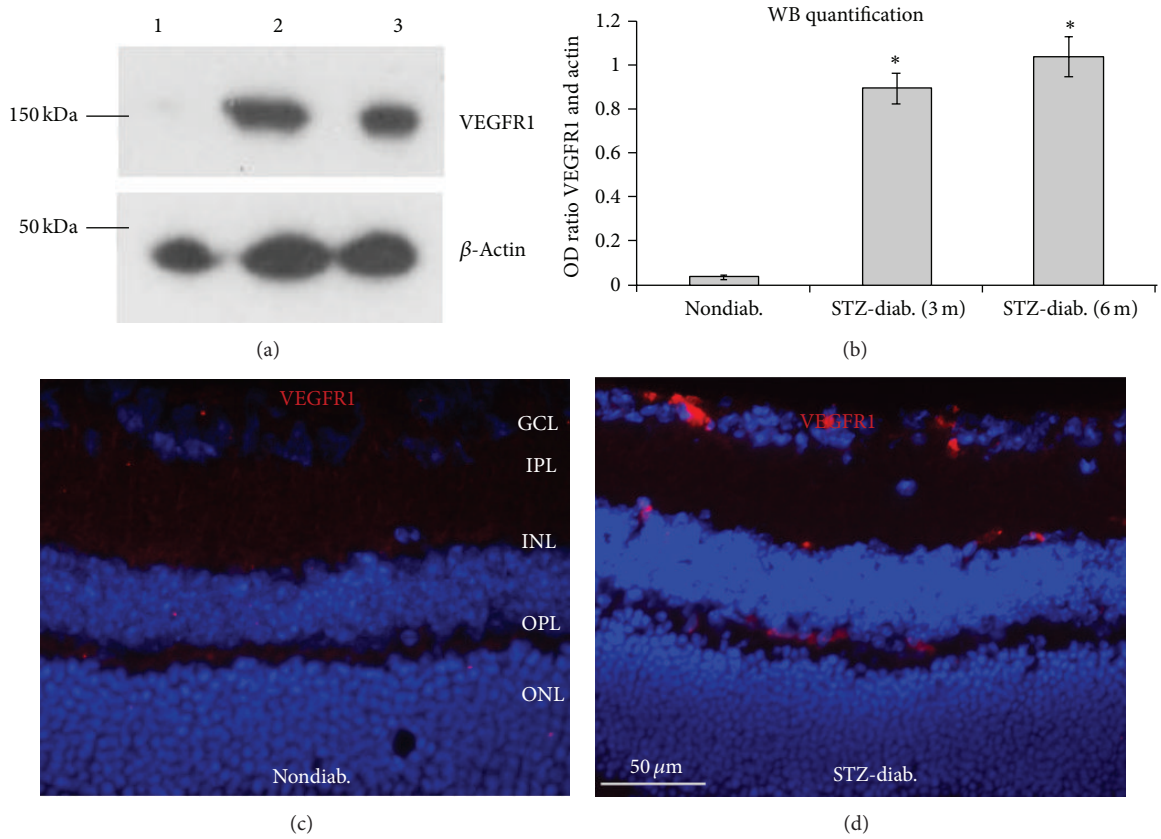


Figure 1: Increased expression of VEGFR1 protein in the retina of STZ-induced mice. The STZ-induced diabetic mice with 3 or 6 months of diabetes duration were used for this analysis. (a) Western blots (WB) results. β-Actin was used for loading control. (b) The WB quantification results ($n = 4$). $^*P < 0.05$ versus nondiabetic controls. ((c) and (d)) IF results showed that VEGFR1 protein localized in the blood vessels of the diabetic mouse retina (d) but was not detected in the nondiabetic control mouse retina (c). GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer plexiform layer. Scale bar: 50 μm.

observed that the leukocytes presented in capillary as well as arteries and veins in the diabetic retina (Figures 2(b)–2(d)). It was obvious that the cells in the capillary vessels transverse the whole lumen space, thus becoming an obstacle for blood flow. On some occasions, the cells aggregated at the root of branches from the larger vessels to the smaller capillaries (Figure 2(c)), preventing the blood from circulating into the capillaries.

3.3. VEGFR1 Blockade Inhibits ICAM-1 Upregulation in DR. ICAM-1 plays an important role in retinal leukostasis. We examined the effects of VEGFR1 blockade on ICMA-1 expression. In nondiabetic mice, ICAM-1 immunoreactivity was generally weak in the majority of blood vessels; very few blood vessels showed strong immunoreactivity (Figure 3(a)). ICAM-1 immunoreactivity was strong in all ICAM-1(+) blood vessels in diabetic mice (Figure 3(b)). ICAM-1 Immunoreactivity was weaker in the MF1-treated mice than in rat IgG-treated ones. The signal intensity of ICAM-1 immunoreactivity was faint in the majority of blood vessels but strong only in very few blood vessels (Figure 3(c)). MF1 treatment apparently resulted in a reduction of ICAM-1 immunostaining intensity compared with the mice treated with rat IgG in the vasculature. Quantification further showed the significant differences among the three treatment groups (Figure 3(d)).

3.4. VEGFR1 Blockade Inhibits Vascular Leakage in DR. Albumin staining was used to examine the sites of vascular leakage and the effects of VEGFR1 blockade. Double-labeling immunofluorescence staining showed that the albumin protein colocalized well with the endothelial cell marker CD31/PECAM-1 in nondiabetic mice, suggesting its localization in the blood vessels without leakage (Figures 4(a)–4(c)). By contrast, albumin immunoreactivity was not restrained to the CD31 (+) vessel areas in the diabetic mice treated with Rat IgG (Figures 4(d)–4(f)). In the diabetic mice treated with MF1, the staining results showed that albumin protein colocalized largely with CD31 (Figures 4(g)–4(i)), which was very similar to those from the nondiabetic mice. Quantification showed the intensity ratio of albumin and CD31 immunoreactivity was significantly different among the three groups (Figure 4(j)). In addition, the degree of vascular leakage was further measured by a quantitative BRB assay. The results showed that retina to lung leakage ratio (RLLR) and retina to renal leakage ratio (RRLR) were significantly reduced by VEGFR1 blockade (Figure 5).

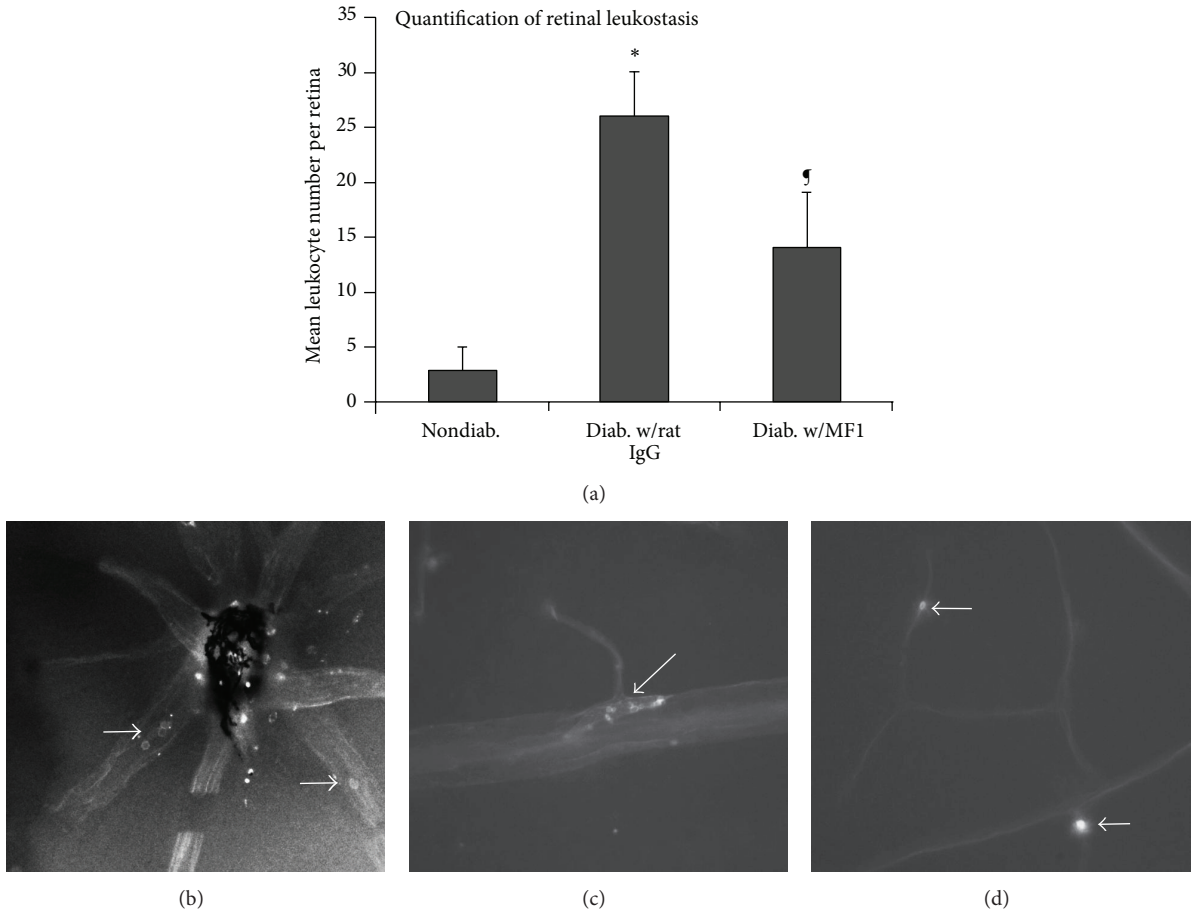


Figure 2: Inhibition of diabetes-caused retinal leukostasis by VEGFR1 blockade. The STZ-induced diabetic mice with two-week diabetes duration were used for this analysis. (a) The quantification showed that the number of leukocytes adhering to the retinal vasculature was significantly increased in the retinas of the diabetic mice compared with the nondiabetic mice but decreased due to VEGFR1 blockade. The total time of MF1 injection was 6 (3x/week). The results were expressed as the mean number ± SD ($n = 5$). $^*P < 0.05$ versus nondiabetic mice. ¶ versus diabetic mice treated with rat IgG. ((b)–(d)) Fluorescein-labeled leukocytes in the retinal vasculature were photographed and converted to black-and-white images, which showed the localization of leukocytes in larger arteries and veins ((b) and (c)) and smaller capillaries (d). Arrows indicated the leukocytes.

3.5. VEGFR1 Blockade Preserves BRB Integrity in Diabetic Retina. We investigated the effects of VEGFR1 blockade on the expression and localization of zonula occludens- (ZO-) 1 and vascular endothelial (VE) cadherin, which are the tight junction and adhesion proteins of BRB. VE cadherin immunoreactivity was very strong and showed a very sharp and blood vessel-like staining pattern in nondiabetic mice, whereas the staining looked foggy and cloudy in diabetic mice. In MF1, the staining showed "clear and vessel-like" patterns similar to those observed in the nondiabetic retinas (Figures 6(a)–6(c)). The quantification showed that the fluorescence intensity of VE cadherin was significantly lower in diabetic mice than nondiabetic mice and MF1 treatment significantly increased its expression compared with the rat IgG control treatment.

In nondiabetic mice, IF staining of ZO-1 showed a "ring and string" pattern, which may reflect that ZO-1 proteins are well localized in the walls of both large retina arteries and veins as well as in the small capillaries (Figure 7(a)); in diabetic mice, the staining looked like short rod and stick, indicating that ZO-1 proteins were displaced in the large vessels and were diminished in the capillaries (Figure 7(b)). In the antibody treated mice, the lumen structures were hardly detected. However, the capillary structures were visible in some ZO-1(+) vessels. Quantification showed that the mean size of the ZO-1(+) vessels was significantly different (Figure 7(d)).

3.6. VEGFR1 Blockade Influences Gene Expression of Cytokine/Chemokine. We investigated the influences of VEGFR1 blockade on the expression of 84 important cytokine and chemokine genes that were contained in an array plate. In total, the expression of 67 genes was found in the retina. The expression of 9 genes was detected only in the diabetic retinas but not in the nondiabetic retinas: cxcr2, cxcl9, itb, ccl8, il1f6, ccl4, cxcl10, cxcl11, and ccl20. Expressions of 11 genes were upregulated by diabetes: cxcl13, ccl5, il18, pf4, il13, abcf1, cxcr5, il10, c3, il16, and il15 (Figure 8(a)), and

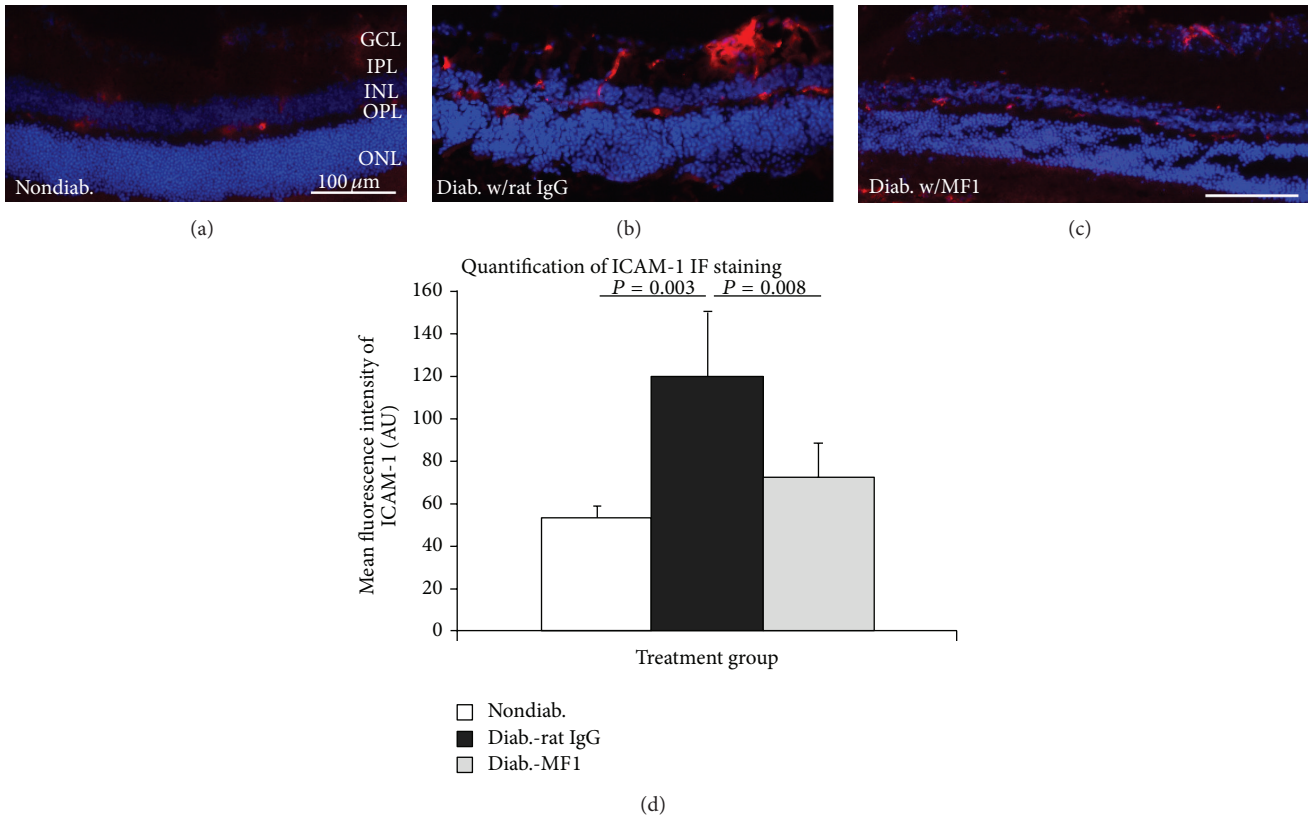


Figure 3: Inhibition of diabetes-caused upregulation of ICAM-1 by VEGFR1 blockade. ((a)–(c)) Immunofluorescence (IF) results from the nondiabetic mice (a) and the STZ-induced diabetic mice that were treated with rat IgG (b) and MF1 (c). (d) Quantification of IF images showed the statistically significant difference. The results were averaged from 4 mice ($n = 4$).

expressions of 31 genes were downregulated by diabetes: such as tnfrsf1a, casp1, ccr7, and cxcl10 (Figure 8(b)). Furthermore, the genes whose expressions were altered by diabetes were selected to investigate the effects by VEGFR1 blockade. Expressions of 10 genes were found to be altered by VEGFR1 blockade compared with rat IgG control: cxcl10, il10, ccl8, il1f6, cxcl15, ccl4, il13, ccl6, casp1, and ccr5 (Figures 8(c) and 8(d)).

4. Discussion

In this study, we first found that VEGFR1 protein expression was increased more than 10-fold in the retinas of diabetic mice compared with nondiabetic mice. This result is consistent with previous reports that showed upregulation of VEGFR1 expression in several other species, such as rat and human [14–16]. We then found that VEGFR1 blockade prevented vascular leakage and retinal leukostasis, degeneration, and disorganization of the tight junction protein ZO-1 and the adhesion molecule VE cadherin in DR. We started treating the mice shortly after the onset of hyperglycemia and long before the occurrence of any complications of DR. Therefore, the results of this study display the preventative effects of VEGFR1 blockade on vascular complications DR. In order to know whether VEGFR1 blockade can also be therapeutically effective or reverse the disease progression, it would be necessary to treat mice at a later stage when complications of diabetes have already taken place because DR is a chronic ischemic and inflammatory disorder and many diabetes-associated complications start appearing at relatively later stages. Finally, we found that VEGFR1 blockade inhibited gene expression of inflammatory marker ICAM-1 and other cytokine and chemokine genes, providing further evidence that VEGFR1 signaling is involved in the regulation of inflammation in DR.

Our findings suggest that targeting VEGFR1 may be a complementary strategy to anti-VEGF therapy, which is widely used to treat vascular disorders, including, age-related macular degeneration (AMD), DR, and DME. This likelihood is also supported from the outcomes from other studies. For instance, VEGFR1 signaling has been suggested to be associated with the diseased cells and tissues more than the normal ones, so inhibiting its activity likely results in less side effects than anti-VEGF therapy [17]. The potency of blocking VEGFR1 appears comparable to that of anti-VEGF antagonists in the experimental models of cancer and ocular angiogenesis [6, 18]. VEGFR1 has been shown to inhibit infiltration of inflammatory cells or orchestrate interplay of VEGF-A, PlGF, and VEGFR2 [11, 19]. In addition, another possible alternative strategy could target the upstream regulators of VEGF, such as prolyl hydroxylase (PHD) and hypoxia inducible factor (HIF) [20]. Because they regulate

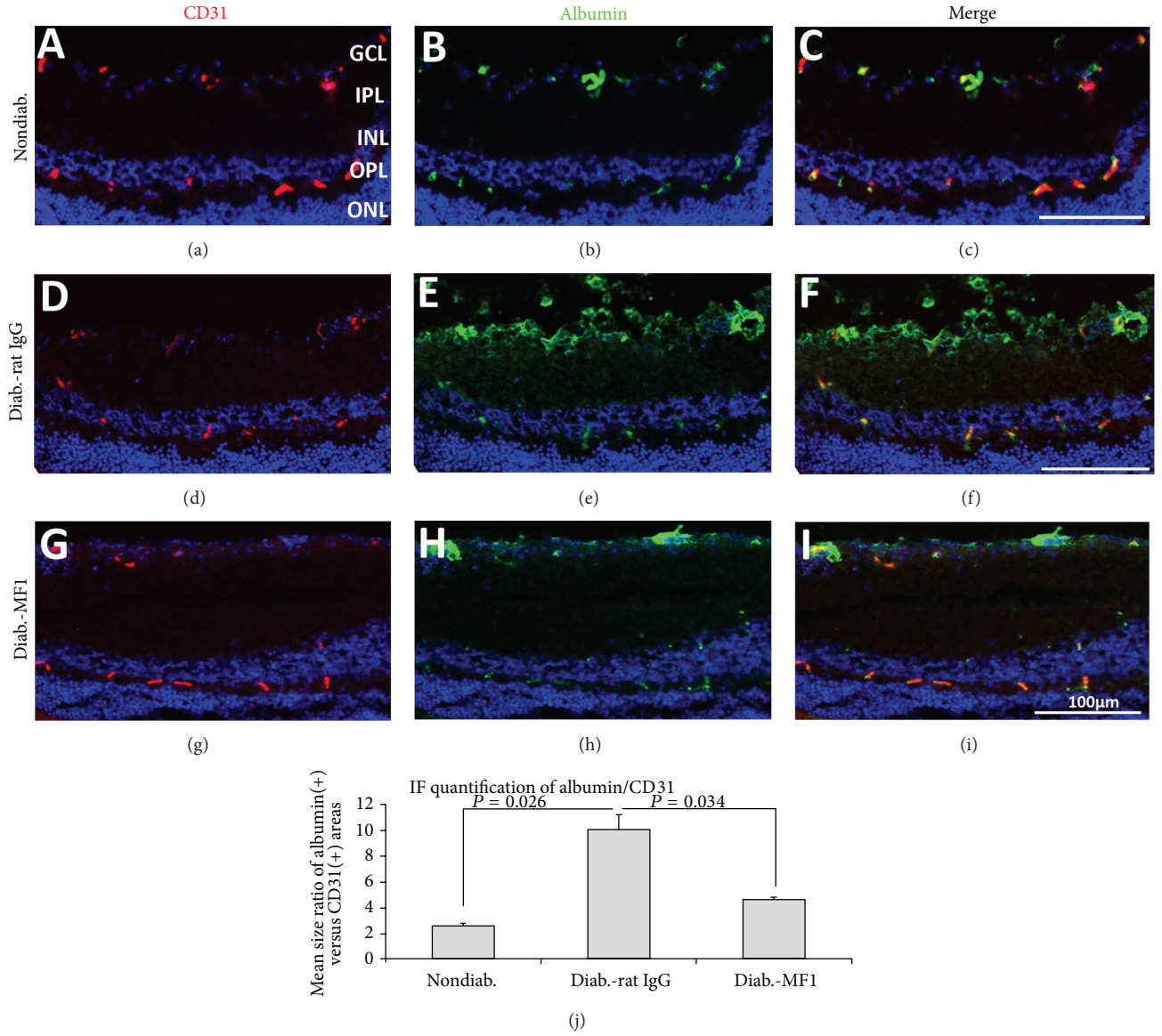


FIGURE 4: Inhibition of diabetes-caused albumin leakage by VEGFR1 blockade. ((a)–(i)) The representative immunofluorescence (IF) images were taken from the nondiabetic control mice ((a)–(c)) and the STZ-induced diabetic mice that were treated with rat IgG ((d)–(f)) or MF1 ((g)–(i)). The diabetic duration was 3 months (or 13 weeks) and the total time of rat IgG or MF1 treatment was 39 (3 times/week). Scale bar: 100 μm. (j) Quantification showed the size ratio of albumin (+) areas versus CD31 (+) blood vessel areas in the three groups. The results were averaged from 4 mice ($n = 4$).

a broad spectrum of gene expressions, inhibition of their activities may lead to a normalization of vasculature instead of impairment and degeneration.

DR is a complex disease involving a multiple of biochemical and molecular changes. In addition to VEGF and its receptor signaling, a number of other molecules and signaling pathways are implicated in the pathogenesis of DR, such as protein kinase C- (PKC-) β/-δ [21, 22], transforming growth factor- (TGF-) β [23], tumor necrosis factor- (TNF-) α [12], β-catenin (a key regulator of Wnt pathway) [24], cyclooxygenase- (COX-) 2 [25], NADPH oxidase (NOX)2 [26], arginase-1/-2 [27], aldose/aldehyde reductase (AR) [28], nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) cells [29], forkhead box protein O1 (FOXO1) [30], inducible nitric oxide synthase (iNOS) [31], and altered *O*-GlcNAc signaling [32]. Understanding these underlying molecular cascades helps develop therapeutic strategies and ushers in clinical trials or applications, especially targeted therapy. The most successful example is VEGF, which was originally discovered as a vascular permeability and mitogen factor [33, 34]. The two VEGF antagonists Lucentis (ranibizumab) and Eylea (aflibercept) have been approved by US Food and Drug Administration to treat the patients with DME. Despite the success, special attention must be paid to the safety and efficacy of treatments in patients, because they do not provide a "cure" and not all patients respond; there

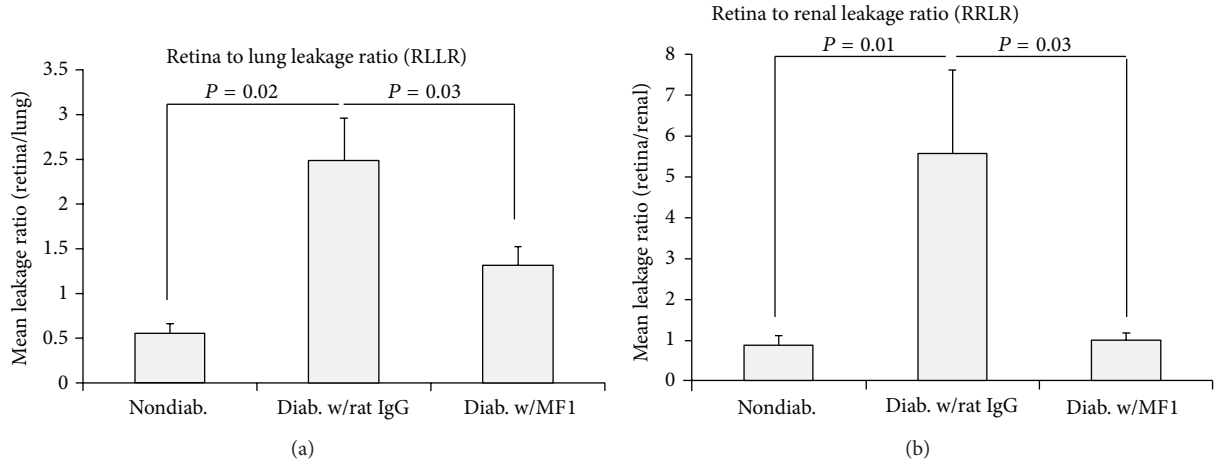


FIGURE 5: Inhibition of diabetes-caused BRB breakdown by VEGFR1 blockade. Nondiabetic mice and STZ-induced diabetic mice that were treated with rat IgG or MF1 were used for quantitative BRB assay. The diabetic duration was 3 months (or 13 weeks) and the total time of rat IgG or MF1 treatment was 39 (3 times/week). The CPM reading of retina, lung, or kidney was normalized by its own tissue mass (CPM/mg). RLLR (retina to lung leakage ratio) and RRLR (retina to renal leakage ratio). The results were averaged from 5 mice ($n = 5$). $^*P < 0.05$.

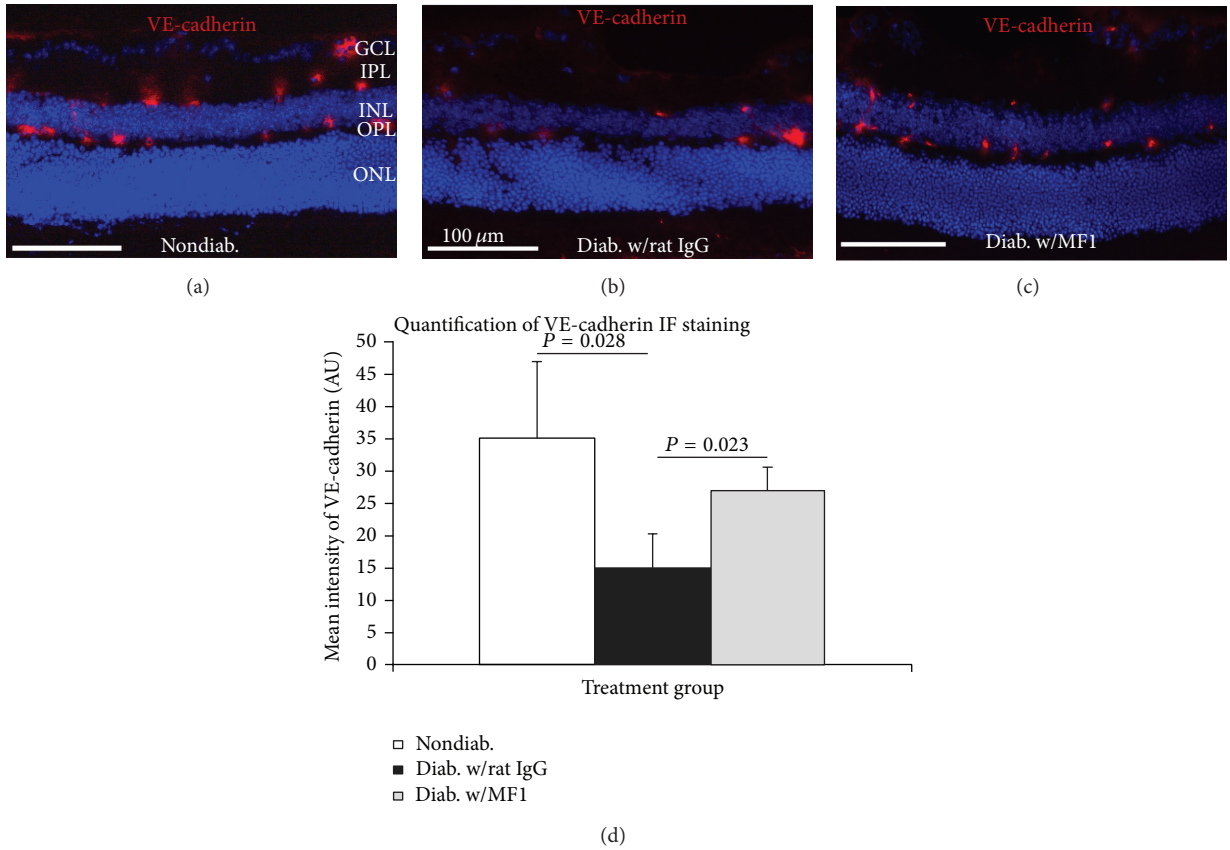


FIGURE 6: Prevention of diabetes-caused abnormal localization and degeneration of VE cadherin by VEGFR1 blockade. ((a)–(c)) The representative immunofluorescence (IF) images were taken from the nondiabetic C57BL6 mice (a) and the STZ-induced diabetic mice that were treated with rat IgG (b) or MF1 (c). The diabetic duration was 3 months (or 13 weeks) and the total time of rat IgG or MF1 treatment was 39. Note the pattern difference: the "foggy and cloudy" pattern for rat IgG treatment group and the "clear and vessel-like" pattern for the nondiabetic and MF1 treatment groups. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer plexiform layer. Scale bar: 100 μm. (d) Quantification showed the intensity difference in the three groups. The results were expressed as the mean ± SD ($n = 4$).

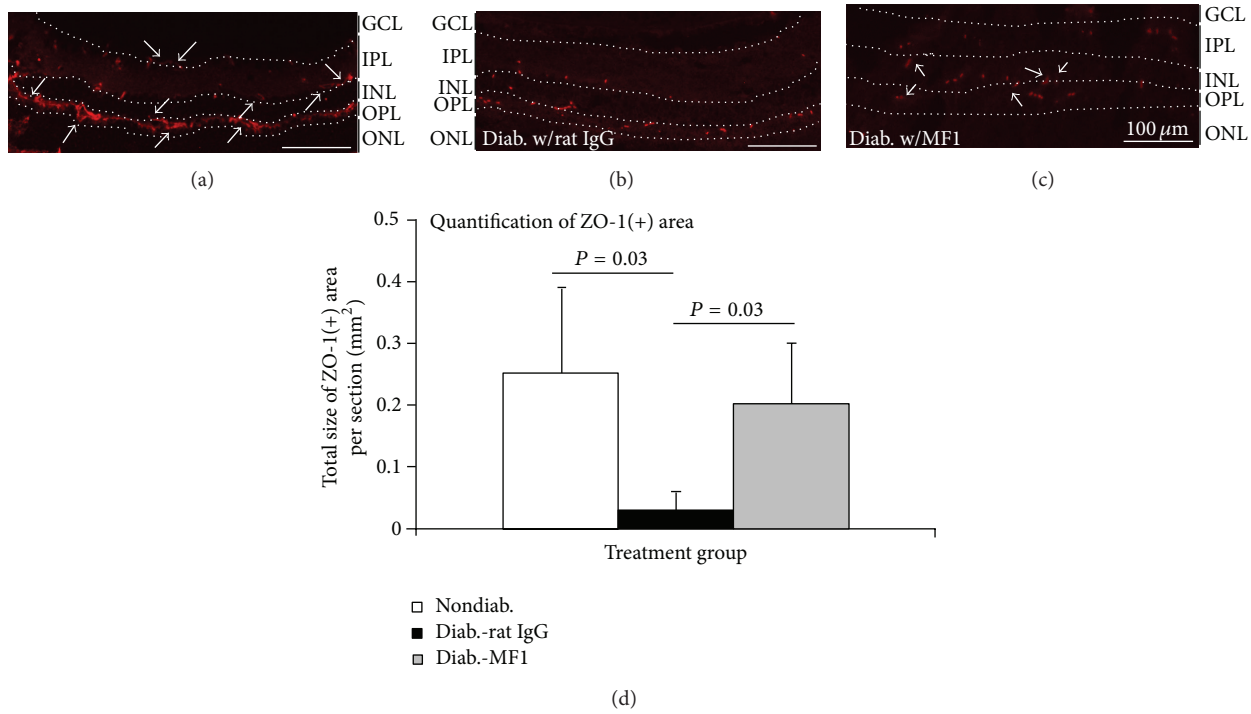
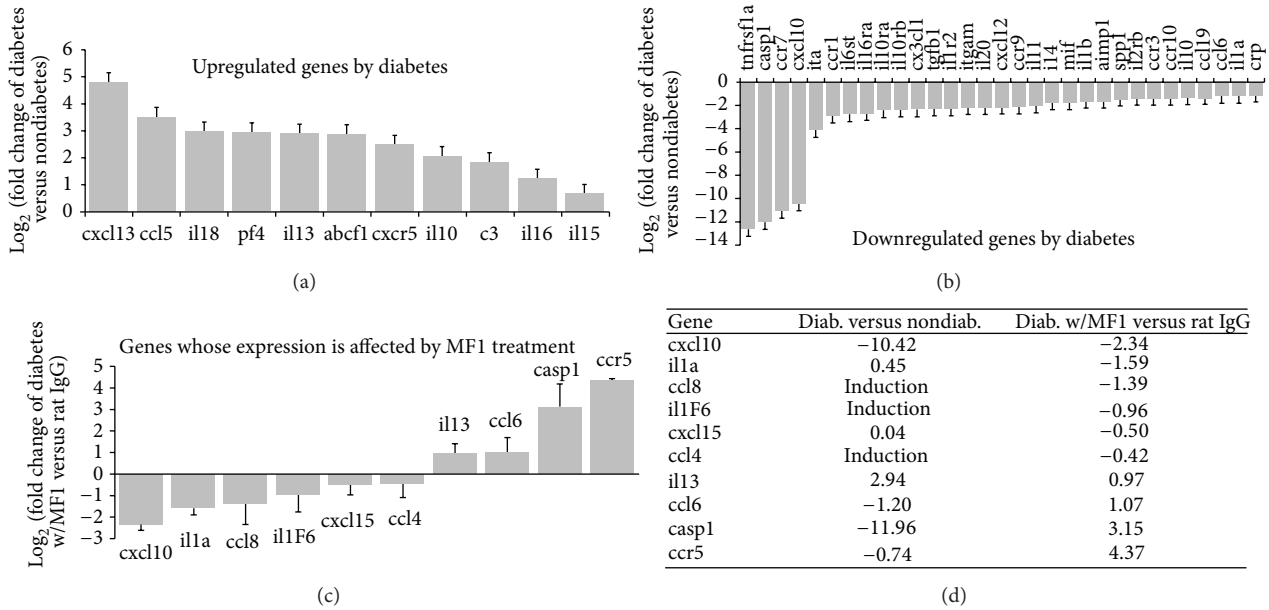


Figure 7: Prevention of diabetes-caused abnormal localization and degeneration of ZO-1 by VEGFR1 blockade. ((a)–(c)) The representative immunofluorescence (IF) images were taken from the nondiabetic mice (a) and the STZ-induced diabetic mice that were treated with rat IgG (b) or MF1 (c). The diabetic duration was 3 months (or 13 weeks) and the total time of rat IgG or MF1 treatment was 39 (3 times/week). The dash lines separate the retinal layers. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer plexiform layer. Scale bar: 100 μm. (d) Quantification showed the difference of mean size of ZO-1(+) areas in the three groups. The results were expressed as mean ± SD ($n = 4$).



