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Innovation in ocular pharmacology

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Innovation in ocular pharmacology

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Editorial: Innovation in ocular pharmacology

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Editorial on the Research Topic
Innovation in ocular pharmacology

An innovative medicinal product can be a new active substance acting against or preventing a disease and developed to improve the quality of patient management.

Thus, product novelty alone is not sufficient to characterize therapeutic innovation, as an improvement of health outcomes is necessary.

Recognizing a true innovation can accelerate the development and adoption of valuable treatment options, encouraging their prioritization to make them quickly available for patients with high unmet medical needs.

This Research Topic collects updated evidence about *Innovation in ocular pharmacology*, a field which rapidly grown over the last few years, leading to the discovery of disease modifying treatments for several eye disorders.

Nevertheless, several unmet needs remain, such as retinal diseases (Cursiefen et al., 2019; Conti et al., 2021; Kaminska et al., 2021), characterized by a devastating impact on the quality of life but also on the healthcare system and the society.

Anti-vascular endothelial growth factor (anti-VEGF) agents represent the mainstay of treatment for neovascular age-related macular degeneration (AMD) (Jonas et al., 2017). However, this therapy can slow the progression but do not cure the disease.

Abdin et al. analysed data from the first year of treatment with brolucizumab for refractory neovascular AMD. This study showed that the drug allows to stabilize visual acuity with less injections in patients with refractory disease, to reduce subretinal fluid and pigment epithelial detachment.

Moreover, the intraocular injection is associated with the risk of several complications, including inflammation, intraocular pressure elevation, hemorrhage, and endophthalmitis, which can lead to significant visual loss (Falavarjani and Nguyen, 2013).

In this regard, Dolar-Szczasny et al. evaluated the onset of ocular inflammation after repeated bevacizumab injections in patients with AMD. The study showed that none of the subjects treated with bevacizumab had detectable inflammation during follow-up, suggesting a good safety profile of the drug.

On the contrary, no specific treatments are currently available for dry AMD. The study of Melecchi et al. compared the efficacy of an oral formulation based on lutein and fish oil, as a

source of omega-3, with a combination of lutein and astaxanthin with Calanus oil (COil), containing omega-3 and their precursors policosanols in a mouse model of dry AMD. Both mixtures demonstrated to exert a significant antioxidant and anti-inflammatory activity, in particular the formulation based on COil. These results support the use of dietary supplements in the prevention and treatment of AMD, suggesting the potential role of fatty acids of COil origin as AMD modifying therapies with higher efficacy compared to fatty acids of fish oil origin.

Anti-VEGF monotherapy represents the first line treatment for Retinal Angiomatous Proliferation (RAP), a form of neovascular AMD. RAP lesions may be difficult to treat and may have a worse response compared with other forms of AMD. In this regard Fallico et al. compared the functional and anatomical outcomes of anti-VEGF monotherapy with the combination of anti-VEGF and Photodynamic Therapy (PDT) in a systematic review with meta-analysis. The study showed that a combined approach with anti-VEGF and PDT could provide better functional and anatomical outcomes in RAP compared with monotherapy.

Diabetic retinopathy (DR) still represents a major cause of impaired vision and blindness. In these patients, oxidative stress, inflammation, and vascular dysfunction lead to retinal ischemia and blood retinal barrier (BRB) impairment (Bucolo and Drago, 2004). Vitamin D3 have been studied in several eye diseases, for its anti-inflammatory, antioxidant and anti-angiogenic activity. The aim of the study of Lazzara et al. was to assess the effects of vitamin D3 on BRB damage using human retinal endothelial cells exposed to high glucose levels. Vitamin D3 demonstrated to preserve the BRB integrity, attenuating cell damage, and reducing the level of inflammatory cytokines. This study supports the protective role of vitamin D3 in DR, characterized by inflammation and BRB disfunction, but also in other retinal conditions.

Diabetic macular edema (DME) is the major cause of vision deterioration in patients with DR. The dexamethasone (DEX) intravitreal implant is a biodegradable device which slowly release DEX for up to 6 months, approved for the management of DME (Mathis et al., 2020). Several studies compared the safety and effectiveness of the DEX implant for DME in nonvitrectomized and vitrectomized eyes. Yuan et al. conducted a systematic review and meta-analysis to compare the improvements of DME with DEX implant in nonvitrectomized and vitrectomized eyes. The study showed no significant differences in terms of anatomical and functional effects between vitrectomized and nonvitrectomized eyes, with good safety profile.

Zerbini et al. focused on the possibility to prevent DR using topical nerve growth factor (NGF). The study showed that retinal neurodegeneration represents an early self-limiting phenomenon in the first stage of the disease, followed by vascular dysfunctions. Topical administration of NGF demonstrated to prevent neurodegeneration, but also the development of the vascular damage of DR.