Gene	Diab. versus nondiab.	Diab. w/MF1 versus rat IgG
cxcl10	−10.42	−2.34
il1a	0.45	−1.59
ccl8	Induction	−1.39
il1F6	Induction	−0.96
cxcl15	0.04	−0.50
ccl4	Induction	−0.42
il13	2.94	0.97
ccl6	−1.20	1.07
casp1	−11.96	3.15
ccr5	−0.74	4.37

(d)

Figure 8: Influence of diabetes and VEGFR1 blockade on gene expression of cytokines and chemokines. The differentially expressed genes between diabetic and nondiabetic retina were identified by cytokine and chemokine PCR arrays $n = 3$ each group. The STZ-induced diabetic mice (3 months diabetes duration) and nondiabetic mice of the same age were used for this experiment. (a) Upregulated genes by diabetes. (b) Downregulated genes by diabetes. (c) Genes whose expression was affected by MF1 treatment were quantified by real-time PCR ($n = 4$ each group). (d) The table showed the fold changes of gene expression between diabetic and nondiabetic mice and between MF1 treatment and rat IgG treatment. The results were the mean value of $\log_2$ (fold changes) ± SD ($n = 4$). Induction means that the gene was detected only in the diabetic mouse retina but not in the nondiabetic mouse retina.

are potential side effects since VEGF is a survival factor for choriocapillary, retinal neuron, and RPE [35–37]. Therefore, Search for more optimal therapeutic strategies is necessary for the improvement of anti-VEGF therapy.

In summary, the results from this study suggest that VEGFR1 is involved in the pathogenesis of DR and a potential target for this disease. The development of VEGFR1 inhibitors is clinically relevant because they could be potentially used for alternative and/or combinatory treatments to anti-VEGF therapy.

Conflict of Interests

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

Authors' Contribution

Jianbo He and Hong Wang contributed equally to this work.

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Review Article

Gene Therapy with Endogenous Inhibitors of Angiogenesis for Neovascular Age-Related Macular Degeneration: Beyond Anti-VEGF Therapy

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Age-related macular degeneration (AMD) is the leading cause of substantial and irreversible vision loss amongst elderly populations in industrialized countries. The advanced neovascular (or "wet") form of the disease is responsible for severe and aggressive loss of central vision. Current treatments aim to seal off leaky blood vessels via laser therapy or to suppress vessel leakage and neovascular growth through intraocular injections of antibodies that target vascular endothelial growth factor (VEGF). However, the long-term success of anti-VEGF therapy can be hampered by limitations such as low or variable efficacy, high frequency of administration (usually monthly), potentially serious side effects, and, most importantly, loss of efficacy with prolonged treatment. Gene transfer of endogenous antiangiogenic proteins is an alternative approach that has the potential to provide long-term suppression of neovascularization and/or excessive vascular leakage in the eye. Preclinical studies of gene transfer in a large animal model have provided impressive preliminary results with a number of transgenes. In addition, a clinical trial in patients suffering from advanced neovascular AMD has provided proof-of-concept for successful gene transfer. In this mini review, we summarize current theories pertaining to the application of gene therapy for neovascular AMD and the potential benefits when used in conjunction with endogenous antiangiogenic proteins.

1. Introduction

Neovascular AMD is the most common cause of severe vision loss in patients over the age of 60 [1, 2]. End stage complications of dry and wet forms of AMD are geographic atrophy or choroidal neovascularization (CNV). Whilst both can lead to vision loss, the wet form is often the more deleterious of the two. CNV originates from the choriocapillaris, with new vessels penetrating through Bruch's membrane and growing into the subretinal pigment epithelium (RPE) and/or subretinal space. Newly formed vessels typically lack normal structural integrity, as evidenced by incomplete basement membrane and/or pericyte content, making them susceptible to leakage and hemorrhage [3]. Such leakage can cause retinal edema resulting in visual distortion and marked diminution of vision when the macula is involved. The recent availability of anti-VEGF monoclonal antibodies has revolutionized the treatment of neovascular AMD by preserving and even restoring vision in patients [4–6]. However, the systemic safety of repeated injections of anti-VEGF agents has raised concern, particularly with regards to reports of increased risk of hemorrhagic stroke [7, 8]. In addition, the loss of efficacy over time has brought into question the long-term benefits of anti-VEGF therapy [9].

The rapid advancement of gene therapy has placed this approach on the doorstep of clinical use in ophthalmology. Given that the eye is a particularly favourable organ for drug delivery, ocular use is likely to be among the most

successful applications of this technique [10–13]. Positive results from a recent clinical trial and animal studies [14–18] suggest that gene transfer deserves more intensive study as a means to achieve local, sustained control of intraocular neovascularization (and possibly excessive vascular permeability) [19]. Indeed, gene-based approaches that can produce safe and long-term expression of one or more endogenous angiogenic inhibitors [20] would be a significant advance in the treatment of neovascular disease.

Gene transfer of endogenous angiogenic inhibitors such as pigment epithelium-derived factor (PEDF), endostatin, and angiostatin has provided beneficial effects in animal models [16] and in a Phase I clinical trial [21]. Other promising candidate transgene products for management of neovascular AMD include vasostatin [22], tissue inhibitor of metalloproteinases-3 (TIMP3) [17], plasminogen kringle 5 (K5) [23], and thrombospondin-1 [24]. This review seeks to briefly summarize current application of gene-based treatments for neovascular AMD and potential alternative treatments involving endogenous angiogenic inhibitors.

2. Pathogenesis and Current Treatment of Neovascular AMD

2.1. Pathogenesis of Neovascular AMD. The retina is metabolically unique in its specialisation for the capture of light and its transduction into an electrical signal. To support this activity there are extremely high energy needs, particularly for effective phototransduction and signal transmission as well as turnover of cellular membranes and phototransduction proteins. Not surprisingly, the retina is the most metabolically demanding of all the body's tissues [25]. The majority of the energy needed in the eye is required for neurotransmission and the maintenance of ionic gradients across the cell membrane. The remaining energy sustains vegetative function. In addition, much of the carbon substrates taken up as glucose into the eye are required for amino acid synthesis to support the turnover of photoreceptor outer segment membrane and membrane bound proteins. The retina also has a specialization, known as the macular, where a high density of cone photoreceptors allows for high spatial acuity. This specialization and the large metabolic burden make the retina particularly susceptible to metabolic insult and diseases that impact upon metabolic processes, such as AMD.

While many potential etiologies and pathological processes have been linked to AMD, our understanding of its development remains incomplete. In addition to aging as the major risk factor for AMD, other risk factors such as smoking, obesity, nutrition, and sunlight exposure have been strongly linked to AMD [26]. More recently, studies of the genetic basis of AMD have revealed variations in genes involved in lipid metabolism, inflammation, and oxidative stress can account for a substantial amount of AMD risk [26, 27].

Early AMD is characterised by the presence of extracellular debris beneath the retina known as drusen [28]. Early AMD can progress to advanced AMD, which has two types that include geographic atrophy AMD and neovascular AMD. Geographic atrophy (GA), or "dry" AMD, is characterized by regional loss of RPE and photoreceptors. Neovascular, or "wet" AMD, is characterized by choroidal neovascularization (CNV), which describes the growth of choroidal blood vessels into retina [1].

Pathologic changes that take place within the choriocapillaris and RPE following stress are believed to give rise to neovascular AMD. Angiogenesis, originating from the choriocapillaris, penetrates through Bruch's membrane and grows inward disrupting the overlying RPE and photoreceptors. These new vessels lack the structural integrity of established vasculature and exhibit incomplete basement membrane and limited pericytes. This gives rise to leakage of fluid and blood product into a region of the eye which is critical for fine vision and if left untreated, focal retinal detachment and loss of vision will ultimately ensue.

Whilst the damage caused by neovascular AMD arises from changes in the choriocapillaris, the key initiating factor is dysfunction of the RPE. The RPE has several specialized functions. That are central to the health of the retina including the secretion of vasoactive factors [29], phagocytosis of photoreceptor outer membranes [30], spatial buffering of ions [31], and epithelial transport to both the choriocapillaris [32] and the subretinal space [33]. A breakdown of the interplay between the RPE and the immunovascular system is thought to be the driving factor for CNV development [34].

The RPE vascular response is triggered by excess secretion of VEGF into the choroidal space [35]. This proangiogenic factor binds to receptors on endothelial cells [36] to initiate the process of CNV. Specifically, there are three types of VEGF receptors present on endothelial cells: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). Binding of VEGF to KDR/Flk-1 plays a key role in angiogenesis; Flt-1 functions as a decoy receptor, and Flt-4 is observed mostly in lymphatic vessels [37]. Several vasculogenic cytokines are also secreted by the RPE and contribute to the development of new vessels [38]. It is important to note that the RPE may not be the only source of proangiogenic factors.

One of the major pathways resulting in VEGF secretion from the RPE is in response to complement factors. The complement system, a component of the innate immune system, is a series of proteins that interact with one another to opsonize pathogens and mount an inflammatory response against infection. In recent years, numerous studies have found associations between sequence variants of complement pathway-associated genes and AMD [34, 39]. Complement has been found to be a constituent of drusen [40] and the presence of these proteins has the ability to induce excess VEGF production from the RPE [41] which works to disrupt epithelial tight junctions [42]. The deposition of complement in the retina is thought to occur secondary to oxidative stress, which is the oxidation of cellular macromolecules. Oxidative stress has been shown to reduce factors that inhibit complement deposition rendering cells susceptible to complement-mediated injury [42].

The immune system plays a role in the development and regulation of CNV and it appears to do so in a synergistic fashion in conjunction with the complement system. Complement factors C3a and C5a have been shown to be responsible for the recruitment of leukocytes to the choroid

[41]. Macrophages are also upregulated and are a key feature of CNV lesions [43]. However, there is conflicting evidence as to whether their migration plays a protective role [44] or represents an exacerbation of disease [45]. Microglia may also play a role in the pathogenesis of CNV. In animal models, the accumulation of these immune cells in the subretinal space appears to amplify the effects of laser-induced CNV [46]. However, in human donor CNV specimens, a change in morphology of microglia is observed but with no increase in number [47]. There may also be a role for nonimmune cells in CNV development, as some one-third of all infiltrating cells in CNV are yet to be classified [48]. This underlines the need for further research into the role of the immune system in the pathogenesis of CNV.

2.2. Current Treatments for Neovascular AMD. Treatment for neovascular AMD has been revolutionised by the availability of intravitreal anti-VEGF agents. Such agents bind VEGF thereby preventing Flt-1 and KDR/Flk-1 signalling and inhibiting the neovascular response. In the treatment of classic CNV, Anti-VEGF agents have been shown to be superior to previous treatment modalities such as verteporfin photodynamic therapy [49, 50]. The most widely used drugs in the treatment of neovascular AMD are ranibizumab (a humanised antibody) and bevacizumab (an antibody fragment), and both bind and remove all bioavailable VEGF-A isoforms. Whilst ranibizumab has approval for ophthalmic use, bevacizumab is often administered "off-label" as a cost-effective alternative. These antibodies have a high affinity for VEGF-A and neutralise it, thus reducing receptor activation and suppressing endothelial cell proliferation and migration [51–53]. When compared, ranibizumab and bevacizumab show similar efficacy in inhibiting endothelial cell growth *in vitro* [54], although another study found ranibizumab was 11-fold more potent than bevacizumab at inhibiting endothelial cell proliferation [55].

Phase III studies (ANCHOR [56, 57] and MARINA [58]) have concluded that monthly administrations of ranibizumab 0.5 mg successfully inhibited the growth of CNV lesions. According to the Comparison of AMD Treatments (CATT) trial [4, 59], monthly injections of ranibizumab 0.5 mg prevented the loss of 15 letters in 94.4% of study participants over a 12-month period. The mean increase in BCVA was 8.5 early treatment diabetic retinopathy study (EDTRS) letters. For bevacizumab 1.25 mg administered via the same protocol, BCVA was stabilized in 94.0% of treated individuals with a mean improvement of 8.0 EDTRS letters.

More recently, aflibercept, a soluble decoy receptor protein with the capacity to neutralize all VEGF-A isoforms, was developed. Results of the VIEW 1 and 2 trials showed that the recommended aflibercept 2 mg treatment protocol (bimonthly injections after 3 monthly injections) was not inferior to ranibizumab 0.5 mg (monthly injections) after 12 months [60]. Further studies assessing the vision improvements and cost benefits of aflibercept over ranibizumab are required.

Anti-VEGF injections may be the standard mode of treatment for choroidal neovascularisation in AMD, but practitioners and patients must bear in mind that certain complications can arise from its administration. Results of the VEGF Inhibition Study in Ocular Neovascularization (VISION) clinical trial show that with intravitreal injection the incidence of endophthalmitis and retinal detachment was 0.16% and 0.08%, respectively [61]. For ranibizumab, the Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration (ANCHOR) and Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA) study groups report presumed endophthalmitis in 1.0–1.4% of patients and serious uveitis in 0.7–1.3% [56, 58]. With all intravitreal injections there is also the possibility of damage to the crystalline lens during the procedure. The CATT study reported that the proportion of patients suffering from serious systemic adverse events was 24.1% in those treated with bevacizumab and 19.0% for ranibizumab [4]. Surprisingly, one study reports an 11% increase in all-cause mortality and a 57% increase in hemorrhagic stroke with intravitreal bevacizumab [8]. In contrast, a retrospective cohort study found no evidence for increased risks of mortality or stroke [7].

The Study of Ranibizumab in Patients with Subfoveal Choroidal Neovascularization Secondary to Age-Related Macular Degeneration (SUSTAIN) study assessed the efficacy of intravitreal ranibizumab for subfoveal choroidal neovascularisation secondary to AMD [62]. Patients were treated with 0.3 mg ranibizumab on a monthly basis for the first 3 months and then were treated on an "as needed" basis thereafter. Whilst 53% of patients respond well to the treatment and maintained their visual improvement over 12 months, 21% exhibited an initial increase in visual acuity for the first 3 months, followed by a steady decrease back to pretreatment levels. A decline in visual acuity with no response to therapy was observed in 26% of patients. At present, when a patient commences treatment with ranibizumab, there is no means to predict which group they may fall into and we do not understand what determines responder status.

These data show that whilst there is promise for an improvement in vision with intravitreal anti-VEGF agents, there are also shortcomings in terms of variable response to therapy as well as loss of efficacy in a subgroup of patients. In addition there are ocular as well as systemic secondary complications associated with the repeated intravitreal administration. Given the excessive costs to the healthcare system and burdens on the patient that have been eluded to earlier, there is a pressing need to look for new treatment modalities that might minimise complications, decrease frequency of administration, and decrease cost.

3. Gene Therapy and the Eye

In recent times, experimental work in gene therapy has gained momentum with many successes in treating both anterior and posterior eye disease. The basic premise of gene therapy involves implanting genetic material into host tissue in order to correct a dysfunctional gene or code for

a therapeutic protein. Whilst gene therapy research typically targets monogenic degenerative diseases, there may be a role for gene therapy in multifactorial degenerative diseases such as diabetic retinopathy and age-related macular degeneration [63, 64].

There are numerous advantages of the eye as a target for gene therapy in comparison to other organs. Firstly, given that localised treatment can be performed instead of intravenous delivery, systemic absorption of gene vectors can be minimized. Once infected, the immune privileged state of the eye limits the provocation of unwanted systemic immune responses [65]. Furthermore, given that the eye consists of a comparatively small volume, minimal amounts of vector may be sufficient to achieve therapeutic levels of transgenes. Another advantage is the anatomy of the eye, which exhibits a high level of compartmentalization making specific cell populations easy to target. Finally, the transparent nature of the optical media permits ease of assessment by various techniques such as electroretinography, optical coherence tomography, and fundus fluorescein angiography.

A number of vectors are available for use in gene therapy; however, recombinant adenoassociated viruses (AAV) have shown great promise owing to their proven safety and exceptional expression kinetics. Belonging to the family Parvoviridae, these small, nonenveloped viruses comprise a linear single-stranded DNA genome. In the context of treating posterior eye diseases such as AMD, AAV vectors exhibit sustained transduction of the RPE, photoreceptors, and ganglion cells [66] with expression lasting several years [67]. Latent infection of AAV is set up due to integration of the virus into a specific locus on human chromosome 19 [68]. This implies that a single administration can offer longer-lasting treatment thereby reducing the need for multiple injections of anti-VEGF agents. What is more, AAV vectors do not induce inflammation or cytotoxicity [69] and studies in humans show negligible adverse effects [11].

Targeting specific cellular populations can be achieved with the advent of hybrid AAV vectors. These involve packaging the AAV plasmid of a particular serotype into the capsid of AAV from another serotype. For example, rAAV2/4 indicates a plasmid of serotype 2 has been encapsulated by that of serotype 4. Whereas rAAV2/4 produces gene expression limited to the RPE [67], rAAV2/7 and rAAV2/8 show promising transduction of photoreceptor cells [70]. Varying the plasmid/capsid serotype also has an effect on expression characteristics. In situations where rapid onset gene transfer is required, rAAV2/5 and 5/5 can produce expression in 3-4 days. If delayed onset is preferable, rAAV2/2 displays gradual levels of transduction efficiency until stable levels are reached in 2–4 months [71]. The repertoire of AAV vectors available can accommodate a wide range of tissue tropisms and expression profiles.

Lentiviral vectors are capable of long-term gene therapy in the eye and do so by integrating into the host genome. Such vectors are best at transducing nondividing cell populations such as the corneal endothelium, trabecular meshwork [72], and RPE [73]. The risk of viral replication via insertional mutagenesis is minimized through the use of highly deleted vectors [74] and self-inactivating vectors [75]. Examples of lentiviral vectors include human immunodeficiency virus-1 (HIV-1) and feline immunodeficiency virus (FIV).

Adenoviral vectors are nonintegrating and have the ability to transduce both dividing and nondividing cells. Gene expression is short-lived, however, due to elicitation of cytotoxic T lymphocyte-mediated immune responses [76]. A variety of nonviral vectors also exist such as DNA nanoparticles [77] and the φC31 integrase system [78] and avoid the safety concerns associated with viral systems.

Figure 1 shows a schematic diagram of gene therapy. Genetic material is incorporated into the DNA of the AAV vector. It is then administered to the eye via a designated route which may be topical, subconjunctival, intracameral, intravitreal, or subretinal. The AAV plasmid/capsid combination is specifically selected to target the cellular population of interest. Once at the target cell, the vector attaches itself to membrane-bound receptors and becomes internalized via the formation of a vesicle. When it reaches the cell nucleus, the vesicle dissolves allowing the virus to deliver the genetic material for gene production.

Numerous clinical trials of gene therapy for retinal disease have been performed. The autosomal recessive disorder Leber's congenital amaurosis (LCA) is in Phase III trials with promising results. Improvements to dark-adapted function and pupillary light reflexes were noted. Most importantly, no significant changes were observed in visual acuity, visual field, or electroretinogram response after exposure to the rAAV2 vector [11].

4. Targeting VEGF via Gene Therapy

Whilst the underlying mechanisms leading to the development of CNV are not fully understood, it is clear that inhibition of VEGF and its receptor is quite effective at arresting choroidal neovascularisation. The next generation of treatment for neovascular AMD must demonstrate wider and longer-term efficacy and reduce the need for frequent administrations, hence reducing costs. It is also imperative that adverse reactions to the treatment are minimized. Whilst still in its experimental and early clinical trial stages, gene therapy appears to possess all of the characteristics necessary to improve upon the current intravitreal anti-VEGF treatment modality.

Animal studies have shown that VEGF over expression can be arrested using gene therapy. Whilst intravitreal gene transfer of antiangiogenic agents has proved to be successful in suppressing experimental CNV [79], the test subject remains at risk of adverse reactions that may arise from invasive intravitreal injections. To overcome this issue, topical administration of angiogenic inhibitors has been trialled and have shown some success in reducing CNV lesions induced by laser rupture of Bruch's membrane [80]. There was, however, the need for a high rate of administration of three times a day, which raises concerns of compliance and systemic absorption via the nasal mucosa. Subconjunctival gene transfer might provide a more localised but less invasive delivery route compared with intravitreal injections and at

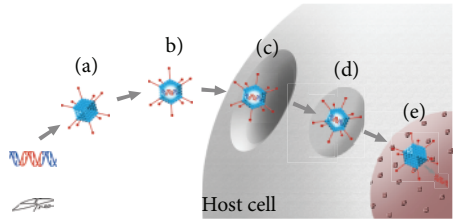

Figure 1: Schematic diagram of gene therapy with AAV. (a) Genetic material is biochemically engineered into the DNA of an AAV vector. (b) AAV is injected into the host. (c) Vesicle formation. (d) Internalization of AAV. (e) Breakdown of vesicle and delivery of genetic material to cell nucleus for protein production.

the same time would negate the need for frequent eye drops and avoid mucosal absorption.

Although anti-VEGF gene therapy provides a way to avoid the limitations of conventional therapy by intravitreal anti-VEGF agents, the issue of systemic safety from long-term neutralization of VEGF remains a concern. This is of particular importance given reports of increased risk of hemorrhagic stroke and RPE atrophy. Therefore, in addition to the development of new delivery routes for gene-based delivery of anti-VEGF agents, there is an intensive search for alternative antiangiogenic agents. Advances in this area are reviewed in the following section.

5. Potential Endogenous Inhibitors of Angiogenesis for Gene Therapy

Angiogenesis is dynamically regulated by the interplay of proangiogenic and antiangiogenic factors. Physiologically, the balance is skewed towards angiogenic inhibitors so that unwanted angiogenesis does not occur [81]. However, this state of homeostasis is disturbed in pathological conditions like neovascular AMD where angiogenic factors counterbalance endogenous inhibitors leading to the aberrant growth of leaky blood vessels. Expression of endogenous inhibitors including PEDF [82] and endostatin [83] in RPE and Bruch's membrane has been found to be reduced in choroid samples from donors affected by AMD. Further immunohistochemical characterisation reveals a decrease in other endogenous inhibitors such as thrombospondin-1 in the RPE, Bruch's membrane, and choriocapillaris [84] where AMD pathology occurs. This suggests that an accumulation of endogenous inhibitors in RPE—Bruch's membrane—choriocapillaris complex could act as a protective barrier for stopping the intrusion of new blood vessels [84]. Apart from suppressing angiogenesis, these inhibitors possess other useful biological functions that make them appealing for gene therapy (Table 1).

5.1. Pigment Epithelium-Derived Factor (PEDF). PEDF belongs to the serine protease inhibitor family and was first isolated from fetal human RPE cells [85]. It is extensively expressed throughout various layers of the human eye including the ciliary epithelium, inner and outer retina, and cornea [85]. Its expression is found to be altered in eyes affected by AMD, specifically in regions where AMD pathology is actively occurring [82]. PEDF is advantageous as a potential target over other endogenous inhibitors due to its neurotrophic and neuroprotective properties. In addition to its antiangiogenic effect on endothelial cells, PEDF has been shown to promote the survival of neuronal cells, preserve their integrity, and protect them from apoptosis [85]. Gene transfer using adenovirus based vectors in mice can successfully produce ocular levels of PEDF protein well above the therapeutic threshold. In one study this led to a regression in oxygen-induced retinal neovascularisation [18], demonstrating the efficiency and efficacy of adenovirus mediated gene transfer. Safety issues were recently addressed by a Phase 1 clinical study, which explored the safety and efficiency of an intravitreal injection of two different titres of an adenovirus vector expressing PEDF in twenty-eight patients with advanced neovascular AMD over 12-month period [21]. A quarter of patients shows mild transient ocular inflammation and six subjects exhibited manageable elevated intraocular pressure [21]. Therefore, gene transfer of PEDF in patients is well-tolerated. Although therapeutic efficacy was not the objective of Phase 1 study, 50% of patients treated with the higher titre of PEDF expressing vector showed a reduction in lesion size at 6 and 12 months following treatment. This is evidence of an extended antiangiogenic effect following a single injection [21]. There have been no further clinical studies on PEDF gene transfer but a recent animal study demonstrated an anti-inflammatory action of recombinant PEDF protein in mice with spontaneous retinal degeneration [86]. Therefore the versatile biological functions of PEDF make it an attractive target for gene transfer therapy.

5.2. Angiostatin. Angiostatin is a cleaved product of plasminogen containing the kringle domains 1–4. It has well-characterised antiangiogenic effects and its therapeutic potential arises from its effectiveness in studies of tumour treatment [87]. Angiostatin promotes apoptosis of proliferating vascular endothelial cells [88] and inhibits proliferation and migration of endothelial cells [89]. The importance of angiostatin in suppressing the growth of retinal neovessels has been documented in a study showing that the local release of angiostatin is an important factor mediating the beneficial action of laser photocoagulation in patients with proliferative diabetic retinopathy [90]. In a murine model of proliferative diabetic retinopathy lentivirus-mediated expression of angiostatin was shown to be a potent inhibitor of neovascularisation [91]. Moreover, systemic administration of recombinant angiostatin in neonatal mice inhibits ischemia-induced growth of retinal vessels with little effect on the normal process of retinal vessel development [92]. This illustrates its selectivity for suppressing pathological and not normal angiogenesis. One study used an adenoviral vector to overexpress kringle domains 1–3 of angiostatin in the neonatal mouse retina. Results showed inhibition of ischemia-induced neovascularisation, as reflected by a

Table 1: Biological actions of endogenous inhibitors of angiogenesis.

Endogenous inhibitor	Functions
PEDF, serine protease	Increases survival of neuronal cell, preserves the integrity of neuronal cells; protects neuronal cells from apoptosis, decreases proliferating endothelial cells, decreases expression of inflammatory molecules like TNFα and iNOS.
Angiostatin, cleaved product of plasminogen containing the kringle domains 1–4	Increases apoptosis of proliferating vascular endothelial cells, decreases proliferation and migration of endothelial cells, decreases recruitment and adhesion of inflammatory cells to the endothelium, and decreases transmigration of inflammatory cells.
Endostatin, fragment of collagen XVIII	Increases apoptosis and decreases migration of cells involved in active neovascularisation, blocks the binding of VEGF to KDR/Flk-1, and decreases spontaneous release of VEGF from endothelial cell culture, structurally supports role of the Bruch's membrane.
TIMP3, inhibitor of matrix metalloproteinase	Increases apoptosis and decreases migration of cells involved in active neovascularisation, blocks the binding of VEGF to KDR/Flk-1, structurally supports role of the Bruch's membrane.
Vasostatin, a derivative from the NH_2 terminal domain of a calcium binding protein calreticulin	Decreases proliferation of endothelial cells, decreases adhesion of leukocytes to endothelium, decreases expression of vascular destabilising factor angiopoietin 2.
Plasminogen kringle 5, cleaved product of plasminogen containing the kringle domain 5	Increases proliferation and decreases migration of endothelial cells, increases apoptosis of endothelial cells, increases infiltration of inflammatory cells.
Thrombospondin-1, glycoprotein	Decreases apoptosis of endothelial cells, decreases expression of inflammatory molecules.

marked reduction in the number of endothelial cells in the retinal layer where neovascular tufts originate [18]. Inhibitory effects of the transgene correlated well with ocular protein expression since its level was found to be well above the therapeutic threshold. Importantly, administration of the adenoviral vector did not result in cytotoxicity [18], highlighting the clinical potential of gene delivery with angiostatin. The highly stable lentivirus-based vector has also been used to deliver angiostatin to rat eyes with an observable decrease in the area of experimental choroidal neovascularisation [16]. Angiostatin has also been shown to suppress the recruitment and adhesion of inflammatory cells to the endothelium, in addition to limiting their transmigration [93]. A 6-month safety study of lentiviral gene delivery of angiostatin in rhesus macaques and rabbits found no change in retinal functional, as evaluated by electroretinography, and no histological structural changes [94]. In summary, angiostatin transgene has significant appeal as a viable therapeutic approach.

5.3. Endostatin. Like angiostatin, endostatin is a potential therapeutic target for treatment of tumour growth owing to its antiangiogenic properties [95]. One such mechanism involves its interaction with VEGF. Endostatin has been shown to prevent the binding of VEGF to its receptor KDR/Flk-1 in endothelial cells [96]. Endostatin also inhibits the spontaneous release of VEGF from human endothelial cell culture [97]. Moreover, endostatin has been shown to suppress VEGF-mediated responses *in vivo* [98]. Lentiviral-mediated overexpression of endostatin in the mouse retina reduced the degree of neovascularisation and vascular leakage, which were both stimulated by locally expressed VEGF transgene [98]. Adenoviral-mediated expression of endostatin was shown to be successful in inhibiting neovascular responses in a mouse model of retinopathy of prematurity [99]. Proapoptotic activity of endostatin also contributes to its antiangiogenic properties. Lentiviral delivery of endostatin induced a decrease in the extent of choroidal neovascularisation, vascular hyperpermeability, and apoptotic cell loss in the neurosensory retina of laser damaged rat eyes [16]. Immunohistochemical characterisation confirms that the proapoptotic activity of the endostatin transgene in neurosensory retina is limited to the laser-damaged eye [16]. This underlines the selectivity of endostatin against pathological growth of vessels. Safety studies have shown no change in retinal structure and function following lentiviral gene therapy with endostatin [94].

Endostatin also has a structural support role, which makes it valuable in gene therapy. It is a proteolytic fragment of collagen XVIII and forms a crucial component of Bruch's membrane [100]. Deletion of endostatin in mice causes a phenotypic change including morphological abnormality of the RPE with an accumulation of sub-RPE deposit formation in the Bruch's membrane that contributes to age-dependent vision loss [100]. Such findings correlate with a reduced expression of endostatin found in Bruch's membrane of human AMD sufferers [83], indicating a requirement of endostatin for a functional Bruch's membrane. Targeted gene therapy with endostatin is therefore a promising therapeutic strategy.

5.4. Tissue Inhibitor of Metalloproteinases-3 (TIMP-3). TIMP-3 is an extracellular matrix component of Bruch's membrane

[101] synthesized by the RPE, choroid and retina [17]. It is the only member of the peptidases that is distributed in the extracellular matrix of the membrane, where it regulates the proteolytic activity of matrix metalloproteinases. The unique location of TIMP-3 suggests a physiological role at the interface of the RPE, Bruch's membrane, and choroid [102]. Indeed mice with a deficiency of TIMP-3 exhibit abnormal development of blood vessels characterised by dilated capillaries at the choroid and augmented activity of matrix metalloproteinases [102]. The abnormal choroidal vascular network in TIMP-3 knockout animals may also be related to the imbalance of angiogenic homeostasis [102] given that TIMP-3 has been shown to possess antiangiogenic activity [103]. Overexpression of TIMP-3 in the eye using gene delivery produces a reduction in laser-induced choroidal neovascularisation [17] and ischemia-induced retinal neovascularisation [99] in rats and mice. Whereas endostatin inhibits the binding of VEGF to KDR/Flk-1, TIMP-3 selectively binds to KDR/Flk-1 but not to Flt-1 [103].

5.5. Vasostatin. Vasostatin is a naturally occurring peptide found in humans and is derived from the NH_2 terminal domain of a calcium-binding protein calreticulin [22]. Recombinant vasostatin has been shown to inhibit the proliferation of human endothelial cells, stimulated by basic fibroblast growth factor (bFGF) [22]. Topical application of recombinant protein to rats subjected to laser photocoagulation also causes a reduction in the area of choroidal lesions [80], underlining its therapeutic potential for suppressing neovascularisation. It has been postulated that the antiangiogenic effect may be due to interference with the signaling of the controversial regulator of angiogenesis angiopoietin 2 [104]. Vasostatin is found to reduce the expression of angiopoietin 2 in inflamed skin, which mediates inflammatory responses including formation of blood vessels, infiltration of inflammatory cells, and adherence of leukocytes to endothelium [104]. Angiopoietin 2 destablilizes blood vessels and has been shown to disrupt early proliferating vessels thereby promoting vessel maturation [105]. Angiopoietin 2 can also induce angiogenesis via the binding of integrins in activated endothelial cells that have a diminished population of Tie2 receptors [106]. The inhibitory mechanism of angiopoietin 2 may explain the selective antiangiogenic effects of vasostatin on endothelial cells of proliferating vessels.

5.6. Plasminogen Kringle 5 (K5). K5 is derived from plasminogen and its antiangiogenic activity appears to be specific for endothelial cells as it inhibits proliferation and migration and promotes apoptosis [107, 108]. Recombinant K5 only suppresses the proliferation of endothelial cells but not vascular smooth muscle cells or fibroblasts under the stimulation of VEGF [108]. When recombinant K5 is given locally via intravitreal injection, either before or during the development of oxygen-induced retinal neovascularisation in rats, the degree of neovascularisation is suppressed [23]. Importantly it reduces the number of vascular endothelial cells in proliferating vessels but not preexisting vessels of rats with oxygen-induced retinopathy [23], supporting its selective action against pathological angiogenesis. K5 may restore angiogenic homeostasis to exert an antiangiogenic effect. Indeed an intravitreal injection of K5 decreases the retinal expression of VEGF while it elevates PEDF in rats with oxygen-induced retinopathy [107]. An interference with the autophagy phase of apoptotic endothelial cells may also contribute to its antiangiogenic activity [109]. Other useful biological actions of K5 include antihyperpermeability and anti-inflammation. Recombinant K5 given through either systemic or ocular administration reduces the extent of retinal vascular leakage in both rat models of oxygen-induced retinopathy and streptozotocin-induced diabetes [110]. The antihyperpermeability effect of recombinant K5 could be related to a reduction in retinal expression of VEGF, which has been shown to cause hyperpermeability in both models [110]. Topical administration of recombinant K5 has also been found to suppress alkali-induced neovascularisation, infiltration of inflammatory cells, and VEGF expression in the rabbit cornea [108], indicating its effectiveness in hampering an inflammation-driven angiogenic response. In addition, nanoparticle-mediated transfer of K5 in the rat retina has been shown to produce an inhibitory effect on experimental CNV [111].

5.7. Thrombospondin-1. Thrombospondin-1 belongs to the glycoprotein family and regulates the structure of extracellular matrix and cellular phenotype associated with tissue remodelling during angiogenesis [112]. The expression of thrombospondin-1 in RPE, Bruch's membrane, and the choriocapillaris in human AMD choroids is found to be less than that of controls [84], suggesting a protective role of thrombospondin-1 in AMD. One of the antiangiogenic effects of thrombospondin-1 appears to be mediated by an induction of apoptotic endothelial cells. Indeed knocking down the expression of thrombospondin-1 in mice resulted in a two-fold decrease in the number of apoptotic nuclei in developing retinal vessels [113]. An increased count of retinal endothelial cells as an index of retinal vascular density is also demonstrated in mice lacking thrombospondin-1 [113]. Moreover, Sorenson et al. [114] recently induced a deletion of thrombospondin-1 in Akita mice that develop spontaneous diabetes and showed an acceleration of diabetes-induced retinopathy in the absence of thrombospondin-1. Collectively, thrombospondin-1 is required for a quiescent and differentiated phenotype of endothelial cells [113]. It is unclear whether an overexpression of thrombospondin-1 in eyes exerts a protective effect against neovascular AMD; however, its anti-inflammatory action [115] will be valuable for suppressing aberrant vessel growth. Therefore, gene transfer studies in animals are warranted to examine a role of thrombospondin-1 in neovascular AMD.

6. Conclusion and Future Perspective

Gene therapy shows great promise in the treatment of eye disease and the prevention of blindness. It is much easier and less costly to manufacture gene therapy vectors than to produce huge amounts of purified protein molecules.

The recent data from animal studies and Phase I clinical trials has indicated that gene therapy of anti-VEGF agent such as sFlt-1, a soluble form of the Flt-1 receptor, provided major benefits in patients with neovascular AMD and other types of ocular neovascularization. These data suggest that long term blockade of VEGF in the retina and choroid by gene transfer is likely to inhibit neovascularization, but it is not yet known if sustained, efficient blockade of VEGF family members will have any adverse effects on normal choroidal vessels and retinal neurons. Moreover, similar to protein-based anti-VEGF treatments, the loss of efficacy of anti-VEGF gene therapy is a clinically significant problem in the battle against neovascular AMD. Thus, an alternative gene-based approach with expression of one or more of the aforementioned endogenous angiogenic inhibitors has excellent potential. Most of the endogenous angiogenic inhibitors have a small molecular size, specifically target endothelial cells, and are effective in preventing the development of neovascularization with no effect on established vessels. Gene transfer of PEDF produced beneficial effects in animal models and a Phase I study has shown an excellent safety profile for intraocular injection of Ad-PEDF. Although therapeutic efficacy is not an objective of Phase 1 studies, patients who received the treatment showed a reduction in lesion size. Moreover, a Phase I single dose trial with a lentiviral vector-mediated expression of two angiogenic inhibitors, endostatin and angiostatin (retinostat), has recently commenced in neovascular AMD patients. Other promising candidates with antiangiogenic properties include vasostatin, TIMP3, K5, and thrombospondin-1. Numerous studies have demonstrated their therapeutic effects, however; further gene transfer studies in animals are needed to build the basis for clinical translation.

Disclosure

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Conflict of Interests

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

Authors' Contribution

Bang V. Bui and Guei-Sheung Liu contributed equally to this work as senior authors.

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Review Article

Laser-Based Strategies to Treat Diabetic Macular Edema: History and New Promising Therapies

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Diabetic macular edema (DME) is the main cause of visual impairment in diabetic patients. The management of DME is complex and often various treatment approaches are needed. At the present time, despite the enthusiasm for evaluating several new treatments for DME, including the intravitreal pharmacologic therapies (e.g., corticosteroids and anti-VEGF drugs), laser photocoagulation still remains the current standard in DME. The purpose of this review is to update our knowledge on laser photocoagulation for DME and describe the developments in laser systems. And we will also discuss the new laser techniques and review the latest results including benefits of combined therapy. In this paper, we briefly summarize the major laser therapeutics for the treatment of diabetic macular edema and allude to some future promising laser therapies.

1. Introduction

In 2011, an estimated 347 million people worldwide were affected by diabetes, and the number is expected to double by 2030. Diabetic macular edema (DME) is a leading cause of visual impairment in such patients [1] and if left untreated >50% of patients lose more than two lines of visual acuity (VA) within 2 years [2]. DME mostly affects working-age adults, imposing significant burdens both on society and on individual patients; these burdens are expected to increase as the prevalence of diabetes rises [3].

The standard therapy for visual impairment caused by DME is focal and/or grid laser photocoagulation. However, this usually simply stabilizes vision. By applying the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria to patients with visual impairment caused by DME, laser therapy reduced the relative risk of loss of 15 VA letters by 50% compared to deferred treatment [4]. The exact mechanisms by which laser photocoagulation effectively treats DME remain unknown. Both laser-induced destruction of oxygen-consuming photoreceptors and oxygen diffusion through the laser scar to the inner retina may relieve internal retinal hypoxia [5, 6].

However, the era of laser therapy is being rapidly replaced by a new pharmacotherapeutic era associated with rapid improvements in VA. Treatments include intravitreal corticosteroids, intravitreal vascular endothelial growth factor (VEGF) inhibitors, and others under current investigation. In some cases where vitreous traction is demonstrated, the treatment of choice is to perform pars plana vitrectomy (PPV). Some such agents have recently been shown to be superior to laser therapy [7, 8]. However, given that several of these newer agents are available, it can be difficult to individualize treatment options, especially when attempting to minimize cost and simplify retreatment cycles (the number of injections). These concerns, together with the absence of long-term effects on VEGF inhibition, mean that laser photocoagulation continues to be the necessary treatment for DME care. Recently, new (and less destructive) laser modalities including subthreshold micropulse diode (SDM) laser treatment and selective retinal therapy (SRT) have been developed. In the present paper, we summarize the various

laser therapeutic options for treating DME and discuss promising laser therapies of the future.

2. Conventional Laser Photocoagulation

Laser photocoagulation was inarguably the important treatment method for DME prior to the advent of intravitreal anti-VEGF agents [9]. The efficacy of focal laser treatment may in part be due to its ability to occlude leaking microaneurysms, but the exact mechanism by which focal photocoagulation reduces DME is unknown.Histopathological studies have revealed that such treatment triggers changes in the retina and the retinal pigment epithelium (RPE) [10, 11]. Some authors have suggested that, following the reduction in retinal tissue associated with photocoagulation, autoregulation decreases retinal blood flow to the macula. Such reduced fluid flow is attributable to improvements in oxygenation after photocoagulation [12]. Biochemical and physiological studies have suggested that the mechanism of resolving edema may involve biochemical changes within the RPE [13, 14]. The effectiveness of grid treatment alone, that is, without direct focal treatment of microaneurysms, implies that retinal photocoagulation has an indirect effect on macular edema [15, 16].

The ETDRS trial [4] was the first rigorous, multicenter randomized trial to explore the benefits of laser therapy for DME. Laser photocoagulation was prescribed for all lesions located within two disc diameters of the macular center. Treatment of lesions closer than 500 microns to the macula was not initially planned. However, if vision was less than 20/40, and if retinal edema and leakage persisted, treatment of lesions up to 300 microns from the center was recommended. Three years after randomization, patients who received focal photocoagulation to treat clinically significant macular edema (CSME) exhibited a 50% reduction in the risk of moderate visual loss, compared to controls (12–24%). However, over the same time period, only 3% of patients exhibited VA gains of three or more lines. The suggested ETDRS guidelines [4] for treating DME via laser photocoagulation emphasize direct laser application to leaking microaneurysms combined with grid treatment of areas of diffuse macular leakage and nonperfusion in thickened retinas, especially in those with nonproliferative diabetic retinopathy (NPDR). As initial pan-retinal photocoagulation (PRP) may worsen macular edema by increasing inflammation and the extent of central retinal blood flow [17], the ETDRS recommended combining PRP and focal laser photocoagulation to treat general DME in selected cases with severe NPDR and early-stage proliferative diabetic retinopathy (PDR). Although effective, conventional ETDRS macular photocoagulation causes visible laser scars that may enlarge once the treatment is finished [18]. In addition, the thermal effects of photocoagulation can trigger complications, including choroidal neovascularization (CNV) [19], subretinal fibrosis [20], and visual field loss (central and paracentral scotoma) [15]. Such damage caused by visible endpoint laser photocoagulation has encouraged many retinal specialists to seek to reduce the duration of laser exposure and to use less visible clinical endpoints than originally proposed by the ETDRS.

Patient outcomes after application of a modified ETDRS laser protocol or mild macular grid laser (MMG) photocoagulation were evaluated in a randomized controlled trial that included 263 eyes with previously untreated DME, in patients who had 12-month follow-up [21]. Reduction in macular thickness was significantly greater in the group treated with the modified ETDRS laser protocol, but no difference was noted in terms of the mean change in best-corrected VA (0 letters in the ETDRS group and −2 letters in the MMG group, $P = 0.10$), suggesting that modified ETDRS focal photocoagulation should continue to be the standard treatment for DME.

Recently, a randomized controlled trial conducted by the Diabetic Retinopathy Clinical Research Network (DRCR.net) protocol B found that focal/grid photocoagulation was more effective and was associated with fewer side effects than intravitreal injection of triamcinolone acetonide in DME patients at both 2 and 3 years of follow-up [22, 23]. The authors suggested that focal/grid laser treatment should remain to be the standard against which other DME treatments are compared. However, some laser-treated patients (10%) in the DRCR.net protocol I study lost 15 letters or more in VA at 2 years of follow-up [24]. Although it is obviously essential to prevent further loss of vision, the need to restore VA via a novel medical or laser therapy has, until recently, been unmet in DME patients.

3. Subthreshold Micropulse Diode Laser Therapy (SDM)

The utility of conventional laser photocoagulation to treat DME has become well established in the time since the ETDRS was reported [4]. However, the procedure produces visible burns in the retina, indicating that the temperature of the tissue is raised to a level sufficient to alter its natural transparency. In other words, photocoagulation, which is currently performed using conventional continuous wave (CW) laser systems, damages the neural retina by inducing the spread of thermal energy from the RPE. Compared to CW treatment, lasers that deliver short pulses ("micropulses") cause less thermal damage in experimental models of retinal photocoagulation. Moreover, the shorter laser exposure times allow effective treatment of the RPE while at the same time inflicting less damage on the neural retina and the choriocapillaries [25]. The outcomes of "invisible" subthreshold micropulse diode laser (SDM) cannot be discerned using ophthalmic imaging methods such as biomicroscopy, fundus fluorescein angiography (FFA), fundus autofluorescence (FAF), or spectral-domain optical coherence tomography (SD-OCT), because SDM-induced retinal damage is absent. Although the mechanism by which SDM effectively treats DME is unknown, the laser may selectively target the RPE and induce changes in the levels of RPE cytokines [26].

SDM system featuring both 810 nm and 577 nm lasers may, in theory, afford a theoretical advantage because the laser burns will selectively affect the deeper layers, sparing

Table 1: Comparison of subthreshold micropulse laser (SDM) systems.

Model name (manufacturer)	PASCAL streamline 577 (Topcon)	IQ810 (Iridex)	2RT (Ellex)
Category	End point treatment	Subthreshold micropulse laser	Retinal rejuvenation therapy
Laser type	Optically pumped semiconductor	Diode	Q-switched green YAG laser
Wavelength	577 nm	810 nm	532 nm (green)
Pulse duration	10 to 1000 ms	CW pulse: 10–9000 ms Micropulse: 0.025–1 ms	3 ns
Power	30–150 mW 150–2000 mW	0–2000 mW	Energy: 0.6–1.2 mJ Fluence: 200 mJ/cm^2
Spot size	60/100/200/400 μm	125 μm	400 μm
Dosimetry	N/A	N/A	N/A

the inner neurosensory retina for the most part. In turn, this should reduce scarring and paracentral scotomas that may arise after treatment [27]. In the micropulse mode, laser energy is delivered via a train of short repetitive pulses (each is typically 100–300 ms in duration) within an "envelope," the width of which is typically 0.1–0.5 s, and the envelope duration is taken to be the exposure duration. The "ON" time is the micropulse duration. The "OFF" time between successive micropulses allows heat to dissipate in tissues and thermally isolates each pulse [28]. Micropulse power settings as low as 10–25% of visible threshold power have been shown to consistently confine photothermal effects to the RPE, thereby sparing the neurosensory retina. The laser power required for optimal SDM treatment can be estimated by comparing the power that causes a visible retinal burn to that which confines histological damage to the RPE over various duty cycles (the frequencies of the micropulse train) [28]. A previous study [29] explored the long-term safety of SDM by evaluating retinal burn risks when FFA and FAF were used to treat 252 eyes (212 with DME; 40 with branched retinal vein occlusion) followed up for as long as 10 years postoperatively. Inadvertent retinal burns were observed in seven eyes (three Asian, three Hispanic, and one Caucasian). All burns occurred in eyes treated using 10% or 15% duty cycles; no retinal damage was found in any eye treated using a 5% duty cycle. Computational tissue temperature models revealed that SDM performed using a 5% duty cycle triggered an adequate thermal rise in RPE cells and was not lethal to other cells (Table 1).

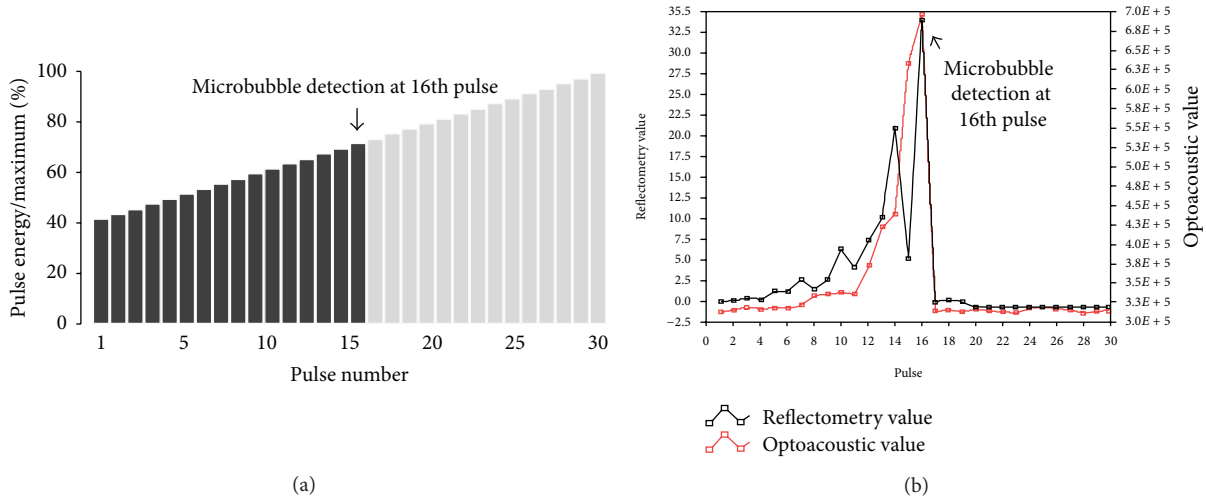
Micropulse laser treatment of DME has been shown to be as effective as conventional argon laser treatment by several authors. Friberg and Karatza [30] reported that almost 70% of patients experienced clinical resolution of DME by 6 months after SDM photocoagulation, and VA either improved or stabilized in 80% of eyes. Luttrull and Musch [31] found that VA was either stable or improved in 85% of eyes at 12.2 months, and DME was reduced in 96% of eyes. Also, no marked adverse effect of the technique has been reported.

Venkatesh et al. [32] conducted a prospective randomized study using multifocal electroretinography (MfERG) to assess the efficacy of SDM-mediated photocoagulation to treat DME. Thirty-three patients (46 eyes) with CSME were randomized to either an SDM (810 nm) laser or the conventional double-frequency Nd:YAG (532 nm) laser. Six months later, it was concluded that both treatments influenced both VA and central macular thickness. However, MfERG data suggested that use of the SDM laser potentially afforded better preservation of retinal tissue and led to better values for various electrophysiological indices. Many commercial micropulse lasers are available at wavelengths of 532 nm, 577 nm, 586 nm, 660 nm, and 810 nm.

In summary, the cited reports show that the SDM laser is as effective as a conventional laser when used to treat DME. Moreover, the attractive safety profile of SDM treatment allows clinicians to offer earlier treatment for DME, thus at a time when such treatment is likely to prevent tissue damage and the development of visual disability.

3.1. Selective Retinal Therapy (SRT). To further reduce adverse effects on the neural retina, it was already suggested in the early 1990s [33, 34] that selective treatment of the RPE should be delivered carefully so as to avoid thermally damaging adjacent photoreceptors or the choroid. Selective retina therapy (SRT) was introduced in the following decade. SRT is thought to cause laser-induced biological stimulation and rejuvenation of the chorioretinal junction [35]. The method differs from SDM in that RPE cells are selectively damaged without affecting the neural retina, the photoreceptors, or the choroid. The goal of SRT is to stimulate RPE cell migration and proliferation into irradiated areas to improve metabolism at diseased sites.

Selective RPE damage is achieved by applying a burst of microsecond laser pulses in the green spectral range; pulse energy is absorbed primarily by melanosomes within RPE cells. If the pulse energy is appropriate, RPE cells are damaged by microvaporization around intracellular melanosomes when the pulse duration is shorter than 5 μs. High peak temperatures that develop around melanosomes during irradiation create short-lived microbubbles that mechanically disrupt RPE cells as the cell volume rises briefly but markedly [36]. Thus, the SRT technique features the use of microsecond-laser pulses to ensure that damage is RPE-selective and to avoid formation of large bubbles associated



(a) (b)

FIGURE 1: (a) The laser pulse energy was increased stepwise with every pulse by 3% of the dynamic range. In the chosen example, laser irradiation was ceased automatically after the 16th pulse due to detection of microbubble formation. (b) The dual dosimetries show that adequate turnoff system works properly (e.g., turnoff at the 16th pulse).

with a risk of photodisruption of the retina or choroid [37]. The effects of this treatment are ophthalmoscopically invisible, and fluorescein angiography is used to identify damage to the RPE layer after treatment. Intact bystander RPE cells migrate and proliferate to cover laser-induced RPE defects, thereby recreating an intact RPE barrier layer within 7 days.

As transient microbubbles are responsible for the desired effects on RPE cells, it is useful to monitor microbubble development. After each burst, microbubble parameters are evaluated to guard against undertreatment (no microbubbles and thus no effect on the RPE) and overtreatment (large bubbles associated with risks of visible effects and large disruptions). As with SDM, SRT does not cause visible changes in the retina, rendering it difficult to determine when the laser dose is adequate. In efforts to solve this problem, two forms of dosimetry are currently under development. The optoacoustic method features real-time temperature monitoring based on the detection of optically excited thermoelastic pressure waves [35]. Reflectometric methods detect light that is backscattered by the RPE during coagulation. Use of the reflectometric technique with controlled pulse energy ramping is both safe and selective [38] (Figure 1).

The ability of this method to selectively damage RPE cells without injuring photoreceptors has been histologically confirmed at various times after treatment [39]. The first SRT clinical trial using an Nd:YLF laser system and a pulse duration of 1.7 μs (100 pulses, at 100 and 500 Hz) revealed the clinical potential of the technique [40]. Subsequently, the SRT laser parameters were successfully refined. The energy delivered was reduced using even shorter pulses, fewer repetitions, and lower repetition rates [41]. At pulse energies of 450–800 mJ/cm^2, RPE defects were angiographically demonstrated by detecting fluorescein leakage. However, neither bleeding nor scotoma, as evaluated microperimetrically, was observed, indicating that neither the choroid nor the photoreceptors (resp.) had suffered any adverse effects. During irradiation, the treated locations are ophthalmoscopically invisible, because the effects are both very limited and confined to the RPE.

The precise mechanism of the therapeutic effect is not understood. Several mechanisms have been suggested. It has been histologically shown that the RPE can regenerate following either conventional laser treatment or SRT, reestablishing a normal RPE monolayer [42]. One theory suggests that the beneficial effects of photocoagulation are associated with the establishment of a new RPE cell barrier, with subsequent restoration of the RPE pump and barrier integrity [6].

Such theoretical considerations have led to the development of SRT laser treatments that selectively affect the RPE. Briefly, both thermal modeling and studies *in vitro* and *in vivo* have shown that the spatial extent of elevated temperature is reduced when multiple laser pulses of short duration are delivered using a low repetition rate. By employing such parameters, the effects of laser exposure are confined to the principal light-absorbing structures such as the intracellular melanosomes of the RPE; the photoreceptor layer, Bruch's membrane, and the choroid are spared [36].

Several pilot clinical studies have demonstrated the efficacy of SRT used to treat DME, central serous chorioretinopathy, and persistent subfoveal fluid accumulation after rhegmatogenous retinal detachment [43–46]. Roider et al. [43] found that SRT was potentially effective and safe when used to treat clinically significant DME; functional and anatomical improvements or stabilization was noted in 84% of patients. Mean BCVA improved from 43.7 letters at baseline to 46.1 letters at the 6-month follow-up ($P = 0.02$), and improvement of >5 letters, or no deterioration, was noted in 84% of eyes. No adverse effects or pain was recorded during or after treatment.

Although SRT has not yet been commercialized, both optoacoustic systems and reflectometry will help define

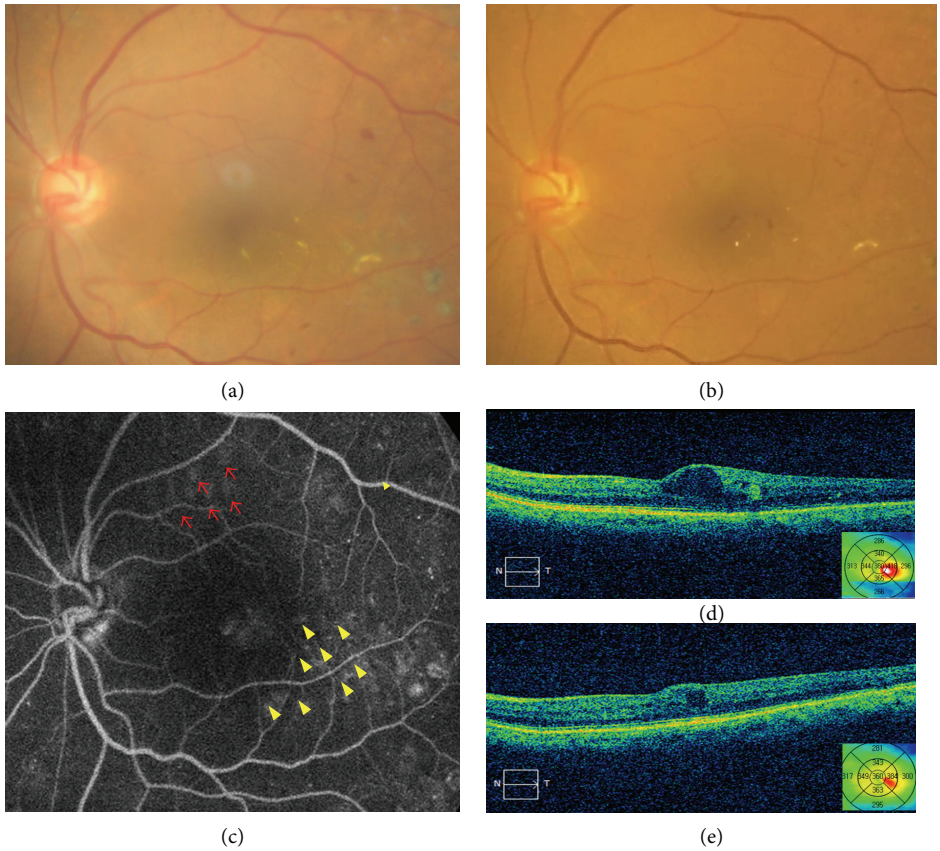


Figure 2: Color fundus photographs showing the reduction in hard exudates before (a) and 3 months (b) after SRT for DME. Laser test spots were applied (red arrow) and SRT treatment was performed (yellow arrowhead). OCT scans showing the reduction of retinal thickness. OCT scan and retinal thickness map before (d) and 3 months after SRT (e).

the energy required for selective RPE damage. Therefore, using a combination of both dosimetric methods to ensure safety, SRT could be an important subthreshold laser treatment modality for DME in the near future (Figure 2).

4. Laser Therapy Combined with Pharmacological Treatment

DME is a chronic disease with variable response and clinical manifestations and it does not appear reasonable that a single treatment may be enough for the entire course of the disease. Above all things, laser photocoagulation, the current gold standard of care in DME patients, usually only stabilizes vision. However, as VA improvements after laser therapy occur only very slowly, the addition of laser treatment to the use of pharmacological agents confers an additional benefit in terms of both VA and patient quality-of-life. The available treatments should also make the most of the beneficial effects of each existing approach, exploiting the opportunity for more successful combined therapy. In particular, it is well known that laser treatment can reduce oxygen consumption and influence the RPE in a complex manner.

The introduction of intravitreal anti-VEGF, corticoids (triamcinolone), and steroid implants in DME treatment altered the current treatment protocols. Several studies have compared the effectiveness of new drugs alone with that of laser therapy alone or combined with drugs.

5. Intravitreal Anti-VEGF Treatment Alone or Combined with Laser Therapy

5.1. Ranibizumab (Lucentis, Genetech, San Francisco, CA). Ranibizumab is a recombinant humanized monoclonal antibody fragment specific for all isoforms of human VEGF-A and has been approved by the Food and Drug Administration (FDA) for intravitreal injection for the treatment of retinal diseases. Ranibizumab has been evaluated as an adjunct to laser photocoagulation in well-conducted prospective studies such as READ-2 [47] and the DRCRnet protocol I [48], as well as the RESTORE [49].

The READ-2 study [47] showed that patients who received ranibizumab alone (group 1) gained an average of 7.4 letters at 6 months as compared to a 0.5-letter loss in patients receiving macular laser therapy only (group 2) and a 3.8-letter gain in patients receiving both laser treatment and ranibizumab (group 3). At 24 months, and after starting groups 2 and 3 on ranibizumab at 6 months, the mean improvements in the best-corrected visual acuity (BCVA) were 7.7, 5.1, and 6.8 letters in groups 1 to 3, respectively. The optical coherence tomography (OCT) findings, however, did

not parallel the visual outcome. The mean foveal thicknesses at 24 months were 340, 286, and 258 μm for groups 1 to 3, respectively. The DRCRnet protocol I trial [48] outcomes indicated that four monthly injections of ranibizumab and then as needed combined with prompt or deferred laser was more effective than prompt laser alone in patients with visual impairment associated with DME (BCVA letter score +9 for the ranibizumab-plus-laser group versus +3 for the laser-alone group; $P < 0.001$).

Recently, DME management has shifted progressively to feature intravitreal drug therapy, usually delivered via injection every 4–6 weeks. To limit the treatment burden associated with frequent injections, several studies have explored combination regimens featuring macular laser photocoagulation and anti-VEGF drug delivery to determine if the number of interventions could be reduced.

Phase III Trials in the Ranibizumab Monotherapy or Combined with Laser versus Laser Monotherapy for Diabetic Macular Edema (RESTORE) study [49]; three monthly injections of ranibizumab 0.5 mg and then as needed either alone or combined with laser therapy was more effective than laser alone in patients with DME. However, no efficacy differences were detected between the ranibizumab alone and ranibizumab-plus-laser arms of this trial. But a subgroup analysis of data from this trial indicated that patients with retinal thicknesses $\leq 300\,\mu$m enjoyed similar outcomes after either laser or anti-VEGF monotherapy, whereas patients with thicker retinas benefited most from anti-VEGF monotherapy.

The results suggest that initial anti-VEGF monotherapy may reduce retinal thickness, thereby improving the substrate for subsequent focal laser application, which is most effective when used to treat relatively thin retinas.

5.2. Bevacizumab (Avastin, Genetech, San Francisco, CA). Bevacizumab was approved by the FDA for the treatment of colorectal cancer. It has been used off label in the treatment of wet AMD and other ocular diseases including DME [50, 51]. A recent prospective randomized controlled clinical trial (the BOLT study) found that bevacizumab has a greater effect than macular laser treatment in patients with center-involving persistent CSME [8]. At 12 months, there was a significant difference in the mean BCVA ($P = 0.0006$). At 2 years, the mean BCVA was also increased in the bevacizumab group compared to the macular laser therapy group ($P = 0.005$).

5.3. Aflibercept (VEGF Trap-Eye, Regeneron Pharmaceutical, NY). Aflibercept is the most recent anti-VEGF approved for clinical use. It is a pan-isoform VEGF-A inhibitor with substantially greater binding affinity to VEGF than either bevacizumab or ranibizumab. The DA VINCI study [52, 53], a phase 2 clinical trial, compared different doses and dosing regimens of aflibercept with laser photocoagulation in patients with DME: aflibercept 0.5 or 2 mg every 4 weeks, 2 mg every 8 weeks, or 2 mg as needed after 3 initial monthly injections or macular laser treatment. At 52 weeks, the mean improvement ranged from 9.7 to 12 letters in the aflibercept groups versus −1.3 for laser group. The mean reduction in central retinal thickness in the aflibercept groups ranged from −165.4 to 227.4 versus −58.4 for the laser group.

6. Laser Therapy Combined with IVTA

Many clinical trials have investigated the effects of intravitreal triamcinolone acetonide (IVTA) alone or combined with laser therapy in DME. The 3-year follow-up reports involved only patients treated with intravitreal triamcinolone 1 or 4 mg or laser photocoagulation. The mean change in VA from baseline to 3 years was +5 in the laser group and 0 in each triamcinolone group. The VA outcomes slightly favored the laser group [23]. In addition, Se et al. [54] randomized 86 eyes with diffuse DME to receive either IVTA or IVTA followed by grid laser treatment. They found improvement in the VA and central macular thickness in both groups after 3 weeks. After 6 months, however, these improvements were maintained in the combined group only, suggesting that laser treatment acted synergistically with IVTA, resulting in an increased duration of the effect attributable to IVTA [54].

The 1-year mean change in the VA from baseline was significantly greater in the ranibizumab + prompt laser and ranibizumab + deferred laser groups, but not in the triamcinolone + prompt laser group, compared with the sham + prompt laser group. By contrast, in pseudophakic eyes, the VA improvement in the triamcinolone + prompt laser group appeared comparable to that in the ranibizumab groups [55].

7. Laser Therapy Combined with Steroid Implants

The major limitation of using IVT as adjunctive therapy for DME is the short duration of action and the need for multiple injections that carry the risk of cataract and glaucoma [56]. The recent availability of corticosteroid implants has allowed new approaches to treating DME with combined therapy [57]. Several intravitreal steroid-releasing implants have been designed to facilitate long-term drug delivery to the macular region. These include nonbiodegradable and biodegradable implants containing dexamethasone, fluocinolone acetonide, and triamcinolone acetonide. The sustained-release biodegradable dexamethasone intravitreal implant (Ozurdex, Allergan, Irvine, CA) is receiving attention from medical professionals.

In the multicenter Ozurdex assessment for DME (MOZART study) [58], the mean improvement in the BCVA from baseline was 7.6 letters at 6 months. A gain greater than 15 letters was found in 27% of the patients at 6 months. In addition, the average CRT decrease was 135 μm at 6 months. The mean rate of injection was 1.2 at 6 months, with an average of 5.4 months for reinjection. Side effects are rare and manageable. The use of injectable, sustained-release steroid implants might be considered as an optional treatment and combined with laser treatment to achieve a beneficial long-term effect in DME.

Anti-inflammatory drugs, especially corticosteroids, can counter the various inflammatory reactions associated with diabetic retinopathy, and anti-VEGF drugs inhibit the effects

of VEGF on retinal and vascular structures. Recently, DME management has shifted progressively towards intravitreal drug therapy, usually delivered via injection every 4–6 weeks. In the case of some sustained delivery implants, the injections can be given at intervals of up to several months.

To limit the treatment burden associated with frequent injections, several studies have explored regimens combining macular laser photocoagulation with anti-VEGF or anti-inflammatory drug delivery to determine if the number of interventions could be reduced [24, 47, 49, 55].

8. Discussion

The treatment of DME is evolving rapidly. The era of laser therapy is being quickly replaced by a new era of pharmacotherapy. Several pharmacotherapies have recently been developed to treat retinal vascular diseases including DME. Several types of intravitreal drugs and sustained delivery devices have undergone phase 3 testing and others are currently being evaluated. The results of clinical trials have shown that the therapeutic effects of intravitreal agents such as anti-VEGF and steroid implants are short-term compared to those of laser therapy. Thus, frequent injections are needed to treat diseases that are chronic and recurrent. Subthreshold laser treatment is easy to deliver and is not associated with any of the serious complications of intravitreal injection (endophthalmitis, retinal detachment, and glaucoma).

Several new lasers used to treat DME are attracting increasing attention, as they are yielding promising results. Laser treatment has been shown to be an effective treatment option, at least compared to ETDRS photocoagulation in patients without pericentral scotoma (which reduces retinal function). In terms of expansion of indications, subthreshold lasers (SDM and SRT) may be valuable for treating subclinically significant DME that is diagnosed early, thus prior to symptomatic and irreversible visual loss, using new high-resolution imaging techniques such as SD-OCT. Such techniques may make it possible to perform safe early interventions to reduce disease risk and the rate of disease progression, in turn reducing inflammation and improving the health of the RPE.

The expansion of retinal phototherapeutic techniques may lead to the development of new treatment strategies and make it possible to manage, or even prevent, DME. Lasers may be used alone or in combination with pharmacological therapies. Such treatment options will play important roles in the complex management of DME.

Conflict of Interests

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

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Clinical Study

Subthreshold Micropulse Photocoagulation for Persistent Macular Edema Secondary to Branch Retinal Vein Occlusion including Best-Corrected Visual Acuity Greater Than 20/40

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To assess the efficacy of subthreshold micropulse diode laser photocoagulation (SMDLP) for persistent macular edema secondary to branch retinal vein occlusion (BRVO), including best-corrected visual acuity (BCVA) > 20/40, thirty-two patients (32 eyes) with macular edema secondary to BRVO were treated by SMDLP. After disease onset, all patients had been followed for at least 6 months prior to treatment. Baseline Snellen visual acuity was used to categorize the eyes as BCVA ≤ 20/40 (Group I) or BCVA > 20/40 (Group II). Main outcome measures were reduction in central macular thickness (CMT) in optical coherence tomography (OCT) and BCVA at 6 months. In the total subject-pool at 6 months, BCVA had not changed significantly but CMT was significantly reduced. Group I exhibited no significant change in CMT at 3 months but exhibited significant reductions at 6 and 12 months. Group II exhibited a marginally significant reduction in CMT at 3 months and a significant reduction at 6 months. In patients with persistent macular edema secondary to BRVO, SMDLP appears to control macular edema with minimal retinal damage. Our findings suggest that SMDLP is an effective treatment method for macular edema in BRVO patients with BCVA > 20/40.