Retinopathy of prematurity (ROP) is a primary cause of blindness in children characterized by abnormal retinal vessel development (Alajbegovic-Halimic et al., 2015). Currently, laser photocoagulation and anti-VEGF agents are the first- and second-line therapies to treat ROP, but they are invasive methods, and no long-term data are yet available (Shulman and Hartnett, 2018; Stahl et al., 2019). RNA therapy has been investigated in several diseases and showed potential as innovative therapy in ophthalmology. Kim et al. reviewed available evidence about noncoding RNAs (ncRNAs) as treatment for ROP.

Finally, two papers focused on ocular surface diseases. Gao et al. compared the effectiveness and safety of different formulations of cyclosporine A (CsA) for the treatment of dry eye disease using a network meta-analysis including high-quality placebo-controlled trials. The results of the study suggest that various formulations of CsA are effective in the treatment of dry eye, but it's difficult to select the optimal one.

After a corneal damage, consequent to various insults including dry eye disease, it is essential to restore the epithelium, the stroma, but also the nervous components. Bucolo et al. performed *in vivo* and *in vitro* studies with six different ocular formulations to evaluate corneal nerve regeneration and corneal wound healing after corneal damage. A new ophthalmic gel containing cross-linked sodium hyaluronate, taurine, vitamin B6 and Vitamin B12 demonstrated to better reduce oxidative stress, to accelerate corneal re-epithelization and to promote nerve regeneration.

Author contributions

LG and GR wrote the manuscript. VP and MT revised and approved the final draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Evaluation of Aqueous Flare Intensity in Eyes Undergoing Intravitreal Bevacizumab Therapy to Treat Neovascular Age-Related Macular Degeneration

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Purpose: To evaluate the effect of repeated intravitreal bevacizumab injections on blood-aqueous barrier permeability in eyes with neovascular age-related macular degeneration (AMD).

Patients and Methods: Forty-eight consecutive patients with neovascular AMD received 3 intravitreal bevacizumab injections (1 mg) every 30–40 days. Subjects were followed for a period of 4 months and were examined at baseline, 1 day and 1 month after each injection. A control group comprised of 19 neovascular AMD patients waiting to begin anti-vascular endothelial growth factor (VEGF) therapy. Anterior chamber (AC) inflammation was evaluated with biomicroscopy and laser flare photometry.

Results: None of the subjects treated with bevacizumab had detectable ocular inflammation during follow-up. An analysis for variance (ANOVA) of the mixed-effects model has shown neither an effect between treatment and control group ($p = 0.921$), nor over the time course of the follow-up ($p = 0.773$). Before treatment, median AC inflammation was 6.7 photons/ms (range: 3.5–18.2 photons/ms). One month after the first, second, and third injections, median laser flare was 6.4, 6.8, and 6.6 photons/ms, respectively, none of which were significantly different from baseline (all $p > 0.05$). Blood-aqueous barrier permeability did not change between injections and was not different from the control group.

Conclusion: Inflammation induced by intravitreal bevacizumab was not detected by examination or flare photometry. This suggests that monthly bevacizumab dosing seems to be safe. The absence of AC inflammation could also reflect the known anti-inflammatory properties of anti-VEGF agents.

Keywords: bevacizumab, neovascular age-related macular degeneration, laser-flare photometry, intraocular inflammation, blood-aqueous barrier integrity

INTRODUCTION

Vascular endothelial growth factor (VEGF) has become the main target for treating neovascular age-related macular degeneration (AMD) in recent years (Plyukhova et al., 2020). As a result, intravitreal injections of anti-VEGF agents are now widely used to halt neovascular AMD progression and, hopefully, improve central visual acuity. Unfortunately, each intraocular injection, even when performed under sterile conditions, carries a risk of vision-threatening complications, including intraocular inflammation, endophthalmitis, intraocular pressure elevation, vitreous hemorrhage, and retinal detachment (Falavarjani and Nguyen, 2013). The most serious of these complications is sterile or infectious endophthalmitis, which can lead to significant visual loss (Dossarps et al., 2015).