1. Introduction

Macular edema is one of the most important causes of impaired vision in patients with branch retinal vein occlusion (BRVO), and, in 1984, the Branch Vein Occlusion Study (BVOS) group demonstrated the efficacy of grid laser photocoagulation to improve visual acuity in patients with macular edema secondary to BRVO [1]. Recently, Campochiaro et al. [2] revealed that antivascular endothelial growth factor (VEGF) therapy for macular edema due to BRVO in patients with best-corrected visual acuity (BCVA) of ≤20/40 yields greater BCVA improvement than conventional laser therapy. Based on previous reports [1–4], most patients with BCVA of > 20/40 are observed without any intervention until visual acuity drops to ≤20/40. However, we have observed numerous chronic-stage patients in whom some degree of macula edema persists long after hemorrhage resolution, though BCVA is maintained at >20/40. These patients often complain of reduced vision or metamorphopsia. Conventional laser therapy [1, 5] inevitably results in retinal scarring [6–8] and reduced macular sensitivity [9] in some patients. Thus, in patients with good BCVA (such as >20/40), conventional grid laser therapy seems overly invasive, and thus undesirable.

Subthreshold micropulse diode laser photocoagulation (SMDLP) is a less invasive treatment than conventional grid laser therapy, designed to produce lesions on the retinal pigment epithelium (RPE) while having minimal effect on the sensory retina and choroid [10, 11]. Pulsed laser ablation is frequently performed during the procedure, without damaging the photoreceptor layer [12]. This treatment has been demonstrated to improve or resolve macular edema without any laser scarring [13].

Several reports have been published on the efficacy of SMDLP for diabetic macular edema and/or macular edema secondary to BRVO [14–23]. In 2006, Parodi et al. first reported a clinical investigation of SMDLP for macular edema secondary to BRVO with BCVA ≤ 20/40 [22]. Since then, only 2 clinical studies from the same study-group have demonstrated the efficacy of this method for macular edema secondary to BRVO with BCVA ≤ 20/40 [22, 23].

A reliably efficacious treatment for persistent macular edema secondary to BRVO in patients with BCVA > 20/40 has not yet been established, and this is the first study of SMDLP for macular edema secondary to BRVO in Japanese patients. The aim of this pilot study was to investigate the efficacy of SMDLP for persistent mild or moderate macular edema secondary to BRVO, including patients with BCVA of >20/40.

2. Materials and Methods

The study reported herein was a single-center, retrospective, and nonrandomized, interventional case series. Thirty-two consecutive patients (32 eyes) with macular edema secondary to BRVO were recruited for SMDLP between the 6th of October, 2003, and the 24th of May, 2012. We obtained approval from the Research Ethics Committee of St. Luke's International Hospital prior to study initiation, and the study followed the tenets of the Declaration of Helsinki.

Prior to treatment, all patients had been followed up for at least 6 months after disease onset, and their macular edema had been confirmed as persistent. Eligibility criteria included BRVO (ischemic or nonischemic) with persistent mild or moderate macular edema with a central macular thickness (CMT) of <600 μm, as determined by optical coherence tomography (OCT). Macular edemas exhibiting CMT values of ≥600 were excluded, because laser energy is not sufficient for treating severe macular edema [20]. All patients had reduced vision or metamorphopsia, due to persistent or increasing macular edema for at least 2 visits after the resolution of dense hemorrhage. Patients who complained of metamorphopsia due to macular edema and had a BCVA of >20/40 or refused additional pharmacotherapy (steroid or anti-VEGF therapy) were also included.

Baseline BCVA ranged from 20/222 to 20/20 on Snellen equivalency (0.35 ± 0.29; mean ± SD). Fluorescein angiography was performed to confirm diffuse dye leakage and rule out focal capillary nonperfusion at recruitment. Subfoveal hard exudate and epiretinal membrane formation were excluded, as were patients with macular hemorrhage precluding sufficient laser ablation. Other exclusion criteria included a history of cataract surgery, any other intraocular surgery within 3 months prior to the study, and previous therapy for macular edema (including subtenon injection of triamcinolone, intravitreal injection of any drug, or macular grid laser photocoagulation) within 6 months prior to the study. During the study period, 34 eyes of patients with macular edema due to BRVO received SMDLP. Of these, 2 eyes with conventional grid photocoagulation, after 1 month of operation, were excluded. Thus, a total of 32 eyes were included in the analysis. All patients in this study had their BCVA, CMT, and TMV evaluated at all time-points up to the study endpoint (baseline, 1 month, 3 months, 6 months, and 12 months).

After providing informed consent, each patient underwent SMDLP. All treatments were performed by the same surgeon, with 33 years of surgical experience in ophthalmology. An 810-nm diode laser photocoagulation device (Iris Medical OcuLight Slx, Iridex Corporation, Mountain View, CA) was used, in "micropulse" operating mode. Laser light was delivered to the involved macular region inside arcade vessels via a slit lamp adapter through a three-mirror contact lens. Laser power for subthreshold treatment was determined for each patient by creating a threshold burn with the lowest energy required to make a visible "test burn" in an appropriate area outside the vascular arcade without retinal edema. The laser was subsequently used at 60%–90% of that energy level in micropulse mode and applied to confluent spots up to 500 μm from the center of the fovea. The test burn was created with continuous wave laser energy (100% duty cycle) for 0.1 s at a diameter of 200 μm. In 13 eyes, laser spots were applied with the 15% duty cycle micropulse mode at 200% of threshold energy, 878.46 ± 215.05 mW (mean ± SD) (750 mW to 1500 mW) for 0.3 s, resulting in the delivery of 90% of the threshold energy. In 19 eyes, laser spots were applied with the 15% duty cycle micropulse mode at 200% of threshold energy, 933.68 ± 417.81 mW (mean ± SD) (360 mW to 2000 mW) for 0.2 s, resulting in the delivery of 60% of the threshold energy.

BCVA and macular parameters were examined at enrollment and at 1, 3, 6, and 12 months after treatment. BCVA was determined with the Snellen chart, and logarithm of the minimum angle of resolution (log MAR) values were calculated. CMT and total macular volume (TMV) were measured using either a Stratus OCT 3000 or a Cirrus HD-OCT (Zeiss Humphrey Instruments, Dublin, CA), with TMV measured in the "fast macular" scan mode (between the 6th of October 2003 and the 23rd of October 2009) or "cube" scan mode (between the 26th of October 2009 and the 22nd of May 2013). According to a report by Abedi et al. [24], CMT values equivalent to those determined by an OCT 3000 instrument can be calculated by subtracting 60 from CMT values determined by a Cirrus HD-OCT instrument. Patients were followed at monthly intervals for at least 3 months, without any additional treatment. Subsequently, additional treatment was limited to SMDLP, which was provided as needed for persistent macular edema and/or reduced BCVA.

Parodi et al. [22] reported that the endpoint of the effect of SMDLP was evaluated at 6 months. Thus, the main outcome measures in this study were decrease in CMT on OCT and BCVA at 6 months. Statistical analyses were performed using the Wilcoxon signed rank test, Friedman test, and Mann-Whitney U test to evaluate these outcomes, while the Friedman test was used to evaluate trends in parameters over time. The SPSS software package (Chicago, Illinois, USA) was used for all statistical analyses, and $P < 0.05$ was used to indicate statistical significance.

Table 1: Sample demographics.

Variable	Treatment group		P value
	Group I: eyes with BCVA ≤ 20/40	Group II: eyes with BCVA > 20/40	
Eyes, n	15	17	
Sex, n (%)			
Male	12 (80.0)	11 (64.7)	0.337
Female	3 (20.0)	6 (35.3)	
Age, mean (SD), years	70.53 (10.78)	63.65 (7.66)	0.044
Hypertension, n (%)	9 (60.0)	10 (58.8)	0.946
Cardiovascular disease, n (%)	5 (33.3)	1 (5.9)	0.047
Diabetes, n (%)	3 (20.0)	4 (23.5)	0.810
BRVO type			
Ischemic	7	6	0.513
Nonischemic	8	11	
Macular BRVO	7	7	0.755
Additional MP treatment, n (%)	8 (53.3)	3 (17.6)	0.034
Baseline BCVA (SD), log MAR	0.5933 (0.2277)	0.1263 (0.0769)	0.355
Baseline CMT (SD), μm	409.266 (87.955)	373.294 (100.268)	0.756
Baseline TMV (SD), mm^3	8.1273 (0.8761)	8.175 (2.8895)	0.952

BCVA: best-corrected visual acuity; BRVO: branch retinal vein occlusion; MP: micropulse photocoagulation; CMT: central macular thickness; TMV: total macular volume.

3. Results

3.1. Demographic Data and Baseline Characteristics. Thirty-two patients (32 eyes, 23 men and 9 women; mean age 66.9 ± 9.74 years; age range 49–88 years) with persistent macular edema secondary to BRVO were enrolled in this study and underwent SMDLP. BRVO onset ranged from 6 to 156 months prior to treatment, with a mean of 34.6 ± 37.5 months. Previous treatments at least 6 months prior to enrollment included subtenon triamcinolone injection in 7/32 eyes (21.9%), intravitreal triamcinolone injection in 2/32 eyes (6.3%), intravitreal bevacizumab injection in 2/32 eyes (6.3%), macular grid laser photocoagulation in 6/32 eyes (18.8%), and vitrectomy in 4/32 eyes (12.5%). Overall, 11/32 eyes (34.4%) had undergone treatment with steroid or anti-VEGF therapy.

BCVA was used to categorize eyes into 2 groups: Group I (15/32 eyes, 46.9%) with BCVA ≤ 20/40 and Group II (17/32 eyes, 53.1%) with BCVA > 20/40. Macular BRVO was present in 14 of the 32 eyes (43.8%) included in this study. Ischemic type, defined by detection of retinal capillary nonperfusion ≥5 disc diameters, was apparent in 13/32 eyes (40.6%). Nonischemic type was apparent in 19/32 eyes (59.4%). Preoperative CMT ranged from 181 μm to 573 μm (mean 390.2 ± 94.9 μm). Characteristics of the patients in the two groups are shown in Table 1.

3.2. Further Treatment. All patients completed 3 months of follow-up, after which additional SMDLP was performed in 11 eyes (31.3%; 8 in Group I, 3 in Group II) within the subsequent 12 months to treat persistent macular edema. A summary of the results in each group, including those who did and did not receive additional SMDLP administration is shown in Table 2.

3.3. Macular Parameters and OCT Findings at 6 Months. At 6 months after SMDLP, a significant reduction in CMT was evident ($P = 0.00026$), as was a significant change in TMV ($P = 0.002$) (Figures 1(a) and 1(b)). Preoperative mean CMT and TMV were 390.2 ± 94.9 μm and 8.15 ± 0.95 mm^3, respectively, while at 6 months they were 303.16 ± 108.15 μm and 7.66 ± 0.61 mm^3 (Table 3). Overall, 25/32 eyes (78.1%) showed a reduction in CMT at 6 months and CMT decreased by at least 20% in 16/32 eyes (50%). In Group I, there was a significant change in CMT at 6 months ($P = 0.009$). In Group II, there was a significant change in CMT at 6 months ($P = 0.015$) (Figure 3(a)).

3.4. BCVA at 6 Months. The change in BCVA (log MAR) at 6 months, from 0.34 ± 0.28 to 0.32 ± 0.34 (20/59 to 20/62 in Snellen equivalence, Figure 1) was not significant. In Group II, 16/17 eyes (94.1%) maintained a BCVA > 20/40 at 6 months (Table 4).

3.5. Changes in BCVA and Macular Parameters Throughout 12 Months of Follow-Up. Mean BCVA was significantly improved at 1 month and at 12 months ($P = 0.004$ and $P = 0.046$ resp.) (Figure 1(a)). Mean BCVA was not significantly improved at 3 months or at 6 months ($P = 0.214$ and $P = 0.119$ resp.). CMT reductions remained significant at 3, 6, and 12 months ($P = 0.014$, 0.00026, and 0.006 resp.) (Figure 1(b)). CMT reduction was not significant at 1 month ($P = 0.172$). Changes in TMV were significant at 1, 6, and 12 months ($P = 0.026$, 0.002, and 0.049 resp.). Change in TMV was marginally significant at 3 months ($P = 0.092$) (Figure 1(c) and Figures 2(a)–2(e)).

BCVA data are summarized in Table 3. BCVA was improved or maintained within 0.2 log MAR in 29/32

Table 2: Summary of data from subjects who did and did not undergo additional SMDLP.

Variable	Treatment group	
	No additional SMDLP	Additional SMDLP
Eyes, *n*	21	11
Sex, *n* (%)		
Male	17 (81.0)	7 (63.6)
Female	4 (19.0)	4 (36.4)
Age, mean (SD), years	68.48 (10.63)	63.82 (7.25)
BCVA (SD), log MAR		
Baseline	0.4062 (0.3071)	0.2297 (0.2108)
1 month	0.3599 (0.3262)	0.1839 (0.2255)
3 months	0.3687 (0.3101)	0.2281 (0.2308)
6 months	0.3777 (0.3788)	0.2132 (0.2255)
12 months	0.3476 (0.3535)	0.1939 (0.2390)
CMT (SD), μm		
Baseline	393.524 (99.622)	383.727 (89.608)
1 month	369.809 (108.959)	377.909 (97.217)
3 months	313.857 (111.305)	391.091 (84.911)
6 months	267.095 (104.461)	372.000 (80.554)
12 months	266.240 (86.214)	400.182 (79.328)
TMV (SD), mm^3		
Baseline	8.1168 (0.7531)	8.2109 (1.2705)
1 month	8.0005 (0.6299)	8.2080 (1.0580)
3 months	7.8533 (0.6394)	8.322 (1.0524)
6 months	7.49 (0.3861)	7.99 (0.8307)
12 months	7.5547 (0.4610)	7.9929 (0.5118)

BCVA: best-corrected visual acuity; BRVO: branch retinal vein occlusion; SMDLP: subthreshold micropulse diode laser photocoagulation; CMT: central macular thickness; TMV: total macular volume.

Table 3: Change in CMT and TMV after subthreshold micropulse diode laser photocoagulation.

	Baseline	1 month	3 months	6 months	12 months
Mean CMT (μm) ± SD	390.16 ± 94.95	372.59 ± 103.55	340.41 ± 108.20	303.16 ± 108.15	312.28 ± 104.90
Mean TMV (mm^3) ± SD	8.151 ± 0.954	8.072 ± 0.791	8.021 ± 0.867	7.657 ± 0.606	7.683 ± 0.508
Macular thickness reduction[a] (%)		7/32 (21.9%)	12/32 (37.5%)	16/32 (50.0%)	14/32 (43.8%)

CMT: central macular thickness; TMV: total macular volume.
[a]Number of eyes with 20% or more reduction in CMT from baseline.

Table 4: Changes in BCVA after subthreshold micropulse diode laser photocoagulation ($n = 32$).

Change in BCVA[a]		1 month	3 months	6 months	12 months
Improved (*n*, %)	Total ($n = 32$)	3/32 (9.4%)	4/32 (12.5%)	3/32 (9.4%)	5/32 (15.6%)
	Group I ($n = 15$)	3/15 (20.0%)	4/15 (26.7%)	3/15 (20.0%)	5/15 (33.3%)
	Group II ($n = 17$)	0/17 (0%)	0/17 (0%)	0/17 (0%)	0/17 (0%)
Unchanged (*n*, %)	Total ($n = 32$)	29/32 (90.6%)	26/32 (81.3%)	26/32 (81.3%)	25/32 (78.1%)
	Group I ($n = 15$)	12/15 (80.0%)	11/15 (73.3%)	10/15 (66.7%)	9/15 (60.0%)
	Group II ($n = 17$)	17/17 (100.0%)	15/17 (88.2%)	16/17 (94.1%)	16/17 (94.1%)
Worsened (*n*, %)	Total ($n = 32$)	0/32 (0%)	2/32 (6.3%)	3/32 (9.4%)	2/32 (6.3%)
	Group I ($n = 15$)	0/15 (0%)	0/15 (0%)	2/15 (13.3%)	1/15 (6.7%)
	Group II ($n = 17$)	0/17 (0%)	2/17 (11.8%)	1/17 (5.9%)	1/17 (5.9%)

BCVA: best-corrected visual acuity; Group I: eyes with BCVA ≤ 20/40; Group II: eyes with BCVA > 20/40.
[a]Change in BCVA is defined as 0.2 or more log MAR (logarithm of the minimal angle of resolution) value. Improved: 0.2 or more log MAR gain; Unchanged: within 0.2 log MAR change; Worsened: 0.2 or more log MAR loss.

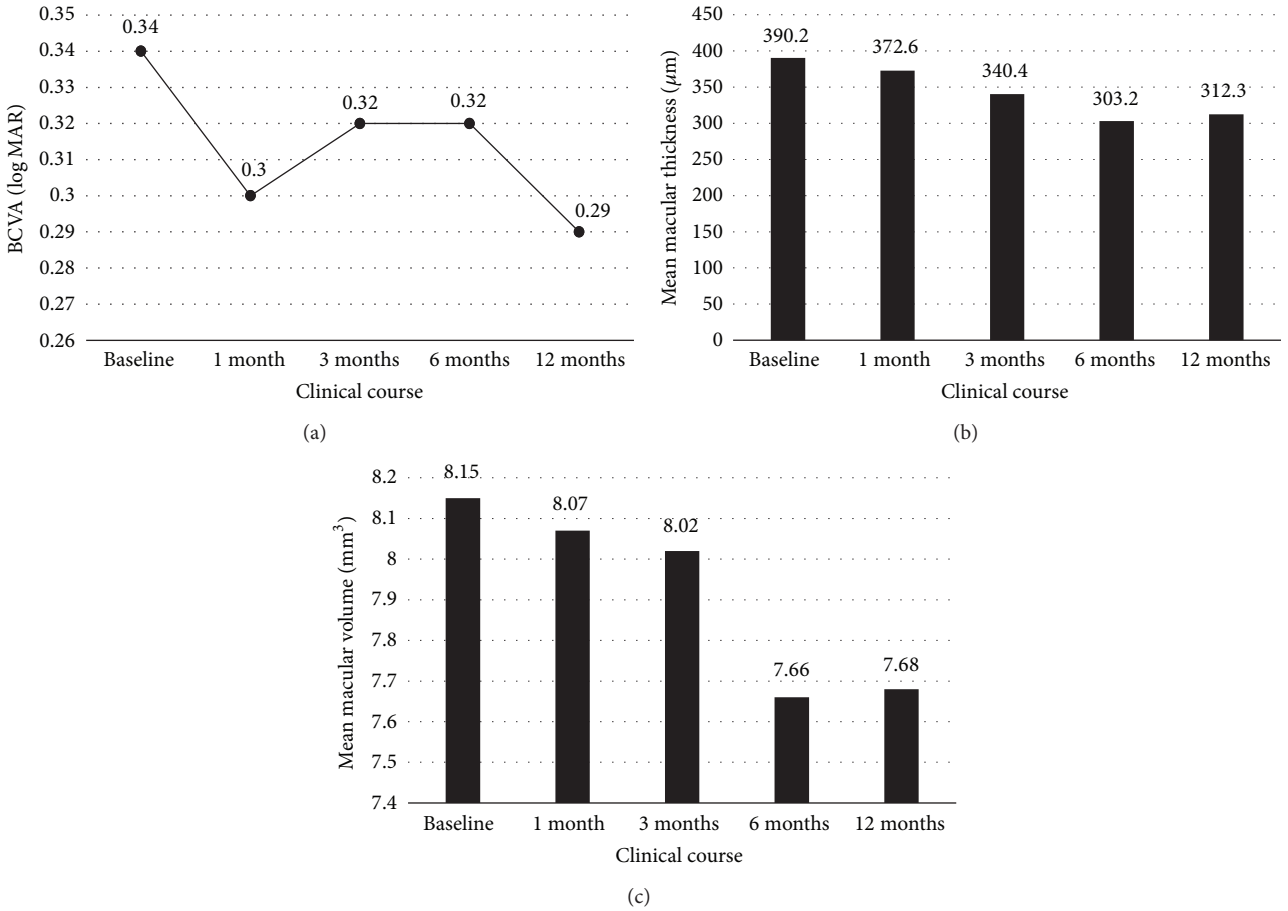


Figure 1: Changes in postoperative parameters: 12-month follow-up of all patients after subthreshold micropulse diode laser photocoagulation (SMDLP). (a) Changes in visual acuity (logarithm of the minimum angle of resolution) over time after SMDLP. Visual acuity showed significant improvements at 1 month and 12 months ($P = 0.004$ and 0.046 resp.). (b) Changes in macular thickness over time after SMDLP. Central macular thickness (CMT) showed a significant decrease at 3 months, and remained stable thereafter ($P < 0.01$). (c) Changes in macular volume over time after SMDLP. Macular volume showed significant reductions at 1, 6, and 12 months ($P = 0.049$, 0.002, and 0.049 resp.).

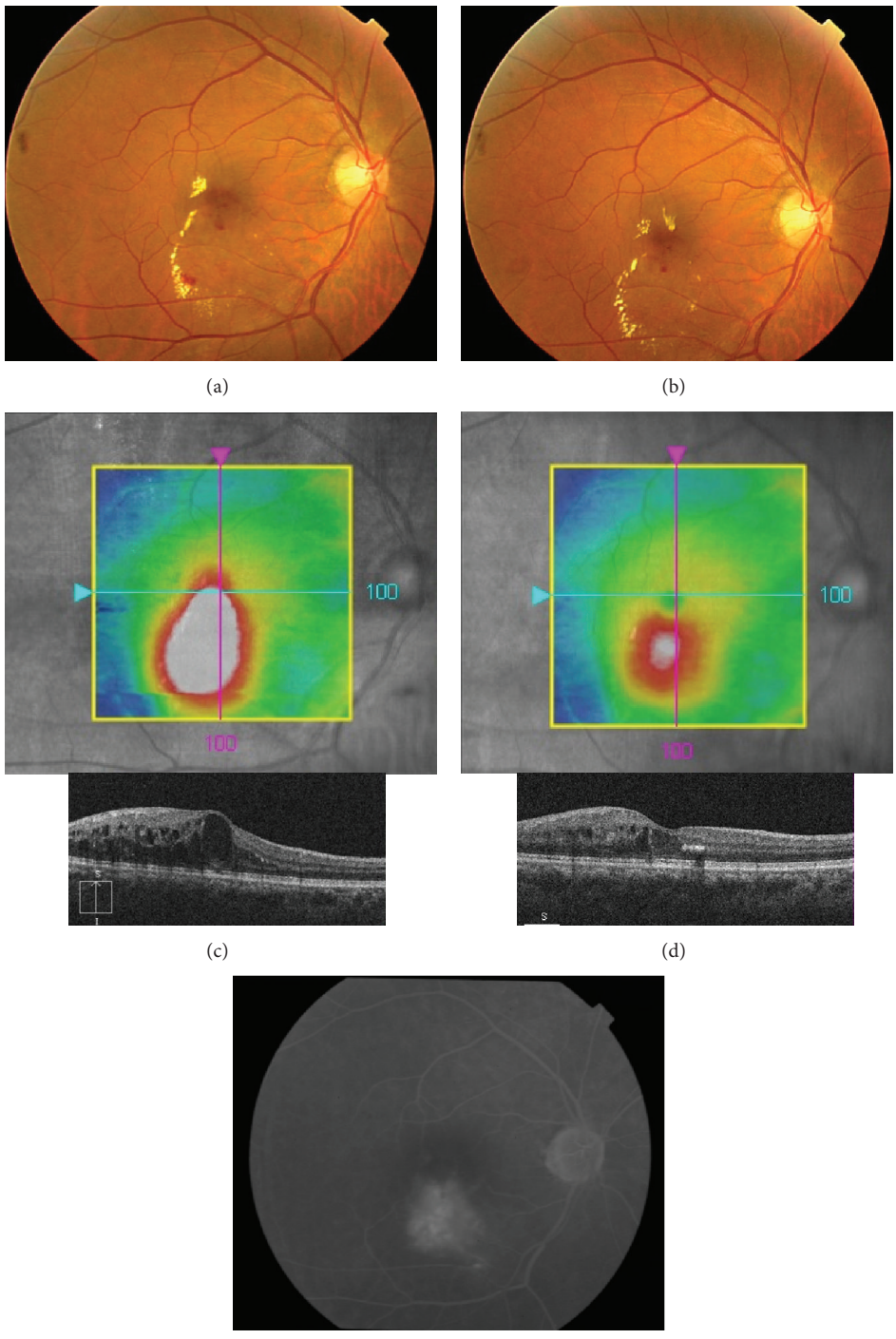
eyes (90.6%) at 6 months, and 30/32 eyes (93.8%) showed improvement or maintenance of BCVA at 12 months. Of the 21/32 (65.6%) eyes without additional treatment, 20/21 (95.2%) showed an improvement in BCVA of ≥0.2 log MAR or maintained it within 0.2 log MAR for 12 months. There was no significant change in BCVA in either group, at 12 months (Group I, $P = 0.129$; Group II, $P = 0.245$) (Figure 3(b)). Data on CMT, TMV, and ≥20% macular thickness reduction rate are shown in Table 3.

3.5.1. Comparison of Macular Parameters in Eyes with BCVA ≤ 20/40 and Eyes with BCVA > 20/40. Clinical courses of CMT and TMV are shown in Figures 3(a) and 3(c), respectively. At no time-point (baseline, 1, 3, 6, or 12 months) did Group I or Group II show any significant difference in CMT or TMV. In Group I, CMT was significantly reduced at 6 months and 12 months ($P = 0.009$ and 0.041 resp.) but not at 1 month or 3 months ($P = 0.281$ and 0.125 resp.). In Group II, CMT was significantly reduced at 6 months ($P = 0.015$) and was marginally reduced at 3 months and 12 months ($P = 0.062$ and 0.098 resp.) and there was no significant reduction at 1 month ($P = 0.368$). In Group I, TMV was significantly reduced at 1 month and 6 months ($P = 0.041$ and 0.028 resp.) but not at 3 months or 12 months ($P = 0.278$ and 0.182 resp.). In Group II, TMV was significantly reduced at 6 months ($P = 0.016$) but not at 1 month, 3 months, or 12 months ($P = 0.201$, 0.173, and 0.208 resp.).

3.5.2. Adverse Events and Macular Changes. When before and aftercolor fundus photographs were compared, no laser scars secondary to treatment were detected. Likewise, fluorescein angiograms showed no evidence of laser spots. No patients complained of ocular discomfort after SMDLP.

4. Discussion

The data from the present study demonstrate that SMDLP can effectively resolve macular edema and maintain visual acuity in Japanese patients with mild or moderate persistent macular edema secondary to BRVO, including patients with



(a) (b) (c) (d) (e)

Figure 2: Persistent macular edema secondary to branch retinal vein occlusion with best-corrected visual acuity (BCVA) > 20/40 treated by SMDLP. (a) Fundus color photograph obtained before SMDLP, showing cystoid macular edema. (b) Fundus color photograph at 3 months after SMDLP. (c) Optical coherence tomography at baseline. (d) Optical coherence tomography at 3 months after treatment. The baseline horizontal scan shows a cystoid area at the fovea that has improved at 3 months. CMT was 525 μm at baseline and 346 μm at 3 months. BCVA (logarithm of the minimum angle of resolution) was 0.046 before SMDLP and 0 at 3 months. (e) Baseline fluorescein angiography revealing diffuse dye leakage in the macular area. SMDLP was applied to the area of diffuse dye leakage.

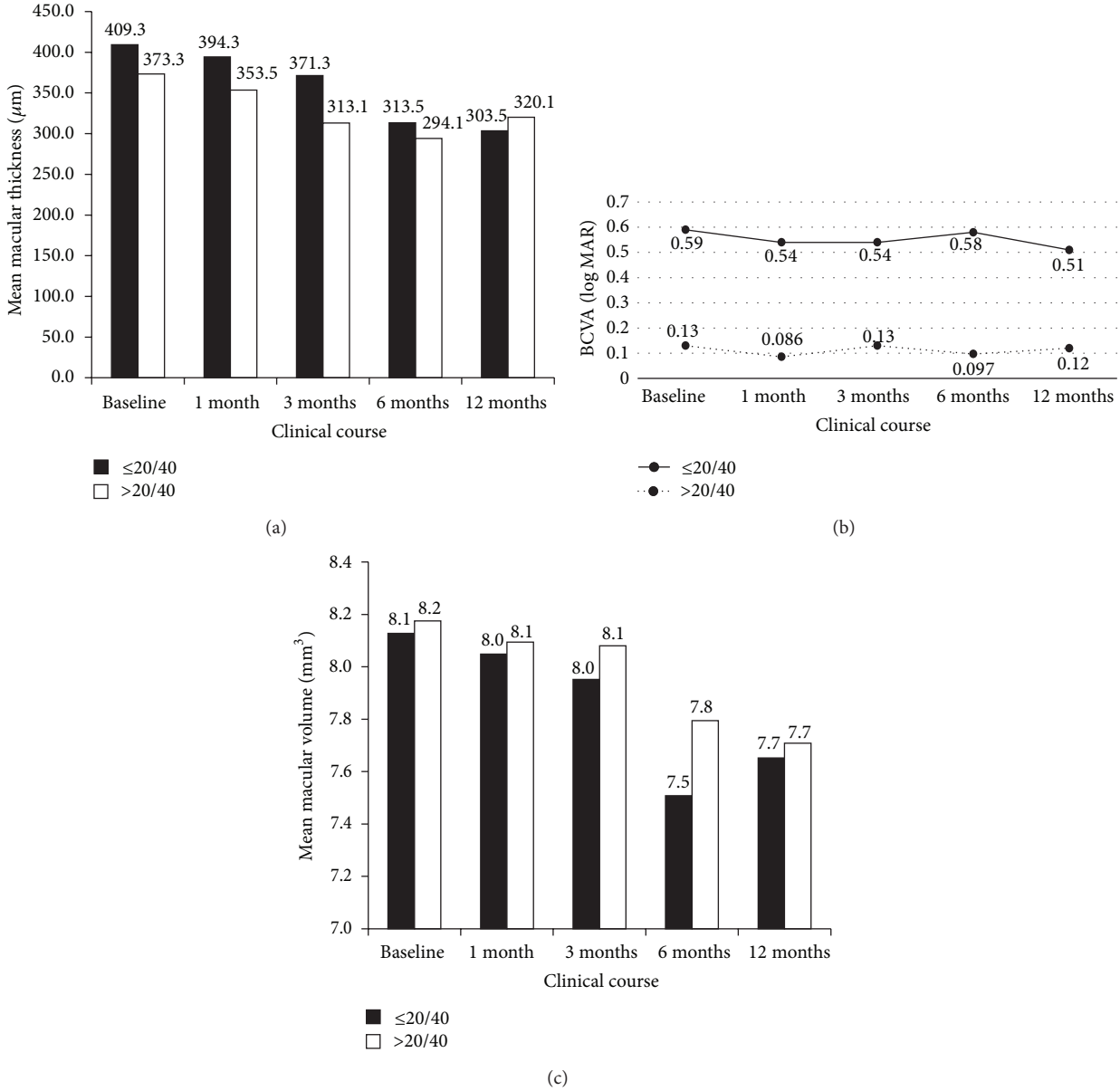


Figure 3: (a) Comparison of CMT in Group I (eyes with BCVA ≤ 20/40) versus Group II (eyes with BCVA > 20/40). No significant differences in CMT were evident between Group I and Group II at any time-point (baseline, 1, 3, 6, or 12 months) after the operation. In Group I, CMT showed significant reductions at 6 months and 12 months ($P = 0.009$ and 0.041 resp.). In Group II, CMT showed a significant reduction at 6 months ($P = 0.015$) and marginally significant reductions at 3 months and 12 months ($P = 0.062$ and 0.098 resp.). (b) Change in visual acuity over time after SMDLP in Groups I and II. There was no significant change in visual acuity throughout 12 months (Group I $P = 0.129$, Group II $P = 0.245$). (c) Comparison of total macular volume (TMV) in Group I (eyes with BCVA ≤ 20/40) versus Group II (eyes with BCVA > 20/40). No significant differences in TMV were evident between Group I and Group II at any time-point (baseline, 1, 3, 6, and 12 months) after the operation. In Group I, TMV showed significant reductions at 1 month and 6 months ($P = 0.041$ and 0.028 resp.). In Group II, TMV showed a significant reduction at 6 months ($P = 0.016$).

BCVA > 20/40. In the total subject-pool in this study, at 3 months after laser treatment, macular edema was significantly reduced ($P = 0.014$) and remained stable thereafter, and TMV was significantly reduced at 6 months ($P = 0.002$). Macular edema secondary to BRVO is typically self-resolving in nature [4, 25].

Hayreh and Zimmerman [25] reported median times to macular edema resolution of 21 months for major BRVO and 18 months for macular BRVO. It has been reported that macular edema due to BRVO naturally improves gradually over 18–21 months [25]. However, by design, all patients in the present study had persistent or recurrent macular edema,

and the median time to progression to persistent macular edema was 34.6 months for BRVO, which was not indicative of a "self-resolving" condition. Therefore, the early response observed suggests that the observed resolution of macular edema was a direct effect of laser treatment, rather than spontaneous resolution. All patients in the present study had been followed for at least 6 months since disease onset, with 34.4% already having undergone treatment such as steroid [25, 26] or anti-VEGF therapy [2, 3]. Therefore, the resolution of edema documented in this study was apparently a direct result of laser therapy, rather than a natural course of healing.

Grid laser treatment has been a standard treatment for BRVO for many years. In 1984, the BVOS group reported that 65% of patients treated by argon laser grid photocoagulation gained more than 2 lines of visual acuity, compared with only 37% of eyes with untreated BRVO at 3 years follow-up [1]. Unfortunately, conventional grid laser treatment delivered with a visible ophthalmoscopic end-point has been implicated in several long-term complications that can severely affect visual function, including scar enlargement [6–8], subretinal fibrosis [27–30], choroidal neovascularization [31, 32], and perimetric sensitivity deterioration [33–38]. In an attempt to minimize the drawbacks of conventional grid laser photocoagulation, several authors have proposed the use of SMDLP for diabetic macular edema, which has shown promising results [14–21]. SMDLP involves the release of micropulses with low energy per pulse, in order to confine that energy to RPE cells, avoiding lateral thermal spreading. Histopathologically, retinal morphological changes are minimal and no immediate biomicroscopic retinal changes are noted after laser application, and there is no laser scarring on long-term follow-up [11]. Current spectral domain OCT scans cannot optimally discern laser ablation sites [13]. In the present study, no laser scars were detected in any patients after SMDLP.

The clinical application of SMDLP was first reported by Friberg and Karatza [14] in 1999. Since then, several clinical studies have demonstrated the efficacy of SMDLP [16–23]. In 2010, Ohkoshi and Yamaguchi [20] reported the efficacy of SMDLP for diabetic macular edema in Japanese patients. In 2010, Lavinsky et al. [21] reported that high density SMDLP was more effective than modified Early Treatment Diabetic Retinopathy Study (ETDRS) laser treatment, in a randomized clinical trial.

Although the efficacy of SMDLP has been proposed for diabetic macular edema, very few reports have been published for BRVO [22, 23]. In 2006, Parodi et al. [22] reported that the efficacy of SMDLP in macular edema due to BRVO with BCVA ≤ 20/40 was similar to that of conventional threshold grid laser treatment but without biomicroscopic or angiographic signs in the SMDLP group, at 12 months and 24 months. In that study however, macular edema was not significantly reduced at 3 months or 6 months.

In 2008, Parodi et al. [23] reported that they could not achieve significant reduction of macular edema due to BRVO with BCVA ≤ 20/40 within 6 months after SMDLP. In the present study, patients with BCVA ≤ 20/40 maintained BCVA and exhibited significant reductions in CMT at 6 months and 12 months. In this study, significant visual improvement was not achieved, although CMT was significantly improved at 6 months. This may be due to the fact that all of the patients had had chronic persistent macular edema for more than 6 months. Patients with early onset macular edema whose natural course of macular edema and BCVA would be self-improving were excluded from this study.

In this study, patients with BCVA > 20/40 maintained BCVA for 12 months and exhibited marginally significant reductions in CMT at 3 months and 12 months and a significant reduction at 6 months. Although most patients with good visual acuity such as BCVA > 20/40 were observed without any intervention in previous studies [1–5, 22, 23] and the results of this study suggest that early intervention with SMDLP may maintain BCVA and reduce macular edema in cases with good visual acuity.

5. Conclusions

In conclusion, in patients with persistent macular edema secondary to BRVO, SMDLP appears to control macular edema, with minimal retinal damage. Our findings suggest that SMDLP is an effective treatment method for macular edema in BRVO patients, including those with BCVA > 20/40. Limitations of this study include lack of randomization, the fact that it was not a prospective trial, and the relatively low number of patients. A randomized study would be necessary to prove the efficacy of SMDLP for macula edema secondary to BRVO.

Conflict of Interests

The authors declare that there are no conflict of interests with regard to the publication of this paper.

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Review Article

Current Treatment of Toxoplasma Retinochoroiditis: An Evidence-Based Review

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Objective. To perform an evidence-based review of treatments for *Toxoplasma* retinochoroiditis (TRC). *Methods*. A systematic literature search was performed using the PubMed database and the key phrase "ocular toxoplasmosis treatment" and the filter for "controlled clinical trial" and "randomized clinical trial" as well as OVID medline (1946 to May week 2 2014) using the keyword "ocular toxoplasmosis". The included studies were used to evaluate the various treatment modalities of TRC. *Results*. The electronic search yielded a total of 974 publications of which 44 reported on the treatment of ocular toxoplasmosis. There were 9 randomized controlled studies and an additional 3 comparative studies on the treatment of acute TRC with systemic or intravitreous antibiotics or on reducing the recurrences of TRC. Endpoints of studies included visual acuity improvement, inflammatory response, lesion size changes, recurrences of lesions, and adverse effects of medications. *Conclusions*. There was conflicting evidence as to the effectiveness of systemic antibiotics for TRC. There is no evidence to support that one antibiotic regimen is superior to another so choice needs to be informed by the safety profile. Intravitreous clindamycin with dexamethasone seems to be as effective as systemic treatments. There is currently level I evidence that intermittent trimethoprim-sulfamethoxazole prevents recurrence of the disease.

1. Introduction

Ocular toxoplasmosis is the commonest cause of posterior uveitis and is usually the result of an acquired infection caused by the protozoan *Toxoplasma gondii* [1, 2]. The most common manifestation of ocular toxoplasmosis is *Toxoplasma* retinochoroiditis which is typically a unilateral, unifocal, large lesion (greater than 1 disc diameter) typically associated with vitreitis that is in the posterior pole in two-thirds of cases [2, 3]. A granulomatous anterior chamber inflammation is frequent, and retina vasculitis (usually arteriolitis) is present in about a third of patients [2–5]. Visual acuity loss during acute toxoplasma retinochoroiditis results from vitreitis or from involvement of the macula or optic nerve. Visual loss may become permanent due to formation of a macular scar or due to optic atrophy so that 24% of patients have vision of 20/200 or less in at least one eye [5, 6]. The scarring resulting from Toxoplasma retinochoroiditis can be associated with severe visual field loss when it occurs close to the optic disc [7].

There is no consensus as to what the best treatment for *Toxoplasma* retinochoroiditis might be. The most recent systematic evidence-based review of the literature considered articles published up to July 2011 [8]. There have been significant additional contributions to the literature since that time and we wished to repeat a systematic evidence-based review of the literature incorporating our observations on the studies reviewed. We therefore performed this updated systematic literature review to evaluate the treatments for toxoplasma retinochoroiditis.

2. Literature Search

A PubMed (National Library of Medicine) search was conducted using the key phrase "ocular toxoplasmosis treatment" and a filter for "controlled clinical trial" and "randomized

clinical trial." Additionally an OVID medline (1946-May week 2 2014) search was conducted using the keyword "ocular toxoplasmosis." Articles were limited to articles published in English. There were no restrictions on age, ethnicity, or geographic locations of patients.

3. Results

We found a total of 974 publications and reviewed the abstracts to select publications reporting on treatment outcomes of *Toxoplasma* retinochoroiditis. We found 29 publications written in English reporting on outcomes of treatment of *Toxoplasma* retinochoroiditis. The studies used various combinations of endpoints to determine the efficacy and safety of the medications. All studies reported improvement in symptoms associated with ocular toxoplasmosis after treatment. Resolution or improvement in ocular findings was seen within varying time points ranging from 6 weeks to 20 months between trials. There were sources of clinical heterogeneity among studies such as duration and severity of ocular toxoplasmosis, age, and previous treatments used by patients. Therapies also varied in their dosages, duration, frequency, and combinations, making it difficult to compare across studies. There were several studies where the scales used for evaluating endpoint parameters were not well-defined and quality of life and subjective assessments of treatments were not found among the reviewed studies.

We used three subheadings to discuss the treatments of *Toxoplasma* retinochoroiditis: systemic antibiotic treatments, intravitreous antibiotic treatments, and treatments to reduce the rate of recurrence of toxoplasma retinochoroiditis.

3.1. Systemic Antibiotic Treatments for Active Toxoplasma Retinochoroiditis. In 1956, Perkins and colleagues published a double-masked, randomized, and controlled study which included 43 patients with *Toxoplasma* retinochoroiditis treated using either a 2-week course of pyrimethamine or placebo, showing statistically significant improvement compared to placebo [9]. Since that time a number of mainly non-comparative case series have been published purporting that clindamycin [10–12], spiramycin [13], azithromycin [14, 15], trimethoprim-sulfamethoxazole [16], atovaquone [17], alone or in combination with pyrimethamine, and/or sulfadiazine are effective in the treatment of toxoplasmosis. Given the self-limiting nature of *Toxoplasma* retinochoroiditis in immunocompetent individuals noncomparative case series have little role in establishing the efficacy of any particular agent, especially compared to established treatments. We found 2 retrospective comparative studies, 2 prospective comparative studies (although there was significant overlap of patients reported in these 2 studies), and 4 randomized controlled studies on the systemic treatment of *Toxoplasma gondii* retinochoroiditis.

3.1.1. Prospective or Retrospective Comparative Studies. A retrospective, comparative, single-centre study published in 1962 by Fajardo et al. [18] compared the efficacy of 3 treatment regimens for *Toxoplasma* retinochoroiditis on 87 patients. The treatments consisted of pyrimethamine (100 mg initially, then 50 mg), sulfadiazine (1 g qid), and methylprednisolone (4 mg tid); spiramycin (2 g qd) and methylprednisolone (4 mg tid); and methylprednisolone (4 mg tid) alone. The authors reported that the interval to inactivity (resolution of inflammation and scarring of the retinal lesion) was shorter in the group treated with pyrimethamine and sulfadiazine with a statistically significant greater proportion of patients becoming inactive within the first 8 weeks compared to the other treatments, with no differences in visual outcomes [18].

Similarly in a retrospective, comparative, and single-centre study published by Nolan and Rosen reporting on 69 patients, the efficacy of 2 treatments for Toxoplasma retinochoroiditis was compared to treatment with corticosteroids or observation [19]. The treatments were either pyrimethamine (100 mg loading dose then 25 mg daily) or spiramycin (1–4 g daily). Pyrimethamine, but not spiramycin, was found to have significantly reduced the healing time [19].

The above results were in contrast to the initial report from a prospective multicenter study from the Netherlands that compared 3 treatment regimens to observation [20]. The treatment regimens consisted of either pyrimethamine (100 mg for 1 day, then 25 mg bid), sulfadiazine (1 g qid), folinic acid (5 mg), and prednisone (60 mg then taper); clindamycin (300 mg qid), sulfadiazine (1 g qid), and prednisone (60 mg, then taper); or trimethoprim-sulfamethoxazole (160–800 mg bid for 2 weeks then 80–400 mg bid). The 106 patients recruited were assigned to treatment depending on the center at which they were treated (not randomly); they were assigned to observation if the lesions were in the periphery. The authors reported no significant differences between treatments or comparing the treatments to observation in terms of duration of inflammatory activity or reduction in size of the lesion. Visual outcomes or rates of recurrence were not reported. The pyrimethamine-sulfadiazine group had the highest frequency of adverse events (52%), including thrombocytopenia, leukopenia, rashes, and fever [20].

The same group from the Netherlands then published an overlapping publication with 149 patients assigned to the groups described above (presumably the 106 patients in their prior publication were included) [21]. Again there was no difference in the duration of inflammatory activity, visual acuity, or rate of recurrence (mean 49% at 3 years) between the treated and untreated groups. The authors reported that there was marked decrease (at least 0.5 disc diameter) in the size of the lesion in 49% of pyrimethamine treated patients compared to 28% in clindamycin-treated patients, 11% of trimethoprim-sulfamethoxazole treated patients, and 20% in the observation group. The difference between the pyrimethamine group and the observation group was statistically significant for this measure. It should be noted that the lesion size was measured from fundus photographs in the treatment groups (as the lesion was in the posterior pole) while for lesions in the observation groups the lesion size was estimated from drawings of the peripheral retina; the comparison may, therefore, have been biased to show greater efficacy in the treatment groups. Moreover, a chi-squared test was used with no attempt to adjust for multiple comparisons. Further, the original publication in 1989 had

found no statistically significant difference and it was only when the additional 33 patients were added that such a difference was found in the 1993 paper by the same group [21]. It is therefore unfortunate that subsequent reviews of the literature on the treatment of ocular toxoplasmosis have given much weight to this finding. In our view, these overlapping papers support the use of observation for peripheral lesions and suggest that all the treatments employed in the study had similar efficacy with pyrimethamine-sulfadiazine having the worse systemic safety profile.

3.1.2. Randomized-Controlled Studies of Oral Antimicrobials for Active Toxoplasma Retinochoroiditis

Triple Therapy versus Steroid Alone. A randomized, placebo-controlled, and double-masked study by Acers [22] compared the efficacy of pyrimethamine (200 mg on day 1, 100 mg on day 2, 50 mg on days 3–15, and 25 mg on days 16–56), trisulfapyrimidines (2 g), and prednisone 40 mg to prednisone 40 mg alone for active toxoplasma retinochoroiditis. Only 20 patients were recruited to the study and randomized 1 : 1 to each of the groups. No difference was found in the time to inactivity or visual acuity between the 2 groups. In the pyrimethamine-trisulfapyrimidine group 30% of patients developed an adverse event (usually nausea, anorexia, or arthralgia), with 1 patient developing severe thrombocytopenia [22]. The study was limited by the low patient numbers. While the study further questions the efficacy of routine systemic antibiotics for *Toxoplasma* retinochoroiditis it cannot be overstated that several studies since have documented that corticosteroid administration without antiparasitic treatment can lead to a fulminant necrotizing retinochoroiditis and worse visual outcomes [6].

Trimethoprim-Sulfamethoxazole versus Triple Therapy. A randomized, single-blinded study by Soheilian et al. [23] on 59 patients compared the efficacy and safety of trimethoprim-sulfamethoxazole (160 mg–800 mg) against classic therapy triple therapy with pyrimethamine (100 mg for 2 days, then 25 mg daily), sulfadiazine (2 g), and folinic acid (5 mg) with both treatment groups receiving adjuvant prednisone. Randomization was 1 : 1. No significant differences were found in terms of lesion size, mean improvement in visual acuity, recurrence rates, and adverse events to drug therapy, although 5 patients (17%) in each group were lost to follow-up. One patient in each treatment group developed an adverse reaction to their respective treatment (both developed a rash). The authors concluded that trimethoprim-sulfamethoxazole was a reasonable alternative to classic triple therapy; [23] however, the study has been criticized for using half the dose of pyrimethamine and sulfadiazine commonly used in clinical practice, as well as the large numbers of patients lost to follow-up and limited numbers of patients recruited to the study.

Azithromycin versus Triple Therapy. Two studies compared regimens with azithromycin against triple therapy with pyrimethamine, sulfadiazine, and folinic acid. In a 2002 randomized, open-label, and controlled study, Bosch-Driessen et al. [24] compared the efficacy of 4 weeks of azithromycin (250 mg)-pyrimethamine (100 mg on day 1, then 50 mg)-folinic acid (15 mg) versus sulfadiazine (4 g)-pyrimethamine (100 mg on day 1, then 50 mg)-folinic acid (15 mg), or the treatment of active toxoplasma retinochoroiditis. Randomization of the 43 patients was 1 : 1. Both groups received adjuvant prednisone. There were no significant differences between treatment groups on the duration of inflammation, change in lesion size, improvement in visual acuity, or risk of recurrence. Adverse effects were more frequent in the sulfadiazine group (64%), requiring discontinuation of treatment in 3 patients (14%). Adverse effects were less common in the azithromycin group (33%), although 1 patient in the azithromycin group died from a cerebral aneurysm during the course of the study. The study provides some evidence that azithromycin with pyrimethamine and folinic acid is a reasonable alternative to sulfadiazine with pyrimethamine and folinic acid [24].

In a more recent randomized, open-label study, Balaskas et al. [25] compared azithromycin (500 mg) to triple therapy consisting of 50 mg pyrimethamine, 4 g of sulfadiazine (3 g if the patient weighed less than 65 kg), and folinic acid (15 mg); both groups received adjuvant prednisone. Patients were randomized 1 : 1 to each of the groups. There was no significant difference in the number of responders to treatment, with all the patients responding to treatment in the triple therapy group and 90% of patients responding to treatment in the azithromycin group. Adverse events such as malaise, dizziness, headaches, and gastrointestinal disturbances were reported by all patients in the triple therapy group compared to none in the azithromycin group. The study was limited by small numbers, having recruited a total of 19 patients [25]. Therefore the question of whether azithromycin is or not as effective as triple therapy remains unanswered, although it appears that it is better tolerated than triple therapy.

There is conflicting evidence as to whether systemic antibiotics are effective in the treatment of toxoplasma retinochoroiditis in the first place, although the preponderance of evidence suggests some effects [9, 18–22]. Pyrimethamine is known to frequently result in bone marrow suppression leading to thrombocytopenia, leukopenia, and anemia [9, 26], while severe hepatotoxicity is a well-known complication of sulfadiazine therapy [26, 27], skin rashes, anorexia, nausea, and lassitude are quite common with either medication [25]. There is some evidence [20, 21, 23–25], including that from randomized clinical trials [23–25], to suggest that trimethoprim-sulfamethoxazole or azithromycin may be no less effective than pyrimethamine-sulfadiazine and both of the former have more adverse effects than the latter. There is also some evidence from a prospective comparative trial to suggest that this may also be true of clindamycin, although the systemic safety profile for clindamycin (mainly gastrointestinal upset) is worse than that of trimethoprim-sulfamethoxazole or azithromycin [20, 21]. Interestingly, a recent meta-analysis of treatment of toxoplasmic encephalitis in HIV-infected patients showed that trimethoprim-sulfamethoxazole was noninferior to pyrimethamine-sulfadiazine [28]. Trimethoprim-sulfamethoxazole is readily available and is the least expensive

of the two so may be the best first-line treatment if the clinician is inclined not to observe *Toxoplasma* retinochoroiditis.

3.2. Intravitreous Treatments for Toxoplasma Retinochoroiditis. Tabbara and colleagues demonstrated the efficacy of periocular clindamycin (subTenon's or retrobulbar) in a rabbit model of *Toxoplasma* retinochoroiditis in the 1970s [29, 30]. Dr. Peyman's group then reported the resolution of inflammation and improvement in the vision following intravitreous clindamycin and dexamethasone (IVTCD) together with systemic sulfadiazine in the first trimester of pregnancy of a patient with a *Toxoplasma* retinochoroiditis lesion in the maculopapillary bundle [31]. Two noncomparative retrospective case series described 6 and 12 patients, respectively, with *Toxoplasma* retinochoroiditis that were treated with IVTCD due to intolerance, contraindication (pregnancy), or lack of response to oral medication and both reported functional and anatomic improvement [32, 33]. Given the generally self-limiting nature of toxoplasma retinochoroiditis case series such as the above do not establish the efficacy of intravitreous treatment for this condition. We found 2 randomized clinical trials evaluating intravitreous clindamycin-dexamethasone for *Toxoplasma* retinochoroiditis [34].

3.2.1. Randomized, Controlled Studies Evaluating Intravitreous Clindamycin-Dexamethasone for Toxoplasma Retinochoroiditis. In a randomized, single-masked trial, Sohleilianet al. [34] investigated the efficacy of intravitreous clindamycin (1 mg) and dexamethasone (0.4 mg) compared to pyrimethamine (75 mg for 2 days, then 25 mg), sulfadiazine (4 g for 2 days, then 2 g daily), folinic acid (5 mg), and prednisone. The 81 patients participating in the study were randomized 1 : 1 to each group and follow-up was available in 84% of patients. In the IVTCD group, 47% of patients required more than one injection; IVTCD could be repeated every 2 weeks based on clinical response at the discretion of the examiner. No significant differences between the two groups in the primary endpoint of lesion size reduction were reported; similarly the authors found no differences between the two groups in improvement in visual acuity, resolution of the vitreous inflammation, or rates of recurrence (5.9% in each group by 2 years). There were 2 serious adverse events in the group treated with triple therapy (1 patient developed a severe rash and another thrombocytopenia necessitating cessation of treatment in both cases); in the group receiving IVTCD there were injection-site related complications (subconjunctival hemorrhage) but no systemic adverse events. Of note, the study discovered that IgM-positive cases responded better to classic therapy and IgM-negative cases respond better to IVCD therapy in terms of lesion size reduction [34]. It should be noted that the dose of pyrimethamine and sulfadiazine used in this study was half the dose commonly used in clinical practice in the United States; additionally, there was a 16% loss to follow-up, analysis was not carried out on an intent-to-treat basis, and the numbers were limited, somewhat limiting the clinical applicability of the study's findings.

A randomized, single-masked study by Baharivand and colleagues [35] compared intravitreous clindamycin (1 mg) and dexamethasone (0.4 mg) with pyrimethamine (75 mg for 2 days, then 25 mg), sulfadiazine (2 mg for 2 days, then 4 mg), folinic acid (5 mg), and prednisone (50 mg) for 6 weeks. Sixty-eight patients were randomized 1 : 1 to each treatment group. There was no significant difference between the two groups in terms of change in visual acuity, lesion size, resolution of inflammation, or recurrence rate. In the IVTCD group 88% of patients received a single injection. There was 1 episode of hepatotoxicity reported in the triple therapy group and there were no adverse drug events in the IVCD group. It should be noted that the dose of pyrimethamine used in this study was half that in common clinical practice in the United States [35].

Despite the limitations of the above studies, the preponderance of the (currently limited) evidence suggests that intravitreous clindamycin and dexamethasone is a reasonable alternative to systemic antimicrobial therapy in patients unresponsive or intolerant to oral anti-Toxoplasma medications or when these are contraindicated due to pregnancy. Further, the current evidence, while weak, does not refute the opinion that it is not unreasonable to use IVTCD as a first line treatment. It should be noted that a significant proportion of patients need IVTCD repeated every 1-2 weeks. The greatest advantage of this treatment is its systemic safety profile, although it should be noted that there has been a case report of a generalized rash following intravitreous clindamycin; therefore patients with a known allergy to oral clindamycin may not be suitable candidates for this treatment [36].

3.3. Treatments to Reduce Recurrent Rates of Toxoplasma Retinochoroiditis. Three approaches have been evaluated to prevent recurrences of toxoplasma retinochoroiditis. The first such approach historically was the application of laser photocoagulation directly on the lesion or in the immediately surrounding retina. For example, in 1966, Spalter et al. [37] presented a case series of 24 patients with a history of recurrent toxoplasma retinochoroiditis whose lesions were surrounded with laser photocoagulation. During a follow-up period ranging from 8 to 33 months there were only 2 recurrences (8%) and these were distant to the lesions treated [37]. However, in a case series of 35 patients that received laser photocoagulation around foci of *Toxoplasma* retinochoroiditis the recurrence rate was 53% in 5 years [38]. Further, in a comparative study of 33 patients treated either with laser around the foci or with triple therapy there was no difference in the rate of recurrence between the two groups [39]. Laser photocoagulation of *Toxoplasma* retinochoroiditis lesions is not a current practice to prevent recurrences given the above evidence.

A second approach was the use of atovaquone or azithromycin to treat acute episodes of *Toxoplasma* retinochoroiditis. Both atovaquone and azithromycin have demonstrated cysticidal activity in preclinical models and it had been hoped that acute treatment with one of these agents would prevent recurrence of toxoplasma retinochoroiditis [40]. Unfortunately, it is clear that this is not the case. The largest series of patients treated with atovaquone was a retrospective case series of 41 patients treated for 6 weeks: the recurrence rate was 27% by 2 years and 75% by 6 years [41]. Similarly, Rothova et al. [15] published a retrospective case series of 11

immunocompetent patients who were treated for toxoplasma retinochoroiditis with a 5-week course of azithromycin; recurrence was noted in 27% of patients within the first year [15]. Further, in a randomized controlled study comparing the combination of azithromycin with pyrimethamine versus sulfadiazine with pyrimethamine there was no statistical difference in the rate of recurrences [24]. Therefore, while atovaquone or azithromycin are reasonable treatment options for treatment of acute *Toxoplasma* retinochoroiditis they have no role in preventing recurrences.

Long-term use of anti-Toxoplasma agents to prevent recurrences has been the third approach evaluated. Indeed, in a prospective, randomized, and open-label trial, Silveira et al. [42] studied the effects of long-term intermittent trimethoprim-sulfamethoxazole (160 mg–800 mg) on recurrence rates of toxoplasmic retinochoroiditis. In this study, 124 patients with a history of recurrent toxoplasma chorioretinitis documented clinically and with positive serology for *T. gondii* were randomized 1 : 1 to an observation group or to receive trimethoprim-sulfamethoxazole every 3 days for 20 consecutive months. The endpoint of recurrence of *Toxoplasma* retinochoroiditis was met by 23.8% of patients in the observation group and 6.6% of patients in the treatment group, a difference that was statistically significant. There were no qualitative differences between recurrences (e.g., amount of inflammation, extent of active retinochoroiditis, etc.) in the 2 groups. It should be noted that 4 patients (15.5%) in the treatment group withdrew from the study due to mild allergic reactions while an additional 2 patients (3.2%) in the treatment group and 4 patients (15.5%) in the control group were lost to follow-up [42].

More recently, Felix et al. [43] published the results of a well-conducted double-masked randomized placebo-controlled study on the effects of trimethoprim-sulfamethoxazole on recurrence rates of toxoplasma retinochoroiditis. In this study, following treatment for active toxoplasma retinochoroiditis with trimethoprim-sulfamethoxazole (160 mg–800 mg) for 45 days, 95 patients were randomized 1 : 1 to treatment with either trimethoprim-sulfamethoxazole or placebo every 2 days. By 12 months there had been no recurrences in the treatment group, while recurrence was noted in 12.8% of patients in the placebo group [43].

In conclusion, there is level I evidence that intermittent use of trimethoprim-sulfamethoxazole (every 2-3 days) following an active episode significantly reduces the risk of recurrence for at least 1 year after the active episode. Considering the low cost of this medication, use of trimethoprim-sulfamethoxazole should be strongly considered in the absence of contraindications.

4. Conclusions

We noted conflicting evidence as to the efficacy of systemic or intravitreous antibiotics in the treatment of *Toxoplasma* retinochoroiditis, with the preponderance of evidence suggesting that they are effective. Whilst acknowledging the limitations of the evidence available it seems that trimethoprim-sulfamethoxazole may the best first-line treatment of *Toxoplasma* retinochoroiditis, with intravitreous clindamycin with dexamethasone an alternative for patients intolerant, unresponsive or with a contraindication (such as pregnancy) to trimethoprim-sulfamethoxazole. There is level I evidence that trimethoprim-sulfamethoxazole taken intermittently reduces the risk of recurrence.

Our review did not discuss the adjuvant use of corticosteroids as this was well covered in a very recent Cochrane review that found no evidence from randomized controlled studies to support their use or indeed support concerns that their use as adjuvants to anti-Toxoplasma treatment may lead to worse outcomes [44].

Conflict of Interests

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

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Research Article

Surgical and Visual Outcome for Recurrent Retinal Detachment Surgery

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Purpose. To evaluate the anatomical and functional outcome of repeated surgeries for recurrent retinal detachment. *Methods*. We retrospectively reviewed 70 cases with refractory retinal detachment of various etiologies that required multiple operations. Anatomical success (attached retina) or failure (totally/partially-detached retina) was assessed biomicroscopically. The BCVA was used for the evaluation of the functional outcome, at presentation and at the end of follow-up. Various pre-, intra-, and postoperative factors were associated with anatomical success or failure as well as with final functionality. *Results*. The mean number of surgeries was 4 (range: 2 to 10). The anatomical success rate was 80% (56 attached cases, 14 detached cases). 29% of the attached cases had a BCVA better than 20/40 (Snellen chart). The number of operations doesn't seem to affect significantly the final visual acuity. The PVR was found to affect both the anatomical and functional outcome ($P = 0.014$ & $P = 0.002$, respectively). *Conclusions*. In the present study, it is suggested that multiple operations for refractory retinal detachment may result in successful anatomic results, with a fare functional outcome at the same time. Eventually, we verified that the existence of PVR worsens the prognosis.

1. Introduction

The advances of vitreoretinal microsurgical techniques of the recent years have facilitated multiple consecutive interventions in a single eye, in cases where the pathology persists. Recurrent retinal detachment (RRD), once a difficult to manage outcome, today represents an indication for repeated surgery. However, the recurrence of a retinal detachment (RD) still represents a factor that is considered to influence unfavorably the final surgical outcome. Several studies have reported the results of specific patients' subgroups that required repeated retinal detachment surgery. However, the overall influence of repeated surgeries is unclear, since only few studies have reported the functional and anatomical results of patients with retinal detachment of various types that required multiple operations. In this paper, we study retrospectively the functional and anatomical results of a series of patients that required repeated operations for the management of retinal detachments related to a variety of pathologies, in the Retina Service of the University Hospital of Geneva.

2. Methods

This is a retrospective observational case series in which we reviewed the records of retinal detachment cases and included 70 patients that have been operated on for recurrent retinal detachment in the Retina Service of the Ophthalmology Clinic, University of Geneva, Switzerland. Patients' age ranged from 17 to 83 years (mean 60 years, SD = 13), 56 were males and 14 were females. All patients were operated

Table 1: Demographics of the study group.

N	Age mean (SD)	Gender	Eye
Total (70)	61 (13)	M: 54 F: 16	OD: 36 OS: 34
Pseudophakics (31)	65 (11)	M: 26 F: 05	OD: 16 OS: 15
Phakics (39)			
HM/GT[a] (14)	59 (12)	M: 12 F: 02	OD: 05 OS: 09
Trauma (7)	46 (15)	M: 07 F: 00	OD: 06 OS: 01
PRRD[b] (18)	60 (12)	M: 11 F: 07	OD: 10 OS: 08

[a]HM: high myopia, GT: giant tears.
[b]PRRD: primary rhegmatogenous retinal detachment, other than trauma, high myopia, or giant tears.

on by two experienced posterior segment surgeons and had a postoperative follow-up of at least 6 months after the last intervention. All postoperative follow-ups were made at the University Eye Clinic of Geneva.

Data collected from the patient records included patient age and gender; etiology of retinal detachment; best corrected visual acuity (BCVA) before the first intervention; intraoperative techniques and maneuvers used in each patient's consecutive operations such as utilization of scleral buckling (SB), vitrectomy, encircling band (EB), retinectomy, endolaser, cryopexy, gas, and silicone oil (sil-oil); intraoperative vitreous entrapment at the sclerotomy sites; presence of an epiretinal membrane (ERM); presence of proliferative vitreoretinopathy (PVR); discovery of new or missed breaks; postoperative complications; number of operations for each patient; anatomical status of the retina at the end of the follow-up; BCVA at the end of the follow-up; intraocular pressure (IOP) at the end of the follow-up time; and total duration of the follow-up period.

Preoperative and postoperative visual acuity was measured with the Snellen chart. For statistical analysis, visual acuity measurements were converted to logMAR values using appropriate calculations [1, 2].

Patients were divided into 4 groups based on the etiology of their primary retinal detachment as shown in Table 1. Patients having a giant tear detachment or detachments due to high myopia were reported as group 1 ($n = 14$; 13 males, 1 female; mean age 60 years). Patients having a pseudophakic detachment were reported as group 2 ($n = 31$ patients; 25 males and 6 females; mean age 46 years). Patients having a posttraumatic retinal detachment were reported as group 3 ($n = 7$; 6 males and 1 female; mean age 65 years). Finally, phakic patients with primary RD were reported as group 4 ($n = 18$; 12 males, 6 females; mean age 59 years). Anatomical success was considered the total attachment of the retina at the end of the follow-up time. Totally or partially detached retina was considered as failure.

The present research has followed the Tenets of the Declaration of Helsinki.

2.1. Statistical Analysis. Patients were divided into two groups based on anatomical success at the end of the follow-up time as defined above: group of attached patients and group of detached patients. The BCVA (in logMAR) at presentation and the end of follow-up was compared with the Wilcoxon Signed Ranks Test for two related samples (as the distribution of the variables was not normal).

Comparison of variables between the two anatomical groups (attached-detached) was assessed with the nonparametric Mann-Whitney U Test.

The effect of preoperative variables, the intraoperative maneuvers, and postoperative complications on the anatomical outcome was tested, after appropriate transformation of the variables, with the Chi-square and Fisher's exact test when applicable. After justifying the significant associations, a multivariate analysis was assessed using logistic regression for the factors having significant impact on the outcome.

Further analysis was assessed for the anatomically successful group (i.e., group with attached retina). In this subgroup, the factors that may allow for a final BCVA greater than 20/40 (0.3logMAR) were investigated. Similarly, for these associations, univariate (Chi-square and Fisher's exact test) and multivariate (logistic regression) analysis was attempted.

For all the above, the PASW Statistics 17.0 was used (©2009 SPSS Inc., Chicago, Illinois, USA).

3. Results

The postoperative follow-up ranged from 6 to 95 months. The mean number of reoperations was 4, ranging from 2 to 10.

3.1. Anatomic Results. Among the 70 cases investigated, 56 (80%) ended up with attached and 14 (20%) with detached retina. The two anatomical groups were compared for the parameters shown in Table 2. Statistical significance was found for the existence of PVR and the performance of retinectomy. When a multivariate analysis was done, using the variables that were found statistically significant in the univariate analysis, only the existence of PVR seemed to have some effect on the anatomical outcome. This effect, however, marginally missed reaching statistical significance ($P = 0.056$).

3.2. Functional Results. The functional outcome, in terms of BCVA, was examined in the group with successful anatomical outcome (attached retina). The average visual acuity at the last follow-up in the group of attached patients was 0.9logMAR (20/160 Snellen) compared to a vision of 1.4logMAR (20/500 Snellen) at their initial presentation. This represents an improvement of 0.5logMAR units and it was statistically significant ($P = 0.039$).

The cut-off of 20/40 Snellen (0.3logMAR) was used to evaluate the factors that may predispose for moderate to high final visual acuity. Same parameters as in previous comparison were used and are shown in Table 3. Among the fifty-six cases with attached retina, sixteen (29%) ended with a BCVA better than 0.3logMAR. The number of operations

Table 2: Pre- and intraoperative factors affecting the final anatomic outcome.

Pre- and intraoperative risk factors	Attached (total $N = 56$) n (%)	Detached (total $N = 14$) n (%)	Chi-square (P value)	Multivariate logistic regression (P value)
N of operations				
2	16 (29)	1 (0.07)	NS[a]	
>2	40 (71)	13 (99.93)		
PVR				
<C2	30 (54)	2 (14)		
≥C2	26 (46)	12 (86)	**0.014**[*]	NS (0.056)
Category				
(1) Pseudophakic	24 (43)	07 (50)		
(2) HM/GT[b]	13 (23)	01 (7)		
(3) Traumatic	06 (11)	01 (7)		
(4) PRRD[c]	13 (23)	05 (36)	NS	
Macular pathology	5 (0.09)	0 (0)	NS	
Retinectomy	26 (46)	11 (79)	**0.039**[*]	NS
Silicon oil	31 (0.55)	12 (83)	NS (0.063)	
Gas	49 (88)	11 (79)	NS	
Cryopexy	38 (68)	9 (64)	NS	
Endolaser	33 (60)	12 (83)	NS (0.071)	
Vitreous entrapment	6 (0.11)	0 (0)	NS	
Secondary breaks	21 (38)	5 (36)	NS	
Scleral paracentesis	6 (0.11)	2 (0.14)	NS	
Encircling band	29 (52)	8 (57)	NS	
Scleral buckling	20 (36)	4 (29)	NS	

[a]NS: nonsignificant result.
[b]HM: high myopia, GT: giant tears.
[c]PRRD: primary rhegmatogenous retinal detachment in phacik patients, other than trauma, high myopia, or giant tears.
[*]Significant difference ($P < 0.05$).

wasn't found to have any association with the improved visual performance. The existence of PVR, the use of silicon oil, and the use of scleral buckling were found to significantly affect the final BCVA. However, when entering the multivariate logistic regression model, only the existence of PVR was proven significant for the final functional outcome.

4. Discussion

This study investigated a series of patients with retinal detachment that required multiple operations, independent of etiology. The aim of the study was to evaluate the potential role of repeated surgery in patients with recurrent disease, in terms of anatomic and functional success. All the eyes had recurrent retinal detachment and underwent further surgery (from two to ten operations). This study attempts to clarify if the increased number of surgeries affects the final outcome and to identify factors that may influence this outcome.