Among anti-VEGF agents, bevacizumab is often used intravitreally to handle several retinal diseases (Falavarjani and Nguyen, 2013; Reibaldi et al., 2014; Dossarps et al., 2015; Platania et al., 2015; Plyukhova et al., 2020; Yousef et al., 2020; Toro et al., 2021). Bevacizumab is used in ophthalmology in an off-label fashion and, thus, remains a somewhat controversial treatment option. Because the approved indications by drug regulatory agencies did not include ocular diseases, a concern regarding the safety profile and risk for emerged considering the potential ocular inflammatory response following intravitreal administration. Safety issue using chronic intravitreal bevacizumab dosing regimen has also arisen because most AMD patients require at least three injections during the first year of treatment (Plyukhova et al., 2020). On this regards it could be useful develop a biodegradable deliver system to inject bevacizumab, avoiding a multiple treatment (Conti et al., 1997).

Secondly, with regard to recent reports on more frequent inflammatory reactions after the use of the newly registered anti-VEGF drug-brolucizumab (Baumal et al., 2020), the issue of side effects has become even more topical. At the beginning of 2020, the American Society of Retinal Specialists (ASRS) alerted ophthalmologists to reported cases of ocular inflammation after brolucizumab injections (Beovu Update for ASRS Members, 2020).

A potent ocular inflammatory response is easily visible by slit-lamp examination after intraocular injection. This is especially true of changes in anterior chamber fluid clarity, but slight fluid changes can be overlooked (Falavarjani and Nguyen, 2013). However, a laser flare photometer can detect even subtle blood-ocular barrier changes that may not be detectable with standard ophthalmological clinical examination (Tugal-Tutkun and Herbort, 2010). Laser flare photometry (LFP) is a non-invasive tool and allows anterior chamber flare (from disruption of the blood-ocular barriers) to be objectively, accurately, and reproducibly quantified. Therefore, LFP allows ocular inflammatory responses induced by medications or surgical procedures to be examined and compared (Ladas et al., 2005; Tugal-Tutkun and Herbort, 2010; Orès et al., 2020).

Here, we use LFP to examine the effect of multiple intravitreal bevacizumab injections on ocular inflammation in neovascular AMD patients.

PATIENTS AND METHODS

The study protocol was reviewed and approved by the Institutional Review Board (IRB) at Medical University, Lublin, Poland (n° KE-0254/208/2013) on July 11th, 2013. Being the LFP a noninvasive diagnostic tool and according the ongoing regulation, the IRB waived the requirement of informed consent. All study conduct adhered to the tenets of the Declaration of Helsinki.

Study Subjects

Consecutive patients diagnosed with neovascular AMD in the Lublin University Department of General Ophthalmology between January and September 2015 were retrospectively considered for inclusion in this cross-sectional analysis. All subjects had AMD with active macular neovascularization (MNV) confirmed with fluorescein angiography (FA), indocyanine green angiography, and optical coherence tomography (OCT). Only patients who required at least three intravitreal bevacizumab injections on a pro re nata treatment regimen and who had received a LFP monitoring during the loading phase, were included. Patients with advanced cataract, a history of uveitis or inflammation, vitreous hemorrhage, neovascular glaucoma, corneal opacities, recent ocular surgery (within 3 months), or prior anti-VEGF injections were excluded.

A control group of active neovascular AMD patients who were waiting to begin anti-VEGF therapy were also included and observed over a 4 months period. The delayed therapy in this group of patients was related to insufficient health availability in our clinic. These patients were informed about alternative medical centers and about the risks of delayed treatment but, due to transportation barriers and reimbursement issues, decided to wait for therapy in our hospital.

Intravitreal Bevacizumab Injections

The use of intravitreal bevacizumab (Avastin, Genentech, Inc., South San Francisco, CA) as an off-label treatment was approved by the local ethics committee and it is part of clinical routine care in our department. All injections were performed under sterile conditions in our operating room after the patient had signed an informed consent for off-label drug administration. All treatments in this study were carried out as part of routine clinical care for neovascular AMD. After the eye was topically anesthetized with proxymetacaine (0.5%, Alcon-Couvrer nv, Puurs, Belgium), it was disinfected with several drops of 5% povidone iodine (Betadine Ophthalm., Alcon Laboratiries Inc.) placed in the conjunctival sac. Next, a 1.25 mg bevacizumab dose in 0.05 ml was injected into the vitreous cavity. The injected bevacizumab was obtained from a 4 ml vial that contained a 25 mg bevacizumab/ml solution. Sterile tuberculin syringes were used under sterile conditions to generate 0.1 ml (2.5 mg bevacizumab) aliquots of the drug just before intravitreal injections were prepared. The dosages were prepared by the doctor performing whole procedure in the surgical theatre. One day before and 5 days after injection, patients prophylactically used a topical antibiotic. Multiple injections were carried out with a pro renata regimen based on the clinical activity of the disease and the availability of the drug.