In this study, 80% of the patients ended up with attached retina. All our patients had one failed RD operation before entering the study. We were able to show that our group, although it underwent multiple operations, ended-up with relatively good anatomical result. Our results are difficult to compare with the literature, because references to eyes that have received multiple operations for RD are mainly indirect. The majority of retinal detachment treatment publications refer either on first operation or on limited number of reoperations for patients with RD of specific etiology or they are focused on specific techniques (such as retinectomy or silicon-oil tamponade and removal) [3–6, 14]. Anatomic success rates in the literature vary from 53 to 81%, in specific patient groups that were treated for recurrent retinal detachment. Only when cases treated successfully with a single operation are included, the percentage of success overcomes 90% [7].

The overall analysis in this study pointed out that the number of operations does not have an unfavorable effect on the final outcome of repeated retinal detachment surgeries. No statistical significance was found when correlating the number of surgeries neither with the anatomical outcome nor with the functional outcome; the BCVA of patients that underwent multiple interventions was not found to be significantly less than those with two operations. Using the univariate correlation, retinectomy and PVR were identified as significant factors for the anatomic outcome; PVR and

Table 3: Pre- and intraoperative factors affecting the BCVA in the anatomically successful cases.

Pre- and intraoperative risk factors	Number of attached subjects $N = 56$ n (%)	BCVA ≥0.3log MAR $N = 40$ n (%)	BCVA ≤0.3log MAR $N = 16$ n (%)	Chi-square (P value)	Multivariate logistic regression (P value)
N of operations				NS[a]	
2	16 (29)	9 (23)	7 (44)		
>2	40 (71)	31 (77)	9 (56)		
PVR				**0.002***	**0.024***
<C2	30 (54)	16 (40)	14 (88)		
≥C2	26 (46)	24 (60)	2 (12)		
Category				NS	
(1) Pseudophakic	24 (43)	18 (45)	6 (37)		
(2) HM/GT[b]	13 (23)	9 (22.5)	4 (25)		
(3) Traumatic	06 (11)	3 (7.5)	3 (19)		
(4) PRRD[c]	13 (23)	10 (25)	3 (19)		
Macular pathology	5 (9)	3 (8)	2 (13)	NS	
Retinectomy	26 (46)	22 (55)	4 (25)	NS	
Silicon oil	31 (55)	26 (65)	5 (31)	**0.022***	0.472
Gas	49 (88)	35 (88)	14 (88)	NS	
Cryo	38 (69)	25 (63)	13 (81)	NS	
Laser	33 (59)	24 (60)	9 (56)	NS	
Secondary breaks	21 (38)	14 (35)	7 (44)	NS	
Vitreous entrapment	6 (11)	5 (13)	1 (6)	NS	
Scleral paracentesis	6 (11)	5 (13)	1 (6)	NS	
Encircling band	29 (52)	20 (50)	9 (56)	NS	
Scleral buckling	20 (36)	11 (28)	9 (56)	**0.043***	0.135

[a]NS: nonsignificant result.
[b]HM: high myopia, GT: giant tears.
[c]PRRD: primary rhegmatogenous retinal detachment in phacik patients, other than trauma, high myopia, or giant tears.
*Significant difference ($P < 0.05$).

the use of silicon oil and scleral buckling were found to be significant factors for the functional outcome. The risk factor that consistently emerged as significant when applying multivariate analysis was the existence of proliferative vitreoretinopathy for both the anatomical and functional result.

In univariate analysis, a negative association was found between the anatomic success (attached retina) and both the existence of PVR ($P = 0.014$) and the performance of retinectomy ($P = 0.039$); among patients that ended up detached, 86% had PVR and 79% underwent retinectomy. Proliferative vitreoretinopathy (PVR) represents the major cause of failure of retinal detachment surgery [3, 8–13]. Expectedly enough, the existence of PVR proved to be the major reason for unfavorable outcome in our study also. In the total of 70 patients, 54% had PVR grade C2 or higher. Among the detached cases, 86% had PVR grade C2 or higher, whereas 46% of the attached cases had PVR grade C2 or higher. Furthermore, we found that retinectomy was a significant factor for anatomical failure. Correlations in literature about retinectomy are contradictory, depending again on the selection of patients. la Heij et al. [6] found that the size of retinectomy matters, correlating retinectomy greater than 180 degrees with anatomic failure. Other investigators [7, 14], on the other hand, showed that retinectomy is important for complicated cases with PVR. These reports together with the findings of our study may indicate that retinectomy may be an indispensible surgical maneuver for difficult cases but the need for its utilization indicates complexity of the case and is associated with worse surgical prognosis.

The number of reoperations was not found to be a significant factor neither for the anatomical nor for the functional outcome in this study. This is in contrast to other studies that consider multiple interventions as significant factor for unsuccessful outcome. Large series with patients that have had multiple operations for retinal detachment [5, 14, 15] found a significant influence of the number of surgeries on the recurrence of the detachment. However, the same authors could not identify the number of operations as a risk factor for poor visual performance (VA less than 6/24). Further research with larger number of cases may be necessary in order to clarify the effect of repeated retinal detachment surgery in anatomical and functional outcomes.

In general, the functional outcome after repeated retinal detachment surgery is considered poor [16] even in cases

that have successful anatomical result. In the present study, we evaluated the functional outcome of the anatomically successful cases choosing a relatively high cut-off value of final visual acuity compared to the majority of relevant studies (0.30logMAR or 20/40 in the Snellen chart) [6, 7, 14, 16, 17]. Even with this high cut-off value we were able to show that even after multiple recurrences and operations, the retina is capable of retaining quite good functionality if it is finally maintained attached. Among our patients with attached retina, 29% had a BCVA better than 0.30logMAR (20/40 Snellen chart) at the end of the follow-up. This is considerably greater than the values reported in other studies with similar criteria (5%-[7]; 13%-[4]). Studies with higher percentages of "good visual acuity" (12%–59%) have all used lower cut-offs, ranging from 0.6logMAR (12%-[14]) and 0.7logMAR (47%-[18]) to 1.00logMAR (51%-[12]; 59%-[17]).

As for the factors that affect the visual outcome in attached cases, the univariate analysis, in addition to the unfavorable effect of PVR, revealed the use of scleral buckling as favorable and the use of silicone oil as unfavorable factor for a final BCVA <0.3logMAR. The silicone-oil tamponade is often reported to result in decreased BCVA [6, 12, 14, 15, 17, 19]. In our study, only the 31% ($P = 0.022$) among patients with anatomic success that underwent silicone oil tamponade had good visual acuity (<0.3logMAR). The usual practice of reserving silicon oil tamponade for the more difficult and complicated cases may be an explanation of the less favorable functional results of these cases. On the other hand, scleral buckling showed a positive influence on the final functional outcome; among the attached patients, those that had undergone scleral buckling achieved a good visual acuity in a proportion of 56% ($P = 0.043$). It is possible that cases that were handled with scleral buckle represent cases that were initially judged as less severe and this may explain the relatively better functional result.

In conclusion, in this study, we were able to show that repeated operations in cases of refractory retinal detachment can result in a high percentage of anatomical success. Among the attached eyes, a considerable percentage retained a good visual acuity. The analysis of our data showed that repeated surgery does not have an unfavorable influence in anatomical and functional outcome. Although PVR seems to be the main factor of anatomical failure and poor functional results, our data support that, in cases of unsuccessful retinal detachment operations, surgeons need to continue with as many additional interventions as technically feasible.

Conflict of Interests

None of the authors have any financial interest relating to this paper.

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Review Article

Sustained-Release Corticosteroid Options

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Sustained-release corticosteroid treatment has shown to be a promising strategy for macular edema due to retinovascular disease (i.e., diabetes and retinal vein occlusion) and for the treatment of noninfectious posterior uveitis. Clinicians now have the option of three sustained-release corticosteroid implants: Ozurdex (Allergan Inc., Irvine, CA) which releases dexamethasone and two devices that release fluocinolone acetonide, Retisert (Bausch & Lomb, Rochester, NY), and Iluvien (Alimera Science, Alpharetta, GA). Each has different physical characteristics and duration effect and has been approved for different indications. Herein we provide a summary of the current clinical knowledge regarding these implants.

1. Introduction

Intraocular corticosteroids are used for a variety of ophthalmologic conditions such as diabetic macular edema [1], posterior uveitis [2], and macular edema secondary to vascular occlusions [3]. Initially, triamcinolone acetonide was used [4], but many of these conditions are chronic and required repeat injections for prolonged periods of time. This is inconvenient to patients and may increase the risk of complications secondary to the injection procedure, including endophthalmitis and vitreous hemorrhage. Clinicians now have the option of three sustained-release corticosteroid implants: Ozurdex (Allergan Inc., Irvine, CA) which releases dexamethasone and two devices that release fluocinolone acetonide, Retisert (Bausch & Lomb, Rochester, NY), and Iluvien (Alimera Science, Alpharetta, GA). Each has different physical characteristics and duration effect and has been approved for different indications. Herein we provide a summary of the current clinical knowledge regarding these implants.

2. Dexamethasone Drug Delivery System (DDS)

Ozurdex (Allergan Inc., Irvine. Ca) is a biodegradable polymer composed of a polylactic acid-glycolic acid matrix that dissolves completely *in vivo* and is eventually converted to carbon dioxide and water [2]. The implant contains 700 μg of dexamethasone which is released to the vitreous cavity over a six-month period. It is administered via a 22-gauge injecting applicator through the pars plana. It can be administered in an office setting. Animal studies have shown that the peak concentration is reached in the retina and vitreous at day 60 and is detectable for 6 months with minimal systemic absorption [5]. After the first two months, the steroid concentration declines until month 4, where it maintains a lower concentration until month 6. The pharmacokinetic profile is similar between vitrectomized and nonvitrectomized eyes [6]. This is important as other medications such as triamcinolone acetonide are less effective in vitrectomized eyes due to faster clearance from the ocular tissues [7]. The implant is contraindicated in patients with periocular infections and advanced glaucoma and in patients whose posterior lens capsule is not intact because of the risk of implant migration into the anterior chamber.

Ozurdex has been approved by the FDA for the treatment of macular edema secondary to branch or central retinal vein occlusion, as well as for the treatment of noninfectious posterior uveitis. Haller et al. [8] published a phase III trial of

Ozurdex for macular edema secondary to branch or central retinal vein occlusion. The study included 1,267 patients and evaluated two concentrations of dexamethasone and a sham injection group. Patients receiving the dexamethasone implant had a statistically significant improvement in vision compared to the sham group. The greatest improvement was seen at day 60 with the 700 μg implant, with 29% of patients achieving a 15-letter improvement in vision. The proportion of eyes achieving at least a 15-letter improvement from baseline was significantly greater in patients receiving the injection at months 1 and 3, but no difference was seen at 6 months [9]. Cataracts were not increased in any group, and although 30% of eyes were treated with intraocular pressure (IOP) lowering medication, the IOP returned to baseline after 6 months of the procedure in all groups. After 6 months, all study patients were eligible to receive an implant if they experienced a drop in vision or persistent macular edema [10]. The same efficacy and a similar effect on the IOP were seen in the 997 patients who received an implant after 6 months. The only difference was seen in cataract progression, which occurred in 29.8% of patients who received two implants versus 5.7% in patients who had previously belonged to the sham group. However, only one patient required cataract surgery.

A systematic review published by Pielen et al. [11] compared anti-VEGF agents (ranibizumab, bevacizumab, aflibercept) versus steroids (triamcinolone and Ozurdex) for macular edema in CRVO or BRVO. All anti-VEGF agents showed a better visual acuity gain compared to steroids at month 12. The downside was that anti-VEGF therapy requires more frequent injections (around 8 injections per year, compared to 2 injections in the steroid group). IOP increase and cataract progression were also significantly higher in the patients treated with steroids compared to patients treated with anti-VEGF agents. Prospective studies comparing ranibizumab versus Ozurdex are ongoing (COMO and COMRADE (http://www.clinicaltrials.gov)). The dexamethasone implant may be of value in vitrectomized eyes, where anti-VEGFs have shown significantly reduced half-life compared to nonvitrectomized eyes in previous reports [12], although some authors argue there is no difference [13].

The phase III trial for Ozurdex for the treatment of noninfectious intermediate or posterior uveitis was published by Lowder et al. in 2011 [14]. This study included 229 patients and compared two concentrations of dexamethasone versus sham. The proportion of patients with a vitreous haze score of 0 at 8 weeks was significantly higher with the 700 μg implant, and this effect persisted through week 26. Best corrected visual acuity was also significantly better in the dexamethasone treated eyes compared to sham. No significant difference was seen in cataract progression or in the proportion of patients with an IOP > 25 mmHg between the groups. The duration of the study was 6 months and no repeat injections were performed. Ozurdex is approved by the FDA for the treatment of posterior segment noninfectious uveitis.

Ozurdex has also been proposed as treatment for diabetic macular edema (DME). A subgroup analysis of 171 patients with DME that received an Ozurdex implant showed that visual acuity gain was significantly higher compared to observation (33% gained 2 or more lines at 90 days versus 12% in the observation group). Some case series have been published stating that Ozurdex is useful in recalcitrant DME [15, 16]. However, results of Allergan-sponsored prospective studies with repeated injections (NCT00168389, NCT00168337) have not been published. Currently, a study is underway to use Ozurdex for DME in vitrectomized patients (NCT01788475). Ozurdex has been approved by the FDA for the treatment of adults with diabetic macular edema who are pseudophakic or who are scheduled for cataract surgery.

3. Fluocinolone Acetonide Implant

Retisert (Bausch & Lomb, Rochester, NY) contains a 0.59 mg pellet of fluocinolone acetonide (FA) in a nonbiodegradable polyvinyl acetate/silicone laminate. It is implanted through a pars plana sclerotomy and secured by a suture in the sclera. This procedure is performed in the operating room. It releases fluocinolone acetonide for up to 3 years [17]. Ocular drug levels are stable over a year with no evidence of systemic absorption. There is no data regarding pharmacokinetics in vitrectomized eyes, although drug levels were measured in rabbit eyes with a C3F8 gas bubble with no significant changes [18]. Similar levels of the drug are seen not only at the level of the RPE but also in the lens and iris-ciliary body. Retisert is approved by the FDA for the treatment of chronic noninfectious posterior uveitis. It is contraindicated in active viral, bacterial, mycobacterial, and fungal eye infection. It is the most expensive of the devices discussed in this paper (around USD $20,000 versus $2,000 for Ozurdex and $8,000 for Iluvien). A comparative cost effectiveness analysis is not yet available.

The pivotal trial evaluating Retisert in the Unites States randomized 278 patients with noninfectious posterior uveitis to an implant containing either 2.1 mg or 0.59 mg of FA [19]. After implantation, uveitis medications and systemic immunosuppression were tapered within a six-week period. The uveitis recurrence rate decreased from 51.4% (includes the two types of implants) to 6.1% in the first 34 weeks after implantation. Eyes that did not receive an implant had an increased rate of recurrences from 20.3% to 42%. Results from three-year followup showed recurrence rates of 4, 10, and 20% at 1, 2, and 3 years [20]. At two years, visual acuity was significantly better in the implanted eyes (that difference was lost at three years). However, 93% of implanted phakic eyes required cataract surgery (compared to 20% in nonimplanted eyes). 37% of eyes required glaucoma surgery and 75% required pressure lowering medications. It has been proposed that the implant loses its effect after about 2.5–3 years, when it can be exchanged for a new implant if inflammation recurs. In some cases the implant can become dissociated from its strut during the procedure [21, 22]. A newly designed implant has been released in March 2013 to decrease the risk of medication reservoir dissociation, but long-term data on whether this complication will occur is unavailable at this time.

A multicenter, prospective clinical trial was performed comparing Retisert to standard of care in posterior uveitis

[23]. Implanted eyes had significantly fewer recurrences (18.2% in the implant group versus 63.5% in the standard of care group). Uveitis recurrences occurred significantly later during followup. The incidence of cataract and glaucoma was similar to previous reports and there were no nonocular complications. Patients in the standard of care group had a 26% incidence of systemic treatment-related adverse effects. Visual acuity decreased between months 15 and 18 in the implant group probably due to the high incidence of cataract but was similar between both groups at month 24, with no decrease from baseline.

The MUST trial was a large prospective trial (255 patients, 479 eyes with uveitis) to compare the efficacy of the FA implant versus systemic immunosuppression with a followup of 24 months [24]. Visual acuity improvement was comparable between both groups, with a gain of 6 letters in the implant group and of 3.2 letters in the systemic therapy group at 24 months ($P = 0.16$). Control of uveitis was more frequent in the implant group (88% versus 71%, $P = 0.0001$). Although the number of patients with macular edema significantly decreased in the implant group at 6 months, the proportion of patients with macular edema was similar between both groups at 24 months. The implant group had a much higher rate of cataract surgery (80% versus 31% in the systemic treatment group) and glaucoma surgery (26.2 versus 3.7% in the systemic treatment group). Systemic infection requiring prescription therapy was lower in the implant group (0.36 events/person-year in the implant group versus 0.60 in the systemic therapy group, $P = 0.034$), but the risk of hospitalization was similar between both groups. Health-related quality of life and health utility scores increased in both groups, slightly favoring implant therapy (not significant).

Retisert has been proposed as treatment for diabetic macular edema. The largest trial randomized 196 participants to the FA implant versus laser treatment [25]. The percentage of eyes that gained 3 or more lines of vision was significantly higher in the implant group versus laser treatment at 6 months (16.8% versus 1.4%, resp.) and two-year followup (31.8% for the implant versus 9.3% for the laser group). At three years the proportion of patients who gained three or more lines of vision was 31.1% in the implant group versus 20% in the laser group (not significant). The implant group had a significantly higher rate of ocular complications with 91% of phakic eyes requiring cataract surgery and 33.8% requiring glaucoma surgery at 4 years. Although the results were comparable to those obtained with ranibizumab (RISE and RIDE studies [26]) where 36.8% to 51.2% of patients gained three or more lines of vision, the safety profile seems to be better with ranibizumab regarding cataract (incidence of 0.8%) with no increase in IOP. Retisert is not FDA approved for diabetic macular edema.

Retisert was evaluated in a prospective case series of 23 patients with CRVO [27], but a significant increase in visual acuity compared to baseline was not seen at three-year followup, despite improvements in central retinal thickness and 50% of eyes gaining 10 or more letters of vision. Ocular adverse events were similar to previous studies.

4. Fluocinolone Acetonide Insert

Iluvien (Alimera Science, Alpharetta, GA) is a smaller, nonbiodegradable cylindrical tube with a central drug-polymer matrix that releases 0.19 mg of fluocinolone acetonide into the vitreous cavity. It is inserted intravitreally via a 25-gauge needle in the same manner as in intravitreal injection and can be done in the office setting. It releases small doses of fluocinolone acetonide for at least 3 years. No systemic absorption has been documented [28].

The efficacy of Iluvien was evaluated in the FAME (Fluocinolone Acetonide for Diabetic Macular Edema) A and B studies which were two randomized, double-masked, sham injection-controlled multinational trials [29, 30]. Patients ($n = 956$) were randomized to one of two fluocinolone acetonide (FA) insert concentrations (0.2 μg/day or 0.5 μg/day) or sham. Patients could receive rescue laser photocoagulation during the study if there was persistent macular edema. After one year they could receive a second implant if their vision decreased or foveal thickness increased. Clinicians were allowed to use other nonprotocol drugs (such as anti-VEGF therapy or intravitreal triamcinolone) if they felt patients were experiencing no improvement. These medications were recorded but patients were not exited from the study. The primary endpoint was a gain of 15 or more letters at 24 months. In each of the insert groups 28% of patients achieved this goal versus 16% in the sham group ($P = 0.002$). Mean change from baseline BCVA was also significantly higher in the insert groups compared to sham. There was a drop in visual acuity between months 9 and 18 in the insertion group due to the development of cataract which later improved with surgery.

Foveal thickness was significantly less in the insert groups compared to sham at all time points except at 36 months. Significantly fewer patients required rescue laser photocoagulation in the insert groups (40%) versus sham (60%), $P < 0.001$. Regarding other nonprotocol rescue treatments (anti-VEGF, intravitreal triamcinolone), they were administered significantly more in the sham group (28.6%) compared to the insert groups (12.5–13.9%). A subgroup analysis showed that the implant was significantly better compared to sham in diabetic macular edema of more than 3 years of onset (34% versus 13.4%), but all groups were similar in edema of <3 years duration. There was no difference in the effectiveness of both concentrations of the insert, so the one with the lowest drug concentration is commercially available.

Regarding adverse events at 36 months, phakic patients who received the 0.2 μg/day implant developed cataracts in 81.7% versus 50.7% in the sham group and 80% required cataract surgery versus 27.3% in the sham group. Raised IOP was present in 37.1% in the insert group versus 11.9% in sham. Incisional IOP lowering surgery was performed in 4.8% of patients in the implant group (0.2 μg/day) versus 0.5% in the sham group.

Iluvien is approved for use in several European countries (Austria, France, Germany, Portugal, and Spain and is pending approval in Italy) for the treatment of impairment of vision associated with chronic DME that is insufficiently responsive to available therapies [28]. It has yet to receive

approval by the FDA for use in the United States. No data is available for the treatment of macular edema from CRVO or BRVO. Pivotal trials are underway to evaluate the effect of Iluvien in the treatment of noninfectious posterior uveitis, although no data is available at this moment.

5. Conclusion

Sustained-release corticosteroid treatment has shown to be a promising strategy for macular edema due to retinovascular disease (i.e., diabetes and retinal vein occlusion) and for the treatment of noninfectious posterior uveitis. Different types of implants are available, with various mechanisms, duration, and side effects. Ample experience has shown the effectiveness of Ozurdex, but in patients with chronic disease punctuated by recurrences, a treatment duration longer than 6 months is desirable. The fluocinolone acetonide implants have demonstrated increased duration of efficacy, but side effects such as cataract and glaucoma including the potential need for surgical treatment are considered. Further studies are needed to determine the indications for each of these implants, as well as long-term results, including the effects of receiving multiple sustained release steroids over time.

Conflict of Interests

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

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Research Article

The Mitochondria-Targeted Antioxidant SkQ1 Downregulates Aryl Hydrocarbon Receptor-Dependent Genes in the Retina of OXYS Rats with AMD-Like Retinopathy

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The mitochondria-targeted antioxidant SkQ1 is a novel drug thought to retard development of age-related diseases. It has been shown that SkQ1 reduces clinical signs of retinopathy in senescence-accelerated OXYS rats, which are a known animal model of human age-related macular degeneration (AMD). The aim of this work was to test whether SkQ1 affects transcriptional activity of *AhR* (aryl hydrocarbon receptor) and *Nrf2* (nuclear factor erythroid 2-related factor 2), which are considered as AMD-associated genes in the retina of OXYS and Wistar rats. Our results showed that only *AhR* and *AhR*-dependent genes were sensitive to SkQ1. Dietary supplementation with SkQ1 decreased the *AhR* mRNA level in both OXYS and Wistar rats. At baseline, the retinal *Cyp1a1* mRNA level was lower in OXYS rats. SkQ1 supplementation decreased the *Cyp1a1* mRNA level in Wistar rats, but this level remained unchanged in OXYS rats. Baseline *Cyp1a2* and *Cyp1b1* mRNA expression was stronger in OXYS than in Wistar rats. In the OXYS strain, *Cyp1a2* and *Cyp1b1* mRNA levels decreased as a result of SkQ1 supplementation. These data suggest that the Cyp1a2 and Cyp1b1 enzymes are involved in the pathogenesis of AMD-like retinopathy of OXYS rats and are possible therapeutic targets of SkQ1.

1. Introduction

Mitochondria-targeted antioxidant SkQ1 (cationic plastoquinone derivative (10-[6′-plastoquinonyl] decyltriphenylphosphonium) is a novel medication that is designed to retard the development of age-related diseases and aging [1, 2]. Plastoquinone, a very effective electron carrier and an antioxidant of chloroplasts, was conjugated with decyltriphenylphosphonium to obtain a cation that easily penetrates the cell membrane [3–5]. SkQ1's geroprotective properties are based on the ability of this reagent to attenuate pathological processes associated with production of reactive oxygen species [4]. The effects of SkQ1 on aging are accompanied by inhibition of development of such age-related problems as cataract, retinopathy, glaucoma, balding, canities, osteoporosis, involution of the thymus, and peroxidation of lipids and proteins [1, 3]. Recently it was shown that addition of SkQ1 to food or treatment with SkQ1 eye drops not only prevents development of retinopathy but also reduces severity of preexisting pathological changes in the retina of senescence-accelerated OXYS rats [6].

OXYS rats are a known animal model of human age-related macular degeneration (AMD) [7, 8]. OXYS rats develop a retinopathy similar to the dry form of human AMD judging by the symptoms, morphology, and some molecular changes. The OXYS retinopathy involves hypoplasia and atrophy of the retinal pigment epithelium and of photoreceptors, formation of drusen, and retinal neovascularization; this retinopathy also correlates with expression of VEGF (vascular endothelial growth factor) [8]. It was reported that the effects of SkQ1 include improvement of functions of the retinal pigment epithelium and a reduction of lipofuscin accumulation

in the OXYS retina [9, 10]. The molecular mechanisms underlying the effects of SkQ1 have yet to be investigated.

AMD etiology includes genetic predisposition, exposure to environmental toxins and free radicals, and low levels of antioxidants. The pathogenesis of AMD is being actively studied but is not fully understood at present; several key factors are known to be involved. Oxidative stress is one of these key factors together with structural and functional changes in the retinal pigment epithelium, inflammation, and activation of the complement cascade. Recently, some genes that take part in the regulation of redox processes were characterized as possible AMD-associated genes. These genes include nuclear factor erythroid 2-related factor 2 (Nrf2) [11] and aryl hydrocarbon receptor (AhR) [12]. After analysis of haplotypes of detoxification genes, *AhR* caught the attention of researchers as a possible risk factor of AMD [12]. Studies of $AhR^{-/-}$ mice further support the potential role of this gene in AMD pathogenesis [13].

AhR is a ligand-dependent transcription factor that recognizes and binds to a wide range of xenobiotics and endogenous compounds. When inactive, AhR is located in the cytoplasm along with its associated proteins. After AhR binds to its ligand, the above-mentioned complex disintegrates, ligand-bound AhR moves to the nucleus, and dimerizes with Arnt (aryl hydrocarbon receptor nuclear translocator). The AhR/Arnt heterodimer then binds to xenobiotic-responsive elements (XREs) in the genomic DNA, and this process causes initiation of transcription of AhR's target genes [14, 15].

AhR controls expression of some components of phase I and phase II of xenobiotic metabolism [14, 15]. The following are phase I enzymes: cytochrome P450 1 subfamily (Cyp1a1, Cyp1a2, and Cyp1b1) and aldehyde dehydrogenase 3A1 (Aldh3a1) as a participant of phase II of the xenobiotic metabolism [16].

Nrf2 is a transcription factor that controls gene expression of antioxidant systems of the cell. In particular, it controls expression of heme oxygenase 1 (Hmox1), thioredoxin reductase 1 (Txnrd1), and glutathione S-reductase (Gsr) [17]. Nrf2, while in a complex with Maf proteins, interacts with antioxidant-responsive elements (ARE) in the promoter region of target genes [18].

Recent findings demonstrate a relationship between AhR-dependent and Nrf2-dependent signal transduction pathways; Nrf2 may be a genomic target of AhR, and the possibility of a cross-talk between the AhR/XRE and Nrf2/ARE signal transduction pathways cannot be ruled out [16, 19]. Some genes are controlled by both AhR and Nrf2. The list includes NAD(P)H: quinone oxidoreductase 1 (*Nqo1*), uracil diphosphate- (UDP-) glucuronosyltransferase 1A6 (*Ugt1a6*), UDP-glucuronosyltransferase (UGT) 1A9 (*Ugt1a9*), glutathione S-transferase (GST) A1 (*Gsta1*), and a number of other isoforms of UGT and GST [16]. Genes that are controlled by AhR, Nrf2, and both AhR and Nrf2 are sometimes called the *AhR-Nrf2 gene battery*.

The aim of this study was to test whether the antioxidant SkQ1 affects transcriptional activity of a redox-sensitive system: the AhR-Nrf2 gene battery. OXYS and Wistar rats received SkQ1 with food between the ages of 1.5 to 3 months, which is the period of active manifestation of signs of retinopathy. It was repeatedly proven previously that SkQ1 in this regimen can prevent the development of retinopathy in OXYS rats [6, 20].

2. Methods

2.1. Reagents. TRI-Reagent and RNA Secure Reagent were purchased from Ambion (USA); the cDNA synthesis MMLV RT kit and PCR kit qPCRmix-HS were from Evrogen (Russia); RNasin and RQ1 DNase were from Promega (USA); oligonucleotides (primers) for analysis of the rat genes *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Gsta1*, *Nqo1*, *Aldh3a1*, *Ugt1a6*, *Ugt1a9*, *AhR*, *Nrf2*, *Gsr*, *Txnrd1*, *Hmox1*, and *Gapdh* were from Syntol (Russia). SkQ1 was synthesized as described earlier [5]. All other chemicals were obtained from other commercial sources and were analytical grade.

2.2. Animals. Male senescence-accelerated OXYS and age-matched male Wistar (control) rats were obtained from the Shared Center for Genetic Resources of Laboratory Animals of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (SB RAS; Novosibirsk, Russia). The rats were kept under standard laboratory conditions (at 22 ± 2°C, 60% relative humidity, and natural light), provided with standard rodent feed, PK-120-1, Ltd. (Laboratorsnab, Russia), and given water *ad libitum*. All experiments in this study were approved by the Institutional Review Board and performed in accordance with the Animal Care Regulations of the Institute of Cytology and Genetics (Novosibirsk) and with the international norms for studies on laboratory animals.

To assess the effects of SkQ1 (from the age of 1.5 months to the age of 3 months) on gene expression, 1.5-month-old male OXYS rats were randomly assigned to 1 of the 2 groups: the standard (control) diet or the diet supplemented with 250 nmol SkQ1 per kilogram of body weight per day (15 rats per group). The age-matched Wistar rats (standard diet) served as a control (15 rats in this group). The rats were euthanized using CO_2 inhalation and killed by decapitation 5 days after the last examination of eyes. The retinas were removed, frozen, and stored at −80°C until analysis.

2.3. RNA Isolation and Reverse Transcription. Total RNA was isolated using the TRI-Reagent isolation kit (Ambion) as per the manufacturer's protocol. The RNA pellets were dissolved in 1 mM sodium citrate buffer pH 6.5, containing 1× RNA Secure Reagent (Ambion). The RNA concentration was measured using UV spectrophotometry. The RNA samples were treated with RNase-free DNase (Promega, USA) according to the manufacturer's instructions. Then the samples were subjected to repeated RNA extraction with a phenol-chloroform mixture and pure chloroform followed by precipitation with propanol. Reverse transcription was performed using the cDNA synthesis MMLV RT kit (Evrogen, Russia) according to the manufacturer's protocol.

Table 1: Primer sequences for analysis of gene expression.

Gene		Sequence
Cyp1a1	Forward	5′-CCAAACGAGTTCCGGCCT-3′
	Reverse	5′-TGCCCAAACCAAAGAGAATGA-3′
	Probe	5′(FAM)-TTCTCACTCAGGTGTTTGTCCAGAGTGCC-(BHQ1)3′
Cyp1a2	Forward	5′-CGCCCAGAGCGGTTTCTTA-3′
	Reverse	5′-TCCCAAGCCGAAGAGCATC-3′
	Probe	5′(FAM)-CAATGACAACACGGCCATCGACAAG-(BHQ1)3′
Cyp1b1	Forward	5′-GGCATCGCACTTGTACTTCG-3′
	Reverse	5′-CACCAGAGCCTGATGGATGG-3′
	Probe	5′(FAM)-TCTCGCCATTCAGCACCACCACGG-(BHQ1)3′
Gsta1	Forward	5′-ACTACATTGCCACCAAATACAACCT-3′
	Reverse	5′-CACTCCTTCTGCATACATGTCGAT-3′
	Probe	5′(FAM)-ATGGGAAGGACATGAAGGAGAGAGCCC-(BHQ1)3′
Nqo1	Forward	5′-TTGAGTCATCTCTGGCGTATAAGG-3′
	Reverse	5′-GGTCTGCAGCTTCCAGCTTT-3′
	Probe	5′(FAM)-AGGCCGCCTGAGCCCGGATA-(BHQ1)3′
Aldh3a1	Forward	5′-CCGTGATTATGGGAGGATCATC-3′
	Reverse	5′-TGGGCTACTTTCTGGTTGTCAAT-3′
	Probe	5′(FAM)-TGACCGTCACTTCCAGCGGGTCA-(BHQ1)3′
Ugt1a6	Forward	5′-CCTTGGACGTGATTGGCTTT-3′
	Reverse	5′-GCAGCCATAGGCACAACTTTTATA-3′
	Probe	5′(FAM)-CTGGCCATCGTGTTGACGGTGGT-(BHQ1)3′
Ugt1a9	Forward	5′-GAGGCTTTGGGCAGAATTCC-3′
	Reverse	5′-TTTGCAAGGTTCGATGGTCTAGTT-3′
	Probe	5′(FAM)-CAGACGGTCCTGTGGCGCTACACC-(BHQ1)3′
AhR	Forward	5′-TGGACAAACTCTCCGTTCTAAGG-3′
	Reverse	5′-GATTTTAATGCAACATCAAAGAAGCT-3′
	Probe	5′(FAM)-CAGCGTCACGTACCTGAGGGCCA-(BHQ1)3′
Nrf2	Forward	5′-AGCAACTCCAGAAGGAACAGGAGA-3′
	Reverse	5′-CTTGTTTGGGAATGTGGGCAACCT-3′
	Probe	5′(FAM)-TCCCAATTCAGCCAGCCCAGCACA-(BHQ1)3′
Hmox1	Forward	5′-TTACACACCAGCCACACAGCACTA-3′
	Reverse	5′-CATGGCCTTCTGCGCAATCTTCTT-3′
	Probe	5′(FAM)-FAMTGAGCTGCTGGTGGCCCACGCATATA-(BHQ1)3′
Txnrd1	Forward	5′-TTTACTCAGCAGAGCGGTTCCT-3′
	Reverse	5′-TGCACATTCCAAGGCGACAT-3′
	Probe	5′(FAM)-AAGACCCTAGTGGTTGGCGCGTCCT-(BHQ1)3′
Gsr	Forward	5′-CTTCGACAATACGGTCGCCATTCA-3′
	Reverse	5′-AATCTATAAAGCTGGCGCAGGACG-3′
	Probe	5′(FAM)-AGTGGGCCTCTGGGAGGAACCAATCA-(BHQ1)3′
Gapdh	Forward	5′-CAAGGTCATCCATGACAACTTTG-3′
	Reverse	5′-GGGCCATCCACAGTCTTCTG-3′
	Probe	5′(FAM)-ACCACAGTCCATGCCATCACTGCCA-(BHQ1)3′

2.4. Real-Time PCR. The PCR primer sequences used are presented in Table 1.

Gapdh served as an internal control (housekeeping gene). The gene expression patterns were analyzed using the iCycler CFX96 real-time PCR detection system (Bio-Rad Laboratories, USA) based on the TaqMan principle. Aliquots from all cDNA samples were mixed, and the "average" solution was used for preparation of calibration curves, which were used for measurement of relative cDNA levels of genes under study and of a reference gene in experimental samples. The reaction mixture contained the qPCRmix-HS buffer (Evrogen, Russia); a primer mix consisting of 0.5 μL of 5 μM probe, 1 μL of a 10 μM forward primer, and 1 μL of a 10 μM reverse primer; and 2000 ng cDNA. The reaction was conducted under

the following conditions: heating at 95°C for 3 min, then 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 30 s.

In each experiment, we added samples of cDNA under study with primers specific to a target gene (in triplicate for each cDNA sample) to wells of 1 multiwell plate, and similar samples with primers specific to a comparison gene were added to other wells of the same multiwell plate (also in triplicate). From these cDNA samples, we took identical amounts of cDNA to build a standard curve (this was an absolute quantification method using a standard curve). We used serial dilutions of the standard cDNA from 1 : 3 to 1 : 27. To wells of 1 multiwell plate, we added 2-3 repeats of reactions containing primers specific to a target gene and similar samples with primers specific to a comparison gene (2-3 repeats). Using the resulting standard curves, we quantified the original amount of cDNA (relative to the standard cDNA), and this value was normalized to the amount of cDNA of the comparison gene (*Gapdh*) [21]. For each cDNA sample, PCR was repeated at least twice.

2.5. Statistical Analysis. All calculations were performed using the *STATISTICA* software package (StatSoft, Inc., USA). All data were analyzed using two-way ANOVA and the Newman-Keuls *post hoc* test. The independent variables were genotype (Wistar, OXYS) and treatment (controls, SkQ1). One-way ANOVA was used for individual group comparison. The data are presented as mean ± SEM. The results were considered statistically significant if the P value was less than 0.05.

3. Results

In this work, we analyzed mRNA expression of AhR and AhR-dependent genes, Nrf2 and Nrf2-dependent genes, and AhR+Nrf2-dependent genes in phases I and II of xenobiotic metabolism.

Figure 1 shows mRNA levels of AhR and AhR-dependent genes of phase I (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*) and phase II (*Aldh3a1*). Our data show that the genotype had no influence on the *AhR* mRNA level (Figure 1(a)) in the retina ($F_{1,22}$ = 1.02, P = 0.32), but this parameter was affected by SkQ1 ($F_{1,22}$ = 16.00, P = 0.0007). SkQ1 supplementation decreased the *AhR* mRNA level both in OXYS rats (1.9-fold; $P < 0.012$) and in Wistar rats (1.7-fold; $P < 0.17$).

At the same time, the retinal *Cyp1a1* (Figure 1(b)), *Cyp1a2* (Figure 1(d)), and *Cyp1b1* (Figure 1(e)) mRNA level was affected by genotype ($F_{1,26}$ = 7.54, P = 0.011; $F_{1,24}$ = 6.69, P = 0.016; $F_{1,25}$ = 4.43, P = 0.047, resp.). *Post hoc* analysis showed that the retinal *Cyp1a1* mRNA level (Figure 1(b)) was ~50% lower in OXYS rats than the Wistar strain (P = 0.01). *Cyp1a2* (Figure 1(d)) and *Cyp1b1* (Figure 1(e)) mRNA expression was 2-fold (P = 0.03) and 1.7-fold (P = 0.02) higher in OXYS than in Wistar rats, respectively.

SkQ1 supplementation downregulated only *Cyp1a1* mRNA expression (2-fold; P = 0.03) in the retina of Wistar rats (Figure 1(b)), whereas in the OXYS retina, the *Cyp1a1* mRNA expression remained unchanged (Figure 1(b)). In the retina of OXYS rats, the *Cyp1a2* (Figure 1(d)) and *Cyp1b1* (Figure 1(e)) mRNA levels were decreased 2.2-fold (P = 0.03) and 1.7-fold (P = 0.02), respectively, as a result of the dietary SkQ1 supplementation. *Cyp1a2* (Figure 1(d)) and *Cyp1b1* (Figure 1(e)) mRNA expression remained unchanged in Wistar rats between SkQ1 supplementation and control.

According to two-way ANOVA there were no statistically significant differences in the mRNA expression of *Aldh3a1* (Figure 1(c)) either between the 2 strains ($F_{1,25}$ = 3.7, P = 0.07) or between SkQ1 supplementation and control ($F_{1,25}$ = 2.5, P = 0.13). There were no statistically significant differences in the mRNA expression of *Aldh3a1* (Figure 1(c)) either between the 2 strains or between SkQ1 supplementation and control.

Figure 2 shows mRNA levels of genes controlled by both AhR and Nrf2: *Nqo1*, *Gsta1*, *Ugt1a6*, and *Ugt1a9*. Two-way ANOVA showed that *Nqo1* mRNA level (Figure 2(a)) was affected by genotype ($F_{1,26}$ = 4.48, P = 0.04) but was not affected by SkQ1 supplementation ($F_{1,26}$ = 1.67, P = 0.21). One-way ANOVA showed a decreased *Nqo1* mRNA level in the retina of control Wistar rats compared to OXYS rats (1.9-fold; P = 0.02).

There were no statistically significant differences in the mRNA level of *Ugt1a6* (Figure 2(c)), *Gsta1* (Figure 2(b)), and *Ugt1a9* (Figure 2(d)) between the 2 strains ($F_{1,25}$ = 0.25, P = 0.62; $F_{1,26}$ = 2.75, P = 0.11; and $F_{1,26}$ = 0.56, P = 0.47, resp.) or between SkQ1 supplementation and control ($F_{1,25}$ = 3.8, P = 0.06; $F_{1,26}$ = 0.54, P = 0.47; and $F_{1,26}$ = 0.01, P = 0.92, resp.).

Figure 3 shows mRNA levels of *Nrf2* and Nrf2-dependent genes: *Gsr*, *Hmox1*, and *Txnrd1*. There were no statistically significant differences in the mRNA level of *Nrf2* (Figure 3(a)) and of Nrf2-dependent genes *Gsr* (Figure 3(b)) and *Hmox1* (Figure 3(d)) either between the 2 strains ($F_{1,26}$ = 0.77, P = 0.39; $F_{1,26}$ = 1.18, P = 0.29; and $F_{1,24}$ = 3.57, P = 0.07, resp.) or between SkQ1 supplementation and control ($F_{1,26}$ = 0.001, P = 0.997; $F_{1,26}$ = 1.42, P = 0.24; and $F_{1,26}$ = 0.17, P = 0.68, resp.).

Two-way ANOVA analysis showed that the mRNA level of the Nrf2-dependent gene *Txrnd1* (Figure 3(c)) was not affected by genotype ($F_{1,26}$ = 1.65, P = 0.21) but was affected by SkQ1 supplementation ($F_{1,26}$ = 5.55, P = 0.026). One-way ANOVA revealed a difference between the level of *Txrnd1* (Figure 3(c)) in the retina of untreated Wistar and OXYS rats (1.5-fold; $F_{1,12}$ = 13.5, P = 0.003). SkQ1 supplementation downregulated *Txrnd1* mRNA expression (1.7-fold; $F_{1,13}$ = 5.85, P = 0.03) in the retina of OXYS rats, whereas in the Wistar retina, the *Txrnd1* mRNA expression remained unchanged ($F_{1,13}$ = 0.39, P = 0.54).

4. Discussion

The retina is among the types of tissue that are at high risk of damage by reactive oxygen species, whereas oxidative stress is a major contributor to the pathogenesis of retinopathy and AMD [22]. Our previous research showed that the mitochondria-targeted antioxidant SkQ1 can reduce clinical manifestations of retinopathy in OXYS rats. Nonetheless, the molecular mechanisms behind SkQ1's beneficial

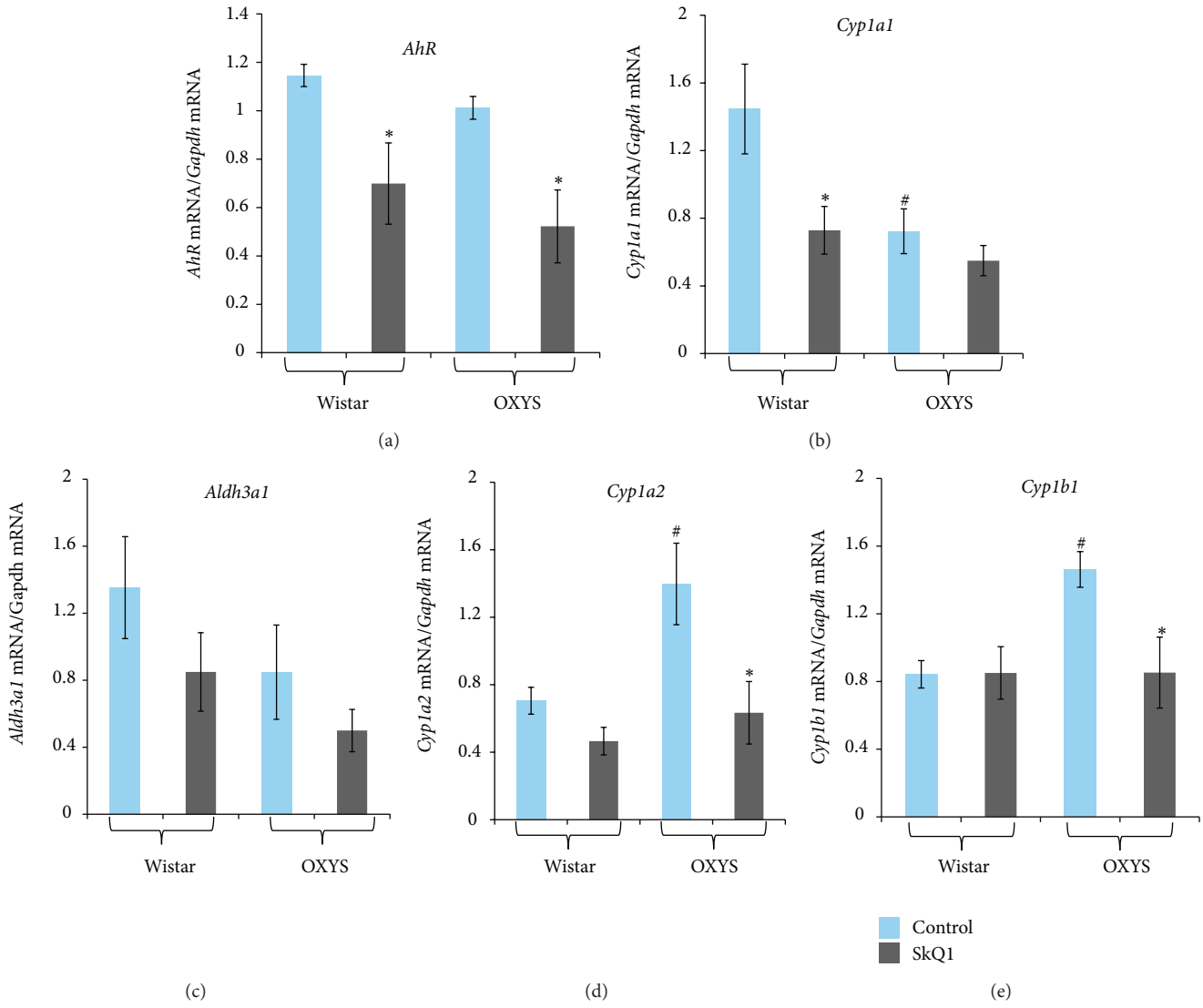

Figure 1: *AhR* (a), *Cyp1a1* (b), *Aldh3a1* (c), *Cyp1a2* (d), and *Cyp1b1* (e) mRNA levels in the retinas of 3-month-old Wistar and OXYS rats treated with SkQ1. All data are normalized to the expression level of a housekeeping gene (*Gapdh*). Values are presented as mean ± SEM ($n = 6$ to 8). Significant differences between groups are marked with $^{*}P < 0.05$.

effects are not well understood. Using an animal model of retinopathy—OXYS rats—in the present work, we explored the influence of SkQ1 on mRNA expression of *AhR*, *Nrf2*, and their dependent genes; those genes can regulate oxidative and antioxidant processes in the cells.

Our present data suggest that only *AhR* and *AhR*-dependent genes of phase I of xenobiotic biotransformation were strongly sensitive to SkQ1 supplementation. The decrease of the *Ugt1a6* mRNA level and low *Nqo1* mRNA expression in OXYS rats (compared to the Wistar strain) can be explained in part by AhR dependence of these genes. The observed effect of SkQ1 on *Txrnd1* mRNA expression was a surprise because direct links between Txrnd1 and AhR are unknown, whereas *Nrf2* and Nrf2-dependent genes *Gsr* and *Hmox1* were not affected. The absence of effects of the antioxidant SkQ1 on the genes responsible for major endogenous antioxidant systems suggests that the antioxidant activity of SkQ1 is not linked to the effects of SkQ1 on the transcription factor Nrf2 or on enzymes of phase 2 of the xenobiotic metabolism. This is not surprising; the mechanism of action of SkQ1 does not imply involvement of transcription factors or regulation of gene expression in general.

Nonetheless, we see the effects of SkQ1 on the mRNA expression of the transcription factor AhR and on mRNA levels of genes of phase I metabolism of xenobiotics that are activated by AhR. These findings are supported indirectly by the data on the influence of SkQ1 on P450 cytochrome activity in the rat liver (Grishanova et al., unpublished observations).

Differences in the level of mRNA of AhR-dependent genes are observed not only under the influence of SkQ1 but also between untreated Wistar and OXYS rats. For example, the observed level of *Cyp1a1* mRNA is lower in OXYS

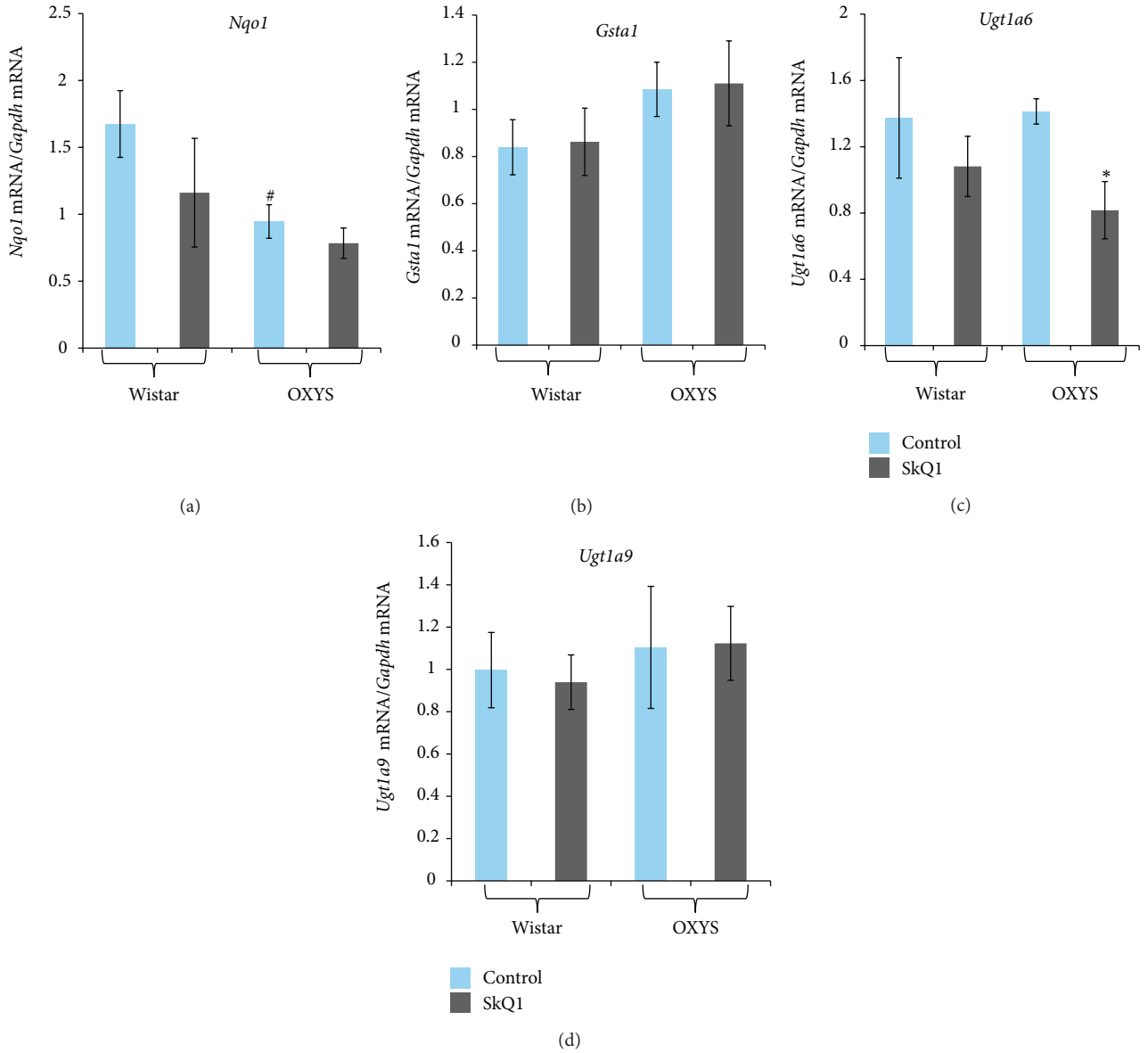

Figure 2: *Nrqo1* (a), *GstA1* (b), *Ugt1a6* (c), and *Ugt1a9* (d) mRNA levels in the retinas of 3-month-old Wistar and OXYS rats treated with SkQ1. All data are normalized to the expression level of a housekeeping gene (*Gapdh*). Values are presented as mean ± SEM ($n = 6$ to 8). Significant differences between groups are marked with $^{*}P < 0.05$.

rats than the Wistar strain. This situation can be explained by the significant decrease in *Cyp1a1* expression under oxidative stress [23], which constitutes one of the stages of the pathogenesis of AMD and AMD-like retinopathy in OXYS rats.

On the other hand, at baseline, *Cyp1a2* and *Cyp1b1* mRNA levels are higher in OXYS than in Wistar rats. It is known that some P450 cytochromes can metabolize arachidonic acid to compounds that affect the tone of a vessel wall and arterial blood pressure [24–26]. In particular, Cyp1b1 participates in the synthesis of 12-HETE (12-hydroxyeicosatetraenoic acid), which is known to be cardiotoxic [27]. Arachidonic acid can be metabolized by Cyp1a2 [28]; as a result, production of epoxyeicosatrienoic acids is enhanced. The latter compounds can serve as a source of reactive oxygen species in addition to being vasodilators and proangiogenic factors able to stimulate growth of endothelial and mesangial cells [29].

Retinopathy pathogenesis includes pathological neovascularization. It has been shown that several metabolites of arachidonic acid can activate the above process. For example, cytosolic phospholipase A(2) has proangiogenic properties and stimulates pathological retinal angiogenesis [30]. Furthermore, retinal neovascularization is associated with increased 12-lipoxygenase expression and with enhanced production of 12-HETE, 15-HETE, and 5-HETE [31]. Accordingly, the elevated *Cyp1a2* and *Cyp1b1* mRNA level in OXYS rats compared to the Wistar strain is likely to reflect (at least partially) the process of pathological neovascularization in OXYS rats.

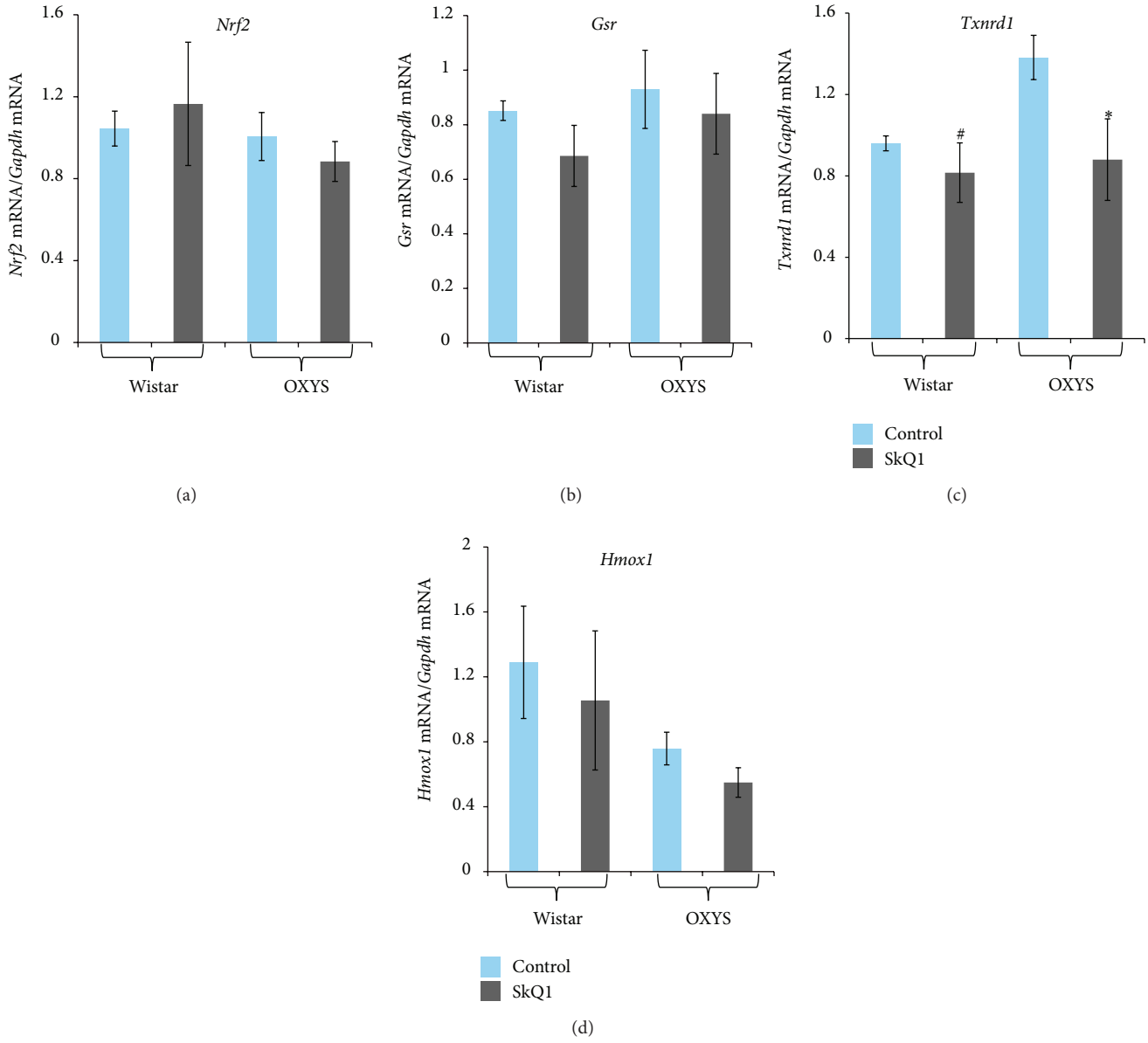

Figure 3: *Nrf2* (a), *Gsr* (b), *Txnrd1* (c), and *Hmox1* (d) mRNA levels in the retinas of 3-month-old Wistar and OXYS rats treated with SkQ1. All data are normalized to the expression level of a housekeeping gene (*Gapdh*). Values are presented as mean ± SEM (n = 6 to 8). Significant differences between groups are marked with $^{*}P < 0.05$.

In this work, the effects of SkQ1 supplementation—where they are present—consist of downregulation of mRNA expression of the relevant genes. First, it is the reduced mRNA level of AhR both in OXYS and in Wistar rats. It is possible that the reduction in mRNA expression of AhR-controlled genes (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*) and the partially AhR-controlled gene *Ugt1a6* is mediated by SkQ1's influence on functioning of the AhR enzyme. Nonetheless, it is of course impossible to rule out direct action on the protein molecules of P450 cytochromes. Undoubtedly, detailed elucidation of such a mechanism of action of SkQ1 would be interesting, and further research is needed.

5. Conclusion

Our results point to the involvement of Cyp1a2 and Cyp1b1 in the pathogenesis of AMD-like retinopathy in OXYS rats. Cyp1a2 and Cyp1b1 can be considered possible therapeutic targets for novel treatments of AMD; it is plausible that these enzymes are targets of SkQ1 when it is administered systemically.

Abbreviations

AhR: Aryl hydrocarbon receptor
Aldh3a1: Aldehyde dehydrogenase 3A1

AMD: Age-related macular degeneration
ARE: Antioxidant-responsive elements
Arnt: Ah receptor nuclear translocator
BHQ: Black hole quencher
Cyp1a1: Cytochrome P450 1A1
Cyp1a2: Cytochrome P450 1A2
Cyp1b1: Cytochrome P450 1B1
FAM: 6-Carboxyfluorescein
Gsr: Glutathione-S-reductase
Gsta1: Glutathione S-transferase A1
Hmox1: Heme oxygenase 1
Nqo1: NADPH-quinone oxidoreductase
Nrf2: Nuclear factor, erythroid derived 2
Txnrd1: Thioredoxin reductase 1
Ugt1a6: UDP-glucuronosyltransferase 1A6
Ugt1a9: UDP-glucuronosyltransferase 1A9
VEGF: Vascular endothelium growth factor
XRE: Xenobiotic responsive element.

Conflict of Interests

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

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Research Article

Novel Lutein Loaded Lipid Nanoparticles on Porcine Corneal Distribution

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Topical delivery has the advantages including being user friendly and cost effective. Development of topical delivery carriers for lutein is becoming an important issue for the ocular drug delivery. Quantification of the partition coefficient of drug in the ocular tissue is the first step for the evaluation of delivery efficacy. The objectives of this study were to evaluate the effects of lipid nanoparticles and cyclodextrin (CD) on the corneal lutein accumulation and to measure the partition coefficients in the porcine cornea. Lipid nanoparticles combined with 2% HPβCD could enhance lutein accumulation up to 209.2 ± 18 (μg/g) which is 4.9-fold higher than that of the nanoparticles. CD combined nanoparticles have 68% of drug loading efficiency and lower cytotoxicity in the bovine cornea cells. From the confocal images, this improvement is due to the increased partitioning of lutein to the corneal epithelium by CD in the lipid nanoparticles. The novel lipid nanoparticles could not only improve the stability and entrapment efficacy of lutein but also enhance the lutein accumulation and partition in the cornea. Additionally the corneal accumulation of lutein was further enhanced by increasing the lutein payload in the vehicles.

1. Introduction

Carotenoids act as quenchers of oxygen free radicals, blockers of blue-light damage, and inhibitors of lipid peroxidation [1]. Intense exposure to ultraviolet radiation and high oxygen tension render the cornea particularly vulnerable to oxidative damage. The role of oxidative stress has been studied in the corneal diseases including keratitis, keratoconus, infection, and inflammation [2]. Antioxidants such as tocopherol and epigallocatechin gallate can protect cornea from free radicals in the animal model [3, 4]. Lutein is reported to suppress the development of endotoxin-induced uveitis in the rat model by inhibiting the NF-κB dependent signaling pathway and the subsequent production of proinflammatory mediators [5]. Lutein also has neuroprotective effects against the neural damage of retina caused by inflammation [6]. The pathogenic mechanism of macular degeneration and the protective role of lutein have been extensively investigated [7, 8]. However, ocular lutein delivery remains challenging because of the isolated structure of eye. The conventional eye drops are restricted to deliver lipophilic drug due to the existence of the anatomical barriers such as cornea and sclera [9]. Occasionally, intravitreal injections cause side effects such as endophthalmitis, cataract formation, and retinal detachment [10]. There is a need to develop novel drug delivery carriers capable of increasing ocular bioavailability and decreasing the side effects. Nanoparticles may overcome the physiological barriers and deliver the drug to the target by virtue of their nanoscale and functionalization [11]. Among several nanoparticles, lipid nanoparticles such as nanoscaled lipid carriers (NLCs) have been recognized as an interesting and promising topical delivery vehicle for lipophilic drugs [12].

NLCs are especially useful in ocular drug delivery as they can enhance the ocular retention and corneal absorption and can also improve the ocular bioavailability [13]. These enhancing mechanisms include the adhesiveness of nanoparticles to the cornea and penetration by lipids on the epithelial layer. The lipids in NLCs have other advantages like biocompatibility, high drug payload, and degradation protection.

Cyclodextrins (CDs) contain several glucose units that form a hydrophobic central cavity for lipophilic drugs and a hydrophilic outer surface for water solubility [14]. CDs are functional additives in the formulations to enhance drug stability and to decrease drug irritation [15]. CDs also act as a permeation enhancer at the cornea by increasing the drug retention at the surface of the corneal epithelium [16]. Hydroxyalkylation of βCD can increase the water solubility of parent βCD by converting βCD into its amorphous and noncrystallizable derivatives and can also reduce hemolytic and renal toxicities of parent CDs [17]. Modified lipid nanoparticles not only can modulate the biodistribution of the loaded drug but also control the absorption rate of drugs administered [18]. For example, solid lipid nanoparticles of paclitaxel (PTX) modified with HPβCD can enhance cellular accumulation of the drug into p-glycoprotein expressing cells [19]. The cysteine-polyethylene glycol stearate modified nanostructured lipid carrier has the mucoadhesive properties on the surface of rabbit eyes. The encapsulated cyclosporine in the hybrid carriers can remain on the ocular surface nearly up to 6 hours [20]. The coating can improve drug absorption due to its ocular adhesiveness or permeability enhancer properties. These results suggest modified nanoparticles as promising vehicles for ocular delivery.

Since the cornea is one of the main barriers for the ocular transport, therefore the distribution of lutein in the cornea is important for the evaluation of topical delivery efficacy. Besides this limited literature data for corneal partition coefficient are available for lutein. Our primary objectives were to evaluate the distribution profile of lutein in the porcine cornea and to study the formulation effects on the corneal partition coefficient of lutein by using NLCs as the vehicles. The porcine cornea was established as an in vitro model for the characterization of lutein accumulation and distribution. Also the novel NLCs were analyzed for their morphology, lutein encapsulation efficacy, and cytotoxicity.

2. Materials and Methods

2.1. Reagents and Chemicals. The lutein (Lutemax) is kindly provided by DKSH (Taipei, Taiwan). Cyclodextrins are purchased from Wako Pure Chemical Industries (Osaka, Japan). Transcutol HP is obtained from Gattefossé (Lyon, France). Tween 80 and Span 60 are supplied by Kanto Chemical (Tokyo, Japan). All other chemicals and reagents are purchased from Sigma-Aldrich unless otherwise stated.

2.2. Preparation of Lutein-Loaded NLC. Lutein-loaded NLCs and lutein-loaded NLCs combined with CD were manufactured by a hot sonication method. Briefly, lutein was first dissolved in diethylene glycol monoethyl ether (Transcutol) at the concentration of 2%. Lutein in Transcutol (1.2 g) was mixed with decanoic acid (60 mg) and Span 60 (120 mg) at 80°C in a dry bath. Tween 80 (180 mg) and CD (30~180 mg) were separately dispersed in 4.44 mL of distilled water. Finally, the mixture solution was sonicated for 5 min using a probe-type sonicator. Samples were stored in the dark for further characterization. Bare NLC and NLC-CD were made by the same method with no lutein addition. Composition of the developed vehicles including NLC and NLC + HPβCD is shown in Table 3.

2.3. Characterization of NLC. The size and surface property of MNP were characterized by TEM. A drop of diluted sample was dispersed onto a 100-mesh copper grid, and then the excess drop was removed with a filter paper. The sample containing copper grid (CF200-Cu, Electron Microscopy Science, Washington, DC) dried for two hours at 55°C prior to TEM analysis. The morphology of the various MNP was observed by TEM (JEOL JEM 2000 EXII, Tokyo, Japan). NLC samples were diluted 1 : 25 with Milli-Q water and dried on carbon film (CF200-Cu, Electron Microscopy Science, Washington, PA, USA) for 12 hours. After being stained with a 1% solution of phosphotungstic acid (Merck, Darmstadt, Germany) for 30 seconds and vacuum-dried in the incubator, samples were then analyzed by TEM. The average particle size and zeta potential in different formulations were characterized by using Zetasizer Nano ZS 90 (Malvern, Worcestershire, UK) at a fixed angle of 90° and a temperature of 25°C. The smaller the PI, the more uniform the size distribution of dispersion. Zeta potential characterizes the surface charge of particles, which is an indicator of the long-term stability. Zeta potential values of ±30 mV and above represent a stable formulation. Samples were diluted with water to a suitable concentration before the analysis of size distribution.

2.4. Estimation of Lutein Partition Coefficient. To investigate possible drug penetration or retention in the cornea, the following experiments were performed to measure partition coefficient and accumulation rate of lutein in the cornea or sclera. The tendency of lutein into scleral tissue was estimated by measuring its partition coefficient between porcine sclera and our formulation at the temperature of 32°C. Lutein dissolved in the vehicle for the partition experiments at a concentration of 2000 μg/mg. The vehicle (2 g) was added to a previously weighed amount of porcine (sclera (18–22 mg), cornea (25–30 mg)) in a 10 mL vial. The lutein vehicle and the ocular tissues were incubated at the shaker with 200 rpm agitation for 4 h. Three scleral tissues were randomly withdrawn from the vial, rinsed with phosphate buffer saline, wiped with paper, and weighed. The ocular tissue was then homogenized and the lutein accumulated in tissue was extracted by using 2 mL of the extraction buffer (10% tetrahydrofuran and 90% methanol). The homogenized solution was centrifuged at 10000 rpm, filtered by a nylon filter with 0.22 μm pore size, and analyzed by HPLC to determine total lutein content (W_e, μg) in the extraction buffer. Each experiment was replicated at least four times. The drug concentration [C_c] in the cornea

after the incubation period was calculated as previously described with some modification [21]:

$$C_c = \frac{W_d}{W_c}, \quad (1)$$

where W_d is the lutein amount in the cornea tissue and W_c is the weight of the cornea.

Finally, the partition coefficient (K) was then calculated using the formula:

$$K = \frac{C_c}{C_i}, \quad (2)$$

where $[C_i]$ is the initial concentration of lutein in the vehicle.

2.5. Estimation of Lutein Entrapment Efficiency. The prepared nanoparticles were separated from the free lutein using a Sephadex G-50 (GE Healthcare) resin for measurement of entrapment efficiency. The lutein-loaded vehicle of 0.1 mL was separated by using 2 mL of resin. The part of the outflow with opalescence and metered volume to 6 mL were collected and measured by HPLC. The entrapment efficiency (EE) of lutein in the nanoparticles was calculated according to the following equations [22]:

$$\text{EE} = \frac{W_e}{W_i} \times 100\%, \quad (3)$$

where W_i represents the initially added amount of drug and W_e drug represents the amount of drug entrapped in the nanoparticles. The HPLC system (Jasco, Tokyo, Japan) consists of a pump, a UV detector, and a Microsorb-C18 column (Varian, Lake Forest, CA, USA). The mobile phase was composed by 10% (v/v) tetrahydrofuran and 90% (v/v) methanol. The flow rate was 1.0 mL/min and the effluent was monitored at 450 nm as previously described [23]. The lutein is well separated at the retention time 3.85 minutes.