TABLE 1 | Anterior chamber flare before and 1 day and 1 month after each intravitreal bevacizumab injection.

	n° (eyes)	Anterior chamber flare (photons/ms)					*p*
		Min	Max	Mean	SD	Median	
Before treatment	48	3.5	18.2	7.51	3.42	6.70	
1 day after 1st injection	47	2.9	16.1	7.34	3.18	6.80	0.738
30 days after 1st injection	46	1.0	17.0	7.07	3.26	6.40	0.419
1 day after 2nd injection	47	3.2	16.9	8.03	3.07	7.10	0.419
30 days after 2nd injection	41	2.6	27.1	7.74	4.24	6.80	0.842
1 day after 3rd injection	38	2.7	22.5	7.60	3.86	6.50	0.882
30 days after 3rd injection	48	3.1	17.2	6.98	2.62	6.60	0.333

Min, minimum value; max, maximum value; SD, standard deviation; p, p *value:* p*-values estimated with linear mixed-effects model.*

Clinical Examinations

All patients were carefully and prospectively examined before, one day and one month after each of the three intravitreal bevacizumab injections during the loading phase. Treated and control subjects were monitored for a range of 4 months.

Ocular inflammation was qualitatively and quantitatively assessed by slit-lamp and fundoscopic examinations and a laser flare photometer, respectively (Kowa FM-500, Kowa Company, Ltd., Tokyo, Japan). All evaluations were made following pupil dilation with topical 1% tropicamide (Polfa-Warsaw SA, Poland). The final flare photometry value was automatically calculated by averaging 5 individual measurements. A total of 7 measurements were obtained, but the highest and lowest measurement values were excluded by the flare meter. All measurements were performed in a darkened room after calibrating the flare meter.

Data Analyses

Data are presented as mean, median, standard deviation (SD), minimum value (min) and maximum value (max). Differences between groups were tested for statistical significance using the Mann-Whitney *U* test. For repeated measure data, a linear mixed-effects model was carried out to estimate differences between study groups and different time points. The two factors were adjusted for the baseline value. An analysis of variance (ANOVA) test of the mixed-effects model and the comparison to baseline for the treatment group, are presented as *p*-values. Statistical significance was defined as $p < 0.05$. Statistical analyses were performed using STATISTICA 12 statistical software (StatSoft Polska, Krakow, Poland) and the statistical software R version 4.0.

RESULTS

A total of 48 eyes of 48 patients (20 men, 28 women) were ultimately included in the injection group. Median patient age was 70 years (range: 47–87 years). A total of 19 eyes of 19 patients (8 men, 11 women) were ultimately included in the control group. Median control patient age was 65 years (range: 49–86 years).

None of the 48 patients in the injection group had clinically detectable anterior chamber inflammation during the follow-up period. Before treatment, median LFP measured 6.7 photons/ms (range: 3.5–18.2 photons/ms). This value was not significantly different from baseline one day following the first (median: 6.8 photons/ms, $p = 0.738$), second (7.1 photons/ms, $p = 0.350$), or third (6.5 photons/ms, $p = 0.882$) injection. The same was also true one month following the first (6.4 photons/ms, $p = 0.419$), second (6.8 photons/ms, $p = 0.842$), and third (6.6 photons/ms, $p = 0.333$) injections (**Table 1**). An ANOVA test of the linear mixed-effects model was carried out with neither a significant difference for treatment-control groups ($p = 0.921$) nor for the time points ($p = 0.773$). Mean values of anterior chamber flare were not significantly different between treated women and treated men at any follow-up time point examined (**Table 2**). Furthermore, there were no significant differences in blood-aqueous barrier permeability between treated (injection group) and untreated (control group) subjects at any time point examined (**Table 3**).

DISCUSSION

Vascular endothelial growth factor is a well-known promoter of angiogenesis and has been shown to be involved in the pathogenesis of wet AMD. Although many other methods have been explored, intravitreal anti-VEGF therapy is the only disease-modifying treatment for the retinal neovascular diseases (Plyukhova et al., 2020). The three anti-VEGF agents commonly used to treat retinal neovascular diseases include bevacizumab, ranibizumab, and aflibercept (Plyukhova et al., 2020). Intravitreal injections are simple to perform and the procedure for this treatment has been well established. However, intraocular injections are still considered to be invasive treatment (Avery et al., 2014), particularly for multiple injections. Some concern about intravitreal injection still exists, even though the injection is performed under sterile conditions (Grzybowski et al., 2018). In addition, the topic of inflammatory response has returned with the launch of a new anti-VEGF agent—brolucizumab (Yousef et al., 2020; Toro et al., 2021). For this drug, in phase 3 clinical trials and according to the FDA label, the incidence of ocular inflammation was higher (>4%) than other anti-VEGF agents (<1%) (Dugel, 2017; US Food and Drug Administration, 2019).