2.6. Preparation of the Ocular Tissues and Histological Examination. Porcine eyes are kindly donated by Ya-Hsen Frozen Foods (Taoyuan, Taiwan). The eye balls are freshly collected and stored in ice during transport. The cornea is dissected within 24 hours of slaughter, wrapped in wetted tissue paper, and stored at −80°C in a polypropylene bag and used within one month [24]. Porcine ocular samples were treated with PBS and CD formulation, respectively, for 24 h on a Franz diffusion cell. Thereafter, ocular tissue was fixed in PBS solution containing 10% formalin and cut vertically, dehydrated using ethanol, embedded in paraffin, and stained with hematoxylin and eosin (H&E) staining. These samples were then observed under light microscope (Olympus BX51, Tokyo, Japan) using 100 magnification. For penetration analysis by confocal laser scanning microscopy, the ocular tissue was removed from the lutein solution and rinsed with PBS and then the surface of the sclera was wiped gently. The ocular tissue was directly sandwiched between a glass slide and a cover slip and examined by using confocal microscopy without additional tissue processing. We used a Leica TCS SP2 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) to assay the lutein corneal distribution. Lutein fluorescence emitted at 515 nm was recorded when excited at a wavelength of 488 nm by means of an argon laser. The sample was scanned from the tissue surface (0 μm) to a depth of 352 μm at a 29.3 μm interval.

Table 1: Effect of cyclodextrins (CD) on corneal lutein accumulation in combination with NLC*.

Vehicles	Accumulation rate (μg/g/hr)	Partition coefficient (10^{-3})	Enhance ratio
NLC	10.6 ± 0.4	21.25 ± 0.8	1
NLC + αCD	12.1 ± 3	24.20 ± 6.2	1.14
NLC + βCD	16.2 ± 9	32.40 ± 1.5	1.52
NLC + HEβCD	16.2 ± 3.5	32.45 ± 7.1	1.53
NLC + HPβCD	19.8 ± 0.2	39.80 ± 0.6	1.80

*The CD and lutein content in the tested vehicles is 0.5% and 2000 μg/g, respectively.

2.7. Cell Culture and Cytotoxicity of Vehicles. Bovine cornea endothelia (BCE, type: C/D-1b) cells were purchased from the BCRC (Hsinchu, Taiwan). BCE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured using trypsin-EDTA when reaching 80% confluence. For cytotoxicity experiments, BCE cells were seeded on 48-well plates at a density of 5×10^4 cells/well and allowed to grow for 48 hours. NLCs were diluted to a series of concentrations using DMEM medium. Cells were fixed by 0.2 mL of 4% formaldehyde and the nucleus stained by Hoechst 33342 and then analyzed by INCell Analyzer 1000 (GE Healthcare, Piscataway, NJ) at the end of the incubation. This high throughput analyzer is designed for cellular imaging assays. The fluorescence at 455 nm in the nucleus stained by Hoechst 33342 was recorded after excitation at 350 nm. The cell viability is defined as follows: (the number of nuclei in treated cells/ the number of nuclei in controlled cells) × 100%.

2.8. Statistical Analysis. Statistical analysis of differences between different treatments was performed using the Student's t-test. A 0.05 level of probability was taken as the level of significance. An analysis of variance (ANOVA) test was also used.

3. Results and Discussion

3.1. NLC Composition on Corneal Lutein Accumulation. In order to evaluate the corneal lutein accumulation by CD combined NLC, the accumulation of lutein in the cornea was examined using the porcine cornea. The accumulation rate and partition coefficient of drug in targeting tissue are important for the evaluation of delivery efficacy. We investigated the effects of combination of various CDs with NLCs on lutein accumulation in the corneal tissue during the four-hour period. As shown in Table 1, the corneal accumulation

Table 2: Summary of corneal lutein delivery using CD combined with NLCs.

Vehicles	Lutein content (μg/g)	Accumulation rate (μg/g/hr)	Partition coefficient (10^{-3})	Enhance ratio
NLC	2000	10.6 ± 0.4	21.25 ± 0.8	1
NLC + 0.5% HPβCD	2000	19.8 ± 0.2	39.8 ± 0.55	1.80
NLC + 1% HPβCD	2000	23 ± 1.8	46 ± 3.7	2.17
NLC + 2% HPβCD	2000	52.3 ± 5	104.6 ± 9	4.92
NLC + 3% HPβCD	2000	27 ± 1.6	54.2 ± 3.2	2.55
NLC + 2% HPβCD	4000	142.8 ± 3.0	142.8 ± 5.7	13.44

and partition coefficient of lutein in combination with NLCs increase in the order of α-CD < β-CD < HeβCD < HPβCD. The stimulatory effect of the CD in NLCs for lutein accumulation in the cornea was confirmed. The partition coefficient between the porcine cornea and the vehicles was calculated by assuming the tissue to be homogenous and the diffusion into cornea to be passive. Since the lutein accumulation increased in the same order, therefore similar trend was observed for the partition coefficient of lutein in the porcine cornea. The partition coefficient of NLC + HPβCD was significantly higher ($P < 0.05$) than that of NLC, whereas no significant difference ($P > 0.05$) existed between the two vehicles NLC + αCD and NLC + βCD. The average lutein accumulation rates in cornea for NLC + 0.5% HPβCD and NLC were 19.8 ± 0.2 and 10.6 ± 0.4 μg g^{-1} hr^{-1}, respectively. NLC combined with 0.5% HPβCD could elevate the lutein accumulation 1.8-fold as compared to that of NLC alone. Besides, the addition of CD in the NLC at the concentration of 0.5% did not affect the stability of formulation. CD-based formulations have successfully delivered various drugs into the eye [15, 25]. Among the tested CDs, HPβCD effectively enhanced the lutein accumulation in the porcine cornea. We consequently investigated the effect of amount of HPβCD in NLC carriers on the lutein accumulation. The dosages of HPβCD in NLC on the corneal lutein accumulation are shown in Table 2. The concentration of HPβCD in the NLC up to 2% could enhance the lutein accumulation in the porcine cornea. No further increase in the lutein accumulation is observed for more than 3% HPβCD in the NLCs. The instability of NLC + 3% HPβCD might account for reducing accumulation since the phase separation was observed in this vehicle. Among the tested HPβCD concentration, 2% HPβCD in NLC carrier could significantly enhance the lutein accumulation in the porcine cornea. The average lutein accumulation rate in cornea for NLC + HPβCD is found to be 52.2±1.8 μg g^{-1} hr^{-1}, indicating the lutein accumulation to be 4.91-fold as compared to that of NLC alone. The payload effect of lutein (1000~4000 μg g^{-1}) in the NLC + HPβCD vehicle on corneal lutein accumulation is depicted in Figure 1. Enhancing effect of the lutein concentration on corneal accumulation was observed using the optimal formulation. The amount of lutein permeating into the pig cornea gradually increased when lutein in the vehicles increased from 1000 to 4000 μg g^{-1}. The elevation of the lutein load in the vehicles could improve the driving force needed for the diffusion of lutein into the cornea. Fick's first law postulates that the diffusion flux goes from

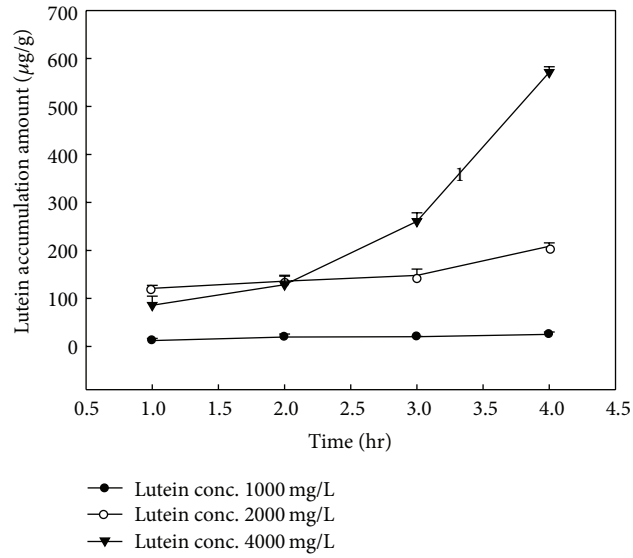


Figure 1: Dose effect of lutein on corneal accumulation using 2% HPβCD combined NLCs.

regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient [26]. As shown in Table 2, the partition coefficient and the accumulation rate of NLC + HPβCD (4000 μg g^{-1} lutein) were significantly higher ($P < 0.05$) than those of NLC + HPβCD with 2000 μg g^{-1} lutein. Similarly, the lutein accumulation rates in cornea for 4000 and 2000 μg g^{-1} lutein in NLC + HPβCD were 142.8 ± 3 and 52.2 ± 1.8 μg g^{-1} hr^{-1}, respectively. The increase in lutein concentration also stimulated the partition coefficient in the porcine cornea. NLC + HPβCD loaded with 4000 μg g^{-1} lutein could elevate the lutein accumulation 13-fold as compared to that of NLC + HPβCD loaded with 2000 μg g^{-1} lutein. Phase separation was induced after 3 weeks storage in the cargo of NLC + HPβCD with 4000 μg g^{-1} lutein. The payload effect of 2000 μg g^{-1} lutein in the NLC + HPβCD vehicle was investigated in the following sections.

3.2. Corneal Lutein Delivery and NLCs Characterization. In order to understand the mechanism of the enhancing effect of HPβCD, the characterization of the four vehicles and lutein distribution pattern in a porcine cornea were performed. The

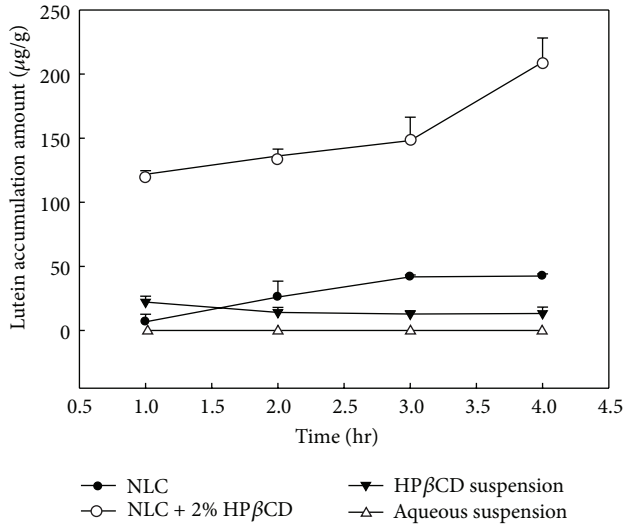
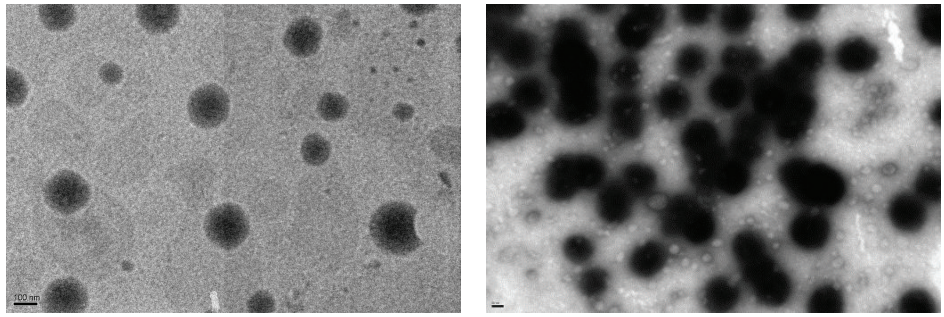


Figure 2: Effect of vehicles on lutein corneal accumulation. The lutein content in all vehicles is 2000 μg/g.

compositions of the four vehicles including NLC, NLC + HPβCD, HPβCD suspension, and aqueous suspension are shown in Table 3. Capric acid, a natural fatty acid, was used as a solid lipid in the NLC. Corn oil and the liquid oil were mixed with capric acid into which the lutein was incorporated. Corn oil was used as a carrier for lutein in the DKSH product. Our NLC system was stabilized by using the two surfactants Tween 80 (with a hydrophile-lipophile balance (HLB) of 15) and Span 60 (with an HLB of 4.7). Combinations of hydrophilic and lipophilic emulsifiers have been proved to maintain a stable dispersion by imparting more rigidity and strength to the binary-surfactant film [27]. In addition, surfactants can reduce the colloidal size by reducing the surface tension and fluidizing the interfacial droplet film. The HLB of surfactant blends (Tween 80/Span 60) were found to be 10.9 by multiplying the weight percentages of surfactants and their individual HLB values. We observed that the droplet sizes in the surfactant-free suspensions were significantly larger than those of the NLC and NLC + HPβCD. Phase separation in the aqueous suspensions was observed after a short storage. The vehicle effect on corneal lutein accumulation is demonstrated in Figure 2. The lutein-loaded vehicles including NLC, NLC + HPβCD, HPβCD suspension and aqueous suspension had significant difference in corneal lutein accumulation. Lutein droplets were formed when lutein was suspended in the water and almost no lutein accumulation in the porcine cornea was observed. The lutein suspended in the HPβCD alone could hardly enhance the lutein accumulation in the cornea. The limited contact area between the big droplets and the cornea contributed to the low tissue accumulation. The partition coefficient of lutein in vehicle of NLC + HPβCD was about 15-fold higher than that of HPβCD suspension. This result clearly indicates the important role of NLCs in the enhancing effect of corneal accumulation. Only HPβCD could not efficiently enhance the corneal accumulation of lutein. As demonstrated in Table 3, the zeta potentials of these vehicles lie in the range −28 to −38 mV. The addition of CD into NLCs would decrease the zeta potential on the surface of NLC. The size distribution of the NLCs is smaller than that of NLC + HPβCD. TEM photographs provided the information for the NLCs microstructure. HPβCD (white aggregation) around the NLCs was observed in the vehicle of NLC + HPβCD as shown in the TEM image. The adhesion of CDs on the surface of NLCs resulted in the increase in particle size. The spherical morphology of lutein-loaded NLCs and NLC + HPβCD could be clearly observed using TEM. From the TEM image (Figures 3(a) and 3(b)), the mean sizes of the NLCs and NLC + HPβCD are found to be 190 and 360 nm, respectively, which is consistent with the size measured by photon correlation spectroscopy (Table 3). The location of the particles is randomly distributed around the NLCs (Figure 3(b)). There are two reasons that may contribute to the existence of the nanoparticles. Firstly, these particles might be the micelles formed by the Tween or Span molecules released into the aqueous phase. The concept of using HPβCD as emulsifying agents in emulsion systems has been proven by several authors. HPβCD can stabilize emulsion systems by complexation of fatty acid residues of the oil phase and by reducing interfacial tension. Previously Klang et al. found that the excess of lecithin aggregates in the bulk water phase was caused by HPβCD molecules which were inserted into the interfacial film of the oil in water droplets [28, 29]. It is likely that the HPβCD may be incorporated with fatty acid residues of the oil phase. Secondly, HPβCD solubilizes the lutein and forms these complexes in the aqueous phase. These particles might be the noncovalent complex of lutein-HPβCD. In both cases, HPβCD can act as a "transporter" and enhances the leave of the lutein from the oil core into the cornea through complex formation. Therefore the lutein had more accumulation in cornea as compared to the HPβCD free vehicles. However, the exact composition of the population in the TEM remains to be investigated. Finally the addition of HPβCD represents an efficient way to promote the permeation of lutein without affecting the corneal barrier function.

The entrapment efficiencies for NLC and NLC + HPβCD are found to be 59% and 68%, respectively. The addition of HPβCD in NLC could enhance the encapsulation of lutein since the cave of HPβCD provided extraordinary space to accommodate lutein. The amount of corneal lutein accumulation using the NLC + HPβCD is found to be significantly higher than that of NLC. There are four factors, namely, enhancement of lutein solubility, large surface of nanoparticles, occlusive effect, and enhancer effect that contributed to the increased corneal delivery by NLC. The first reason is that lutein is hydrophobic and can be readily dissolved in NLC. This causes higher drug loading in the NLCs which in turn increases the concentration gradient towards the cornea. Secondly, the NLCs had more corneal lutein accumulation because the large surfaces ensure close contact and better adhesion on the cornea to deliver the lutein. However, the transport of intact nanoparticles into the cornea is difficult due to the barrier effects of the corneal epithelium [30]. Thirdly, the NLCs form films of densely packed spheres on the surface of the cornea which exert an



(a) (b)

Figure 3: TEM photographs for NLC(A) and NLC + 2% HPβCD (100000x). Scale bar in (a) and (b) represents 100 and 50 nm, respectively. The size of the NLCs was 190 and 360 nm, respectively.

Table 3: Composition and characteristics of vehicles loaded with lutein*.

Composition (%)	Vehicles			
	NLC	NLC + HPβCD	HPβCD suspension	Aqueous suspension
Lutein/Transcutol	0.2/19	0.2/19	0.2/19	0.2/19
Corn oil	0.8	0.8	0.8	0.8
Capric acid	1	1	—	—
Tween 80	3	3	—	—
Span 60	2	2	—	—
HPβCD	—	2	2	—
Water	74	72	78	80
Property				
Mean (nm)	229.8 ± 65	336.8 ± 43	1003 ± 403	945 ± 186
Zeta potential (mV)	−34.3 ± 0.2	−28.0 ± 0.5	−38.1 ± 1.2	−29.2 ± 2.2
Entrapment efficiency (%)	59.0 ± 1.5	68.1 ± 3.3	0.19 ± 0.01	0.06 ± 0.02
Partition coefficient (10^{-3})	21.25 ± 0.8	104.6 ± 0.9	6.6 ± 2.5	0.3 ± 0.05

*The lutein content in all vehicles is 2000 μg/g.

occlusive effect by increasing corneal hydration [13]. This is the fact for increased corneal activity due to occlusive effect. Finally, the enhancer effect that increases corneal delivery by NLC might be due to the surfactants in the formulation. Surfactants which can loosen or fluidize the lipid bilayers on the corneal epithelium can act as permeation enhancers [31]. Formulation stability during storage is important for formulation development [32]. The effect of storage duration on the size and zeta potential of lutein-loaded vehicles was studied at the room temperature for four weeks. As indicated in Figure 4, NLCs could maintain their initial size and zeta potential during 4 weeks of the study period. NLCs with 2% HPβCD could maintain their initial size for 3 weeks. There are two possible locations that HPβCD molecule may occupy in the NLC vehicles. Since HPβCD can form the complex with fatty acid residues of the oil phase, HPβCD may exist in the oil/water interfaces. Another possibility is that HPβCD evenly distributes in the aqueous phase. The partition coefficients of HPβCD in interfaces and water determine its location. The exact location of HPβCD in the NLC formulation merits further investigation. However, the addition of HPβCD in the lipid nanoparticles can promote the accumulation of lutein in the ex vivo cornea model. HP-βCD could enhance the viability of corneal cells in the drug-free or lutein loaded NLCs.

Significant fluctuation of size distribution was observed for the aqueous and HPβCD suspensions during the storage. The size of the CD suspension and aqueous suspension was around 1000–2000 nm which was significantly larger than those of NLC vehicles. Absence of surfactants in these suspension accounted for the instability and large lipid colloids. Chemical transformation of solid lipids during storage is reported to change the structure of lipid core and release the drug [13]. The increase in particle size is a good indicator of instability since the aggregation and sedimentation is easy for these large particles. The zeta potentials for the four vehicles are found to be around −30 mV which could maintain the particles in suspension by repelling each other. The decrease in zeta potential was observed in the samples of NLC, aqueous suspension, and CD suspension during the 28-day storage. No lutein precipitation in the vehicles of NLC and NLC + HPβCD was observed during the storage at 25°C for 28 days. The developed NLCs and NLC + HPβCD are found to be stable when stored below 25°C for 4 weeks. NLCs

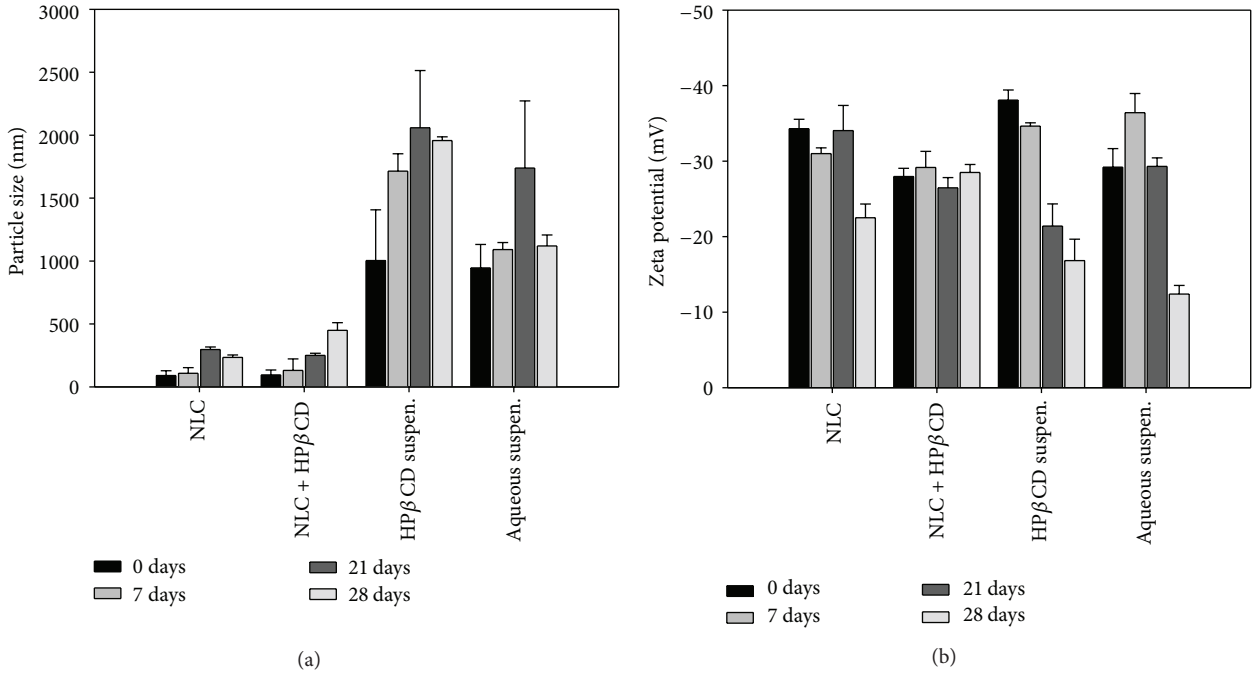


Figure 4: Size stability and zeta potential of lutein loaded vehicles after one month storage, $n = 3$.

also avoid drug expulsion caused by crystallization or lipid transformation of solid lipids nanoparticles upon cooling or storage [33]. The lutein distribution profile in the porcine cornea is evidenced by confocal laser scanning microscopy (CLSM).

3.3. Confocal Observations and Histological Examination of Porcine Cornea. CLSM provides the localization and permeation profile of fluorescent compounds in the transparent cornea without embedding procedures. When lutein is excited by 488 nm laser it emits light of wavelength 515 nm. This distribution of lutein in the cornea for NLC, NLC + HPβCD, HPβCD suspension and aqueous suspension is indicated in Figure 5. The lutein located at various corneal depths (from 0 to 352 μm) was examined using CLSM. A depth of 0 μm represents the corneal epithelium, whereas a depth of 352 μm represents the stroma layer. Lutein in NLC + HPβCD could penetrate the most depth of 176 μm into the cornea and could have the most fluorescence as compared to other vehicles. Since the thickness of corneal epithelium is 54 μm [34], NLC + HPβCD could enter the corneal stroma. The fluorescence intensities of lutein in the skin were ranked in the order of NLC + HPβCD > NLC > HPβCD suspension = aqueous suspension. These results are consistent with corneal lutein accumulation using 4 vehicles shown in Figure 2. CLSM provides the direct evidence of distribution of lutein in the porcine cornea by using lutein fluorescence. The fluorescence intensities of lutein in NLC and NLC + HPβCD at the epithelial layer are found to be 179 and 86 arbitrary units (AU), respectively. The vehicle of

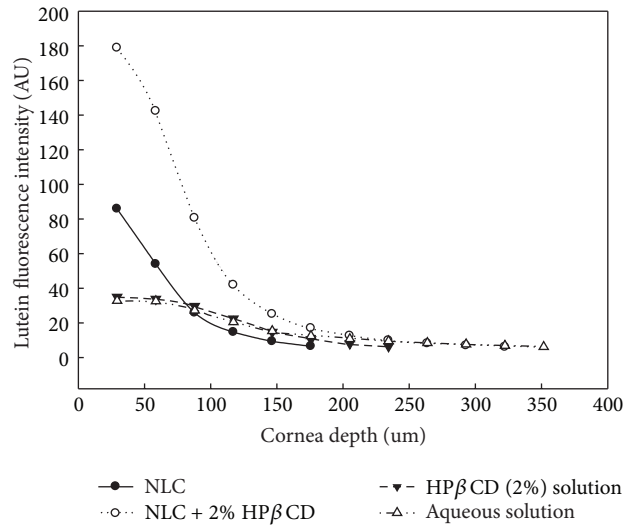
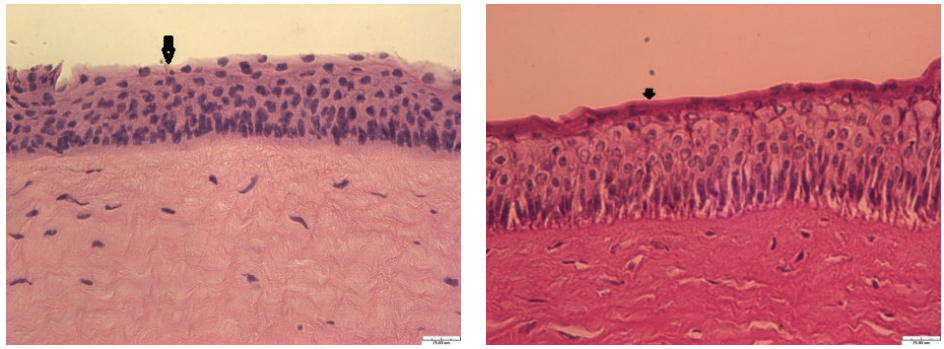


Figure 5: Lutein distribution in porcine cornea after 24 h treatment observed by using confocal laser scanning microscopy. Lutein fluorescence emitted at 515 nm was recorded when excited at a wavelength of 488 nm by means of an argon laser. The sample was scanned from the tissue surface (0 μm) to a depth of 352 μm at a 29.3 μm interval.

NLC + HPβCD exhibited the largest lutein accumulation which provided a driving force for deeper lutein penetration. Very slight lutein penetration from the HPβCD suspension and the aqueous suspension was observed at 29 μm depth. In contrast, lutein could penetrate to the depth of 117 μm in the



(a) (b)

Figure 6: Micrographs of HE staining after 24 h treatment with PBS (a) and NLC + 2% HPβCD (b). Arrow indicates the epithelium. Scale bar represents 25 μm. Porcine cornea after 24 h treatment was fixed, stained, and observed using light microscopy.

corneal stroma with the help of NLC vehicle. The HPβCD or aqueous suspension only had the fluorescence intensity of 35 AU at the outermost epithelium. The distribution profiles made from the CLSM data for lutein within the cornea tissue are observed to be vehicle dependent. The evidence of confocal intensity indicated that the lutein accumulation by NLC + HPβCD in the epithelium was twice higher than that of NLC alone. Since epithelial penetration of lutein is the first step for corneal accumulation, therefore the improved corneal accumulation in the presence of HPβCD may be due to the increase of lutein partition in the corneal epithelium. The lutein accumulation was proved again when HPβCD combined with NLC as the ocular delivery vehicles. In fact, the corneal lutein accumulation is controlled by the parameters such as diffusivity coefficient, path length, and partition coefficient in the corneal tissue. The type of cyclodextrins and lutein payload in the NLCs impacted the partition coefficient and sequentially affected the lutein accumulation. The proposed mechanism of HPβCD combined with NLC in the enhanced lutein accumulation includes the following possibility. In the process of corneal permeation, the drug should be released from the vehicles followed by partition or absorption into the cornea [35]. The diffusion rate of drugs from the vehicle is essential for the corneal accumulation of drugs [36]. The lutein molecules encapsulated into the fat matrix of NLCs had limited diffusion mobility. In contrast, the lutein loaded in HPβCD can be easily released to increase the lutein accumulation rate. Moreover, CD can extract cholesterol, phospholipids, and proteins from the cornea and can reduce the barrier effect of the corneal epithelium [15]. Finally, additional contact surfaces provided by CD might ensure more interact with corneal tissues [20].

3.4. Safety Evaluation of NLCs. The histological examination of the porcine cornea by the NLC + HPβCD was analyzed using Hematoxylin and eosin (HE) staining in order to understand the impact of vehicles on the ocular tissue. Porcine cornea was treated with NLC + HPβCD and control (PBS) cornea for 24 hours before the HE staining. Micrographs of control and NLC + HPβCD treated corneas demonstrated normal histology of cornea as shown in Figures 6(a) and 6(b). Three layers of epithelium, Bowman membrane, and stroma could be clearly observed in the photos. Top of epithelial layer has some deposits of lutein and vehicles as compared to that of PBS treated cornea. Some puffy cells in the boundary between epithelium and Bowman membrane were found which might contribute to the enhanced effect of lutein delivery using the NLC + HPβCD vehicle. Cornea represents a five-layer barrier consisting of lipophilic epithelium (~50 μm), hydrophilic stroma (~450 μm), and lipophilic endothelium (monolayer) as well as Bowman membrane between epithelium and stroma and Descemet's membrane (between stroma and endothelium). Among the layers of cornea, the epithelium and endothelium are considered as lipophilic layers and the stroma is an aqueous layer. The epithelium contributes to resistance to hydrophilic drugs across the cornea [13]. The loose morphology of the epithelium treated by NLC + HPβCD (Figure 6) might act as the channel for the lutein accumulation and delivery into the cornea.

In order to evaluate the formula safety, the bare vehicles and drug loaded vehicles containing 2000 μg g^{-1} lutein were used for the cytotoxicity experiments in BCE cells. The dilution range of vehicles is 0.2~2.5% using DMEM as the diluent. Similarly cell viability was observed for the bare and lutein-loaded vehicles as indicated in Figures 7(a) and 7(b). Lutein payload would not increase the toxicity as compared to the bare vehicles. The viability values for BCE cells at the dose of 1.25% of bare NLC + HPβCD and NLC were found to be 51.2% and 36.7%, respectively. Cytotoxic effects on BCE cells were alleviated when HPβCD added into the NLC in the drug-free or lutein loaded conditions. It has been noted that the toxicity of drug and carriers is decreased when CD incorporated in the formulation. In a previous study, remarkable cytotoxic effects on murine macrophage were found at 0.1% addition of free lipid nanoparticles [37]. The nanoparticles formulated with the stearic acid were cytotoxic at the 1% concentration for mouse J774 macrophages, 3T3 fibroblasts, and human HaCaT keratinocytes [38]. Very limited information is available for the toxicity effects of

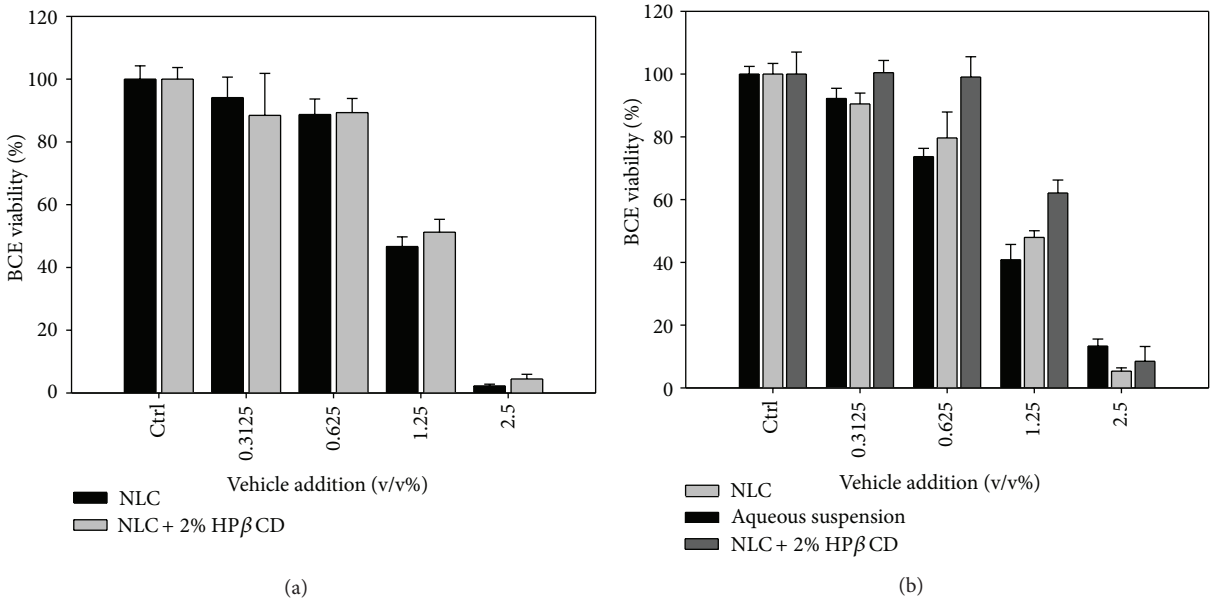


Figure 7: Cytotoxic effect of vehicles on bovine cornea epithelium cells: (a) vehicle only and (b) lutein loaded vehicles.

lipid nanoparticles on ocular cell lines. The results of the cytotoxicity test performed herein on BCE cells point to the importance of the formula ingredients used to prepare the lipid vehicles. Our data demonstrated that HPβCD in the NLC composition could increase the viability of BCE cells (Figure 7).

The physiological cornea condition is significantly different from the ex vivo experiments. However, several research groups choose similar conditions to evaluate the ex vivo drug delivery. For example, the effect of cyclodextrin on corneal permeability of riboflavin is studied in vitro using Franz diffusion cells with a 3-hour period. The steady-state flux and permeability in bovine cornea can be calculated by analyzing the aliquots taken from the receptor chamber every 30 min [24]. Additionally, the permeability coefficient of sodium fluorescein across fresh sclera is calculated by using a 4-hour in vitro diffusion apparatus [39, 40]. These ex vivo results are comparable as they are obtained under similar condition. However, the animal test is necessary for the further evaluation of the developed formulation.

In conclusion, lutein is a hydrophobic antioxidant associated with the macular degeneration. This study demonstrated the lutein localization in the porcine cornea by using the novel nanocarriers. However, lutein could not penetrate the whole porcine cornea under the tested conditions. We have explored the combinatory effect of CD and NLC on the corneal partition, encapsulation efficacy, stability, and distribution of lutein. The corneal accumulation and partition of lutein are improved by the developed vehicle. Also HPβCD enhanced the viability of corneal cells in the drug-free or lutein loaded NLCs. The permeation enhancement and nanoscaled properties of HPβCD combined with NLCs contributed to the enhanced accumulation of lutein in the cornea.

Conflict of Interests

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

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