The most common treatment, in some countries, for ocular neovascular disease is bevacizumab, even though its use is off-label (Berg et al., 2015). Clinical observations have shown that the pharmacological effect of bevacizumab to handle retinal diseases is as safe and effective as to other anti-VEGF agents (Berg et al.,

TABLE 2 | Anterior chamber flare before and 1 day and 1 month after each intravitreal bevacizumab injection in group of women and men.

	Women			Men			*p*
	n°	Mean	SD	n	Median	SD	
Age	28	67.71	11.25	20	69.55	7.94	0.565
Before treatment	28	7.34	3.55	20	7.75	3.31	0.579
1 day after 1st injection	27	7.21	3.51	20	7.52	2.75	0.383
30 days after 1st injection	27	7.01	3.18	19	7.15	3.46	0.647
1 day after 2nd injection	27	8.26	3.04	20	7.72	3.16	0.583
30 days after 2nd injection	25	7.47	3.20	16	8.16	5.59	0.936
1 day after 3rd injection	24	7.41	3.30	14	7.93	4.79	0.987
30 days after 3rd injection	28	6.73	2.78	20	7.33	2.42	0.310

SD, standard deviation; p, p *value:* p*-values estimated with Mann-Whitney U test.*

TABLE 3 | Anterior chamber inflammation in the control group and in eyes treated with intravitreal bevacizumab.

	Laser flare photometry (photons/ms)						*p*
	Control group			Bevacizumab group			
	n° (eyes)	Mean	SD	n° (eyes)	Mean	SD	
Before treatment	17	7.32	3.22	48	7.51	3.42	0.840
1 day after 1st injection	18	8.56	3.75	47	7.34	3.18	0.253
30 days after 1st injection	18	7.03	3.39	46	7.07	3.26	0.893
1 day after 2nd injection	19	7.80	1.98	47	8.03	3.07	0.860
30 days after 2nd injection	19	6.68	2.47	41	7.74	4.24	0.546
1 day after 3rd injection	17	6.49	3.24	38	7.60	3.86	0.233
30 days after 3rd injection	18	7.49	4.76	48	6.98	2.62	0.565

SD, standard deviation; p, p *value:* p*-values estimated with Mann-Whitney U test.*

2015; Martin et al., 2020; Plyukhova et al., 2020). The CATT Research Group performed the largest bevacizumab-ranibizumab comparison study, which included 1,208 patients from 44 centers in the United States. Patients were put on a monthly or as-needed treatment scheme. Endophthalmitis was rare, occurring after 2 of 5,449 injections (0.04%) in 599 patients treated with ranibizumab injections (Martin et al., 2011). A similar incidence (0.07%, 4 of 5,508 injections) was observed in the 586 patients treated with bevacizumab ($p = 0.49$) (Reibaldi et al., 2019). However, one or more serious systemic adverse events occurred in 31.7% of ranibizumab-treated patients and in 39.9% of bevacizumab-treated patients ($p = 0.004$) (Martin et al., 2011).

Endophthalmitis is the most threatening complication associated with intravitreal injection (Reibaldi et al., 2019). However, clinical data suggests that most cases following injection were caused by medications contaminated during dose extraction (Merani and Hunyor, 2015).

Another safety concern surrounding intravitreal bevacizumab use, is ocular inflammation, that is known to occur after multiple intravitreal injections. Some reported cases associated with intravitreal injections suggest sterile endophthalmitis (Chong et al., 2010; Williams et al., 2016). These observations raise concerns of treating ophthalmologists regarding the risks and benefits of neovascular AMD treatments. Higher doses of intravitreal anti-VEGF agents have been shown to significantly increase the risk of intraocular inflammation (Rosenfeld et al., 2005). However, one study assessed anterior chamber inflammation after one intravitreal bevacizumab injection in eyes with exudative AMD and found no inflammatory response (Martin et al., 2012). Furthermore, a significant decrease from pre-injection values occurred in anterior chamber flare 7 days after injection. Our results, in accordance with Yeniad et al. (2011) did not show a decrease in ocular inflammation following injection. It may have been that Kiss et al. administered topical steroids following injection (Kiss et al., 2006), as it was commonly done in some centers. Unfortunately, this was not discussed by authors. In another study, a reduction in laser flare was observed two months after bevacizumab injections. However, only 8 patients were included in this analysis (Errera et al., 2014).

Even though we did not observe a decrease in ocular inflammation, we also did not observe an increase in inflammation. This finding is in agreement with pre-clinical *in vivo* studies that examined the ocular toxicity of four different intravitreal bevacizumab doses. A 5 mg dose of bevacizumab was not toxic to the retina, and only a few inflammatory cells in the vitreous were identified (Manzano et al., 2006; Xu et al., 2010).

The VEGF protein is known to provoke an inflammatory reaction by increasing vascular permeability and activating adhesion of leucocytes to the vascular endothelium (Adamis and Shima, 2005). It is also well-known that eyes with neovascular AMD have markedly increased VEGF levels and higher flare values than normal eyes (Kubota et al., 1994). Indeed, Kubota et al. have shown that flare values in eyes with age-related

macular degeneration were 0.28 ± 0.18 mg/ml, being significant in comparison with the control (0.12 ± 0.05 mg/ml) (Kubota et al., 1994). Therefore, our control group was comprised of eyes with neovascular AMD to minimize baseline differences in ocular inflammation. The delayed therapy in this group of patients was related to insufficient health availability in our clinic and not to an unethical decision. Till November 2015 (our study was concluded in September 2015) treatment of patients with wet AMD has been an epidemiological and economical problem in Poland. Since that date special treatment program financed from public funds started and the situation slowly improved (Figurska et al., 2020). Mekjavic et al. have provided a comprehensive overview of the clinical and economic burden of wet-AMD and DME in Central and Eastern Europe and the status quo associated with their management (Jaki Mekjavic et al., 2019). Patients from our control group were fully aware of risks resulting from delayed treatment but, due to transportation barriers and reimbursement issues, decided to wait for injections in our hospital and be monitored for the time pending the therapy.

Prior studies have compared ocular inflammation in patients treated with intravitreal anti-VEGF for various exudative eye diseases (e.g., non-proliferative diabetic retinopathy, macular edema with branch or central retinal vein occlusion). No differences in anterior chamber inflammation were observed (Yeniad et al., 2011). Various anti-VEGF agents (bevacizumab, ranibizumab, and aflibercept) have also been compared. Blaha et al. found a small, but statistically significant, difference between the change in anterior chamber flare 1 day after intravitreal bevacizumab or intravitreal ranibizumab administration (Blaha et al., 2015). However, the small observed difference was not clinically relevant because no evidence of increased cell or are counts has been observed after routine use of these drugs (Blaha et al., 2015).

Recent reports of a new agent-brolucizumab and its possible side-effects have re-ignited interest in the cause of inflammatory reactions after intra-vitreous anti-VEGF drug administration. The mechanism of inflammation during anti-VEGF therapy remains unclear and is currently under investigation. Various theories suggest an immune response to the active molecule of the drug, other protein by-products within the drug or pH changes. One of the possible hypotheses for the pathogenic mechanism of this spectrum of events that is under investigation is the formation of local anti-bodies (Agrawal et al., 2013; Baumal et al., 2020; Haug et al., 2020). Clarification of the pathogenesis of inflammatory reactions after some anti-VEGF drugs is important also for clinical reasons. It is crucial to distinguish non-infectious from infectious intraocular inflammation, a severe vision-threatening condition that requires urgent evaluation and treatment.

CONCLUSION

In conclusion, our results showed a good safety profile and lack of inflammatory response following multiple intravitreal bevacizumab injections. These observations confirm that multiple intravitreal bevacizumab administrations are safe with no risk for patients with exudative AMD, even though CATT study demonstrated that the proportion of patients with one or more systemic serious adverse events was higher with bevacizumab than ranibizumab. Further prospective studies with an adequate sample size calculation and longitudinal testing are mandatory to confirm our preliminary data.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol was reviewed and approved by the Institutional Review Board (IRB) at Medical University, Lublin, Poland (n° KE-0254/208/2013), on July 11th, 2013. With the LFP being a noninvasive diagnostic tool and according the ongoing regulation, the IRB waived the requirement of informed consent. All study conduct adhered to the tenets of the Declaration of Helsinki.

Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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First Year Real Life Experience With Intravitreal Brolucizumab for Treatment of Refractory Neovascular Age-Related Macular Degeneration

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Purpose: To assess the morphological and functional outcomes within the first year of treatment with intravitreal brolucizumab for refractory neovascular age-related macular degeneration (nAMD).

Methods: This retrospective study included 21 eyes from 19 patients with refractory nAMD followed for 12 months. All patients were switched to brolucizumab after treatment with at least two other anti-vascular endothelial growth factors (VEGF). All eyes received 3x brolucizumab 6 mg/0.05 ml intravitreal injections (IVI) monthly as an upload phase. Then eyes received an IVI every 8 weeks with interval adjustment to every 12 weeks if disease activity was not present. Main outcome measures: best corrected visual acuity (BCVA), central macular thickness (CMT) and retinal fluid distribution. In addition, we reported the adverse event rate.

Results: The number of previous anti-VEGF IVIs/eye was 36 ± 22 before switching to brolucizumab. BCVA (ETDRS) was 51 ± 16 before treatment and 50 ± 19 at week 52 (p = 0.6). CMT was 374 ± 158 µm before treatment and 298 ± 92 µm at week 52 (p = 0.01). The number of IVIs/eye decreased from 9.6 ± 1.9 IVIs in the last year before switching to 6.4 ± 0.9 IVIs in the first year after switching to brolucizumab ($p < 0.001$). The rate of eyes with subretinal fluid and pigment epithelial detachment decreased at week 52. Finally, two cases of intraocular inflammation were observed as adverse events.

Conclusion: In the first year of treatment, intravitreal brolucizumab was able to stabilize visual acuity with significantly less IVIs in patients with refractory nAMD. It also improved anatomic outcomes in these patients, particularly reducing subretinal fluid and pigment epithelial detachment and subsequently central macular thickness. However, two cases of intraocular inflammation were observed as adverse events.

Keywords: neovascular age-related macular degeneration (nAMD), intraocular inflammation, intravitreal brolucizumab, switching therapy, refractory macular edema

INTRODUCTION

Neovascular age-related macular degeneration (nAMD) is an accelerating disease that represents an increasingly high burden for both patients and health care systems in developed countries (Jonas et al., 2017).

The important role of vascular endothelial growth factor (VEGF) in the development of macular neovascularization (MNV) related to nAMD has been confirmed (Augustin and Kirchhof, 2014). Indeed, it rapidly triggers angiogenesis, enhances vascular permeability, takes part in the disintegration of the blood-retinal barrier, and facilitates an inflammatory reaction (Ferrara et al., 2003). Therefore, intravitreal injections (IVI) of anti-VEGF agents would currently be considered as the gold standard therapy for nAMD, with a primary target of improving or preserving visual acuity (Li et al., 2020).

At present, three anti-VEGF agents have been licensed in Europe and the United States to treat nAMD: ranibiziumab, aflibercept and brolucizumab (Markham, 2019; Li et al., 2020).

Brolucizumab (Beovu®, Novartis Pharma GmbH, Nuernberg, Germany) was approved by regulatory authorities in the United States in October 2019 and the European Union in February 2020 for the treatment of nAMD. It is a low molecular weight (26 kDa) single-chain antibody fragment with high affinity against all forms of VEGF-A, better tissue penetration and higher molar concentration (Holz et al., 2016; Dugel et al., 2020; Dugel et al., 2021).

The clinical trial data from HAWK and HARRIER indicate that brolucizumab has comparable best-corrected visual acuity compared to aflibercept, with better anatomic outcomes. There is an opportunity to extend the dosing regimen to 12-week intervals, which could relieve the treatment burden (Dugel et al., 2021).

On the other hand, the HAWK and HARRIER studies showed a comparable safety profile of brolucizumab and aflibercept, except for a higher rate of intraocular inflammatory events (4.4%) in eyes being treated with 6 mg brolucizumab compared with aflibercept (0.8%) (Dugel et al., 2021). In addition, some clinical studies have reported intraocular inflammatory events with retinal vasculitis and retinal vascular occlusion with occasional severe vision loss after treatment with brolucizumab (Baumal et al., 2021; Holz et al., 2021).

The purpose of the present study was to assess the morphologic and functional outcomes and adverse effects within the first year of treatment with intravitreal brolucizumab for refractory macular edema due to nAMD.

MATERIALS AND METHODS

This retrospective monocentral study included 21 eyes of 19 patients with refractory macular edema due to nAMD followed for 12 months.

Refractory macular edema was defined morphologically as persistent intraretinal fluid (IRF) and/or subretinal fluid (SRF) and/or retinal pigment epithelial detachment (PED) despite treatment with at least two anti-VEGFs. This was identified using spectral-domain optical coherence tomography (SD-OCT).

All patients were switched to brolucizumab after treatment with at least two other anti-VEGFs, including ranibizumab, aflibercept and bevacizumab. All eyes received 3x brolucizumab 6 mg/0.05 ml IVIs monthly for an upload phase. Then, eyes received an IVI every 8 weeks with interval adjustment to every 12 weeks if disease activity was not present (Dugel et al., 2021).

All IVIs were performed between 16 March 2020, and 31 December 2021, at a designated center for intravitreal injections in our Department of Ophthalmology at Saarland University Medical Center (Abdin et al., 2020).

The inclusion criteria were.

1) Eyes with refractory macular edema due to nAMD (as defined above)
2) A minimum follow-up of 12 months.

The exclusion criteria were.

1) History of treatments with photodynamic therapy (PDT).
2) Eyes with macular scarring preventing a change in visual function.
3) Eyes with coexisting vitreoretinal pathology.
4) Intraocular surgery (cataract surgery, pars plana vitrectomy) within the first year of treatment with brolucizumab.

Naïve patients were not included in this study.
Main outcome measures included:

- Best corrected visual acuity (BCVA) as measured on a Snellen decimal scale and converted to approximate ETDRS (Early Treatment Diabetic Retinopathy Study) letter scores (Beck et al., 2003).
- Central macular thickness (CMT) as measured by (Spectralis SD-OCT; Heidelberg Engineering, Heidelberg, Germany) and defined as the mean retinal thickness (μm) between the internal limiting membrane (ILM) and the basement membrane of Bruch (BM) in the central 1 mm of the fovea.
- The number of IVIs before and during the first year of treatment.
- The presence of IRF, SRF, and PED was detected in OCT at baseline and each follow-up visit.

All outcomes were assessed at baseline, then at week 4, 8, 16, 20, 28, 36, 44 and 52 after treatment. The day of the first brolucizumab IVI was considered as the baseline follow-up.

In addition, all patients were followed up 2–5 days after each IVI according to the guidelines of the German Society of Ophthalmology (German Society of Ophthalmology et al., 2021). At each visit, a complete ophthalmic examination, including slit-lamp examination and funduscopy after pupil dilation, was performed to detect any signs of intraocular inflammation (IOI) including retinal vasculitis. Furthermore,

TABLE 1 | Baseline characteristics of the study group (mean ± SD).

Age (years)	**76 ± 8**
Gender (Male:Female)	29%:71%
Eye (Right:Left)	48%:52%
Previous IVIs	36 ± 22
MNV type (1:2)	62%:38%
BCVA (ETDRS)	51 ± 16
CMT (μm)	374 ± 158

IVI, intravitreal injection; MNV, Macular neovascularization; BCVA, Best corrected visual acuity; ETDRS, Early Treatment of Diabetic Retinopathy Study; CMT, Central macular thickness

patients were educated about the symptoms of IOI and advised to report immediately if any ocular or systemic adverse events were noted.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics V.26). A Mann-Whitney *U* test (nonparametric statistics) was performed to examine the effect of time on BCVA, CMT, and the number of injections between each follow-up visit and baseline. A Chi-square test was used to compare the difference in retinal fluid rates before and after treatment. Data was presented as mean ± standard deviation (95% CI). Results were considered statistically significant if the *p* value was <0.05.

RESULTS

The patients' baseline characteristics are summarized in **Table 1**.

The mean BCVA for each time point is displayed in **Figure 1**.

The mean CMT for each time point is displayed in **Figure 2**.

The number of previous anti-VEGF IVIs was 36 ± 22 before switching to brolucizumab (ranibizumab 9.6 ± 9.2 IVIs, aflibercept 22.7 ± 17.5 and bevacizumab 6.9 ± 8.2 IVIs). The number of IVIs/eye before and after switching to brolucizumab is displayed in **Figure 3**.

The percentage of eyes with each type of retinal fluid at each time point are shown in **Figure 4**.

A completely dry macula was observed in seven eyes (33.3%) during the treatment period, two at week 4, one at week 8, one at week 16, one at week 20, one at week 24 and one at week 44. Consequently, the treatment interval was extended to 12 weeks in these eyes. However, recurrence was observed in six eyes after 8.8 ± 3.3 weeks and the treatment interval was again shortened to 8 weeks.

Adverse Events

In four eyes, treatment with brolucizumab was discontinued because of observed adverse effects (**Table 2**).

An 85-year-old woman who had previously received 25 IVIs of aflibercept and 7 IVIs of ranibizumab was switched to brolucizumab for refractory IRF and SRF. She received a total of 3 IVIs of brolucizumab. 8 weeks after the third brolucizumab-IVI, she presented with a headache. Clinical examination revealed an increase in intraocular pressure (IOP) to 50 mmHg, with a normal depth anterior chamber without signs of IOI. The optic disc was within normal limits, and the cup/disc ratio was 0.3. She had no history of glaucoma. The patient was treated with systemic intravenous acetazolamide (500 mg) and local combined eye drops "dorzolamide (hydrochloride) 2% + timolol (maleate) 0.5%" twice daily. IOP improved to 23 mmHg within 3 h. She continued local therapy for 4 weeks. This acute ocular hypertension was probably not due to brolucizumab, but treatment was discontinued as a precaution and later resumed with a previously used anti-VEGF agent.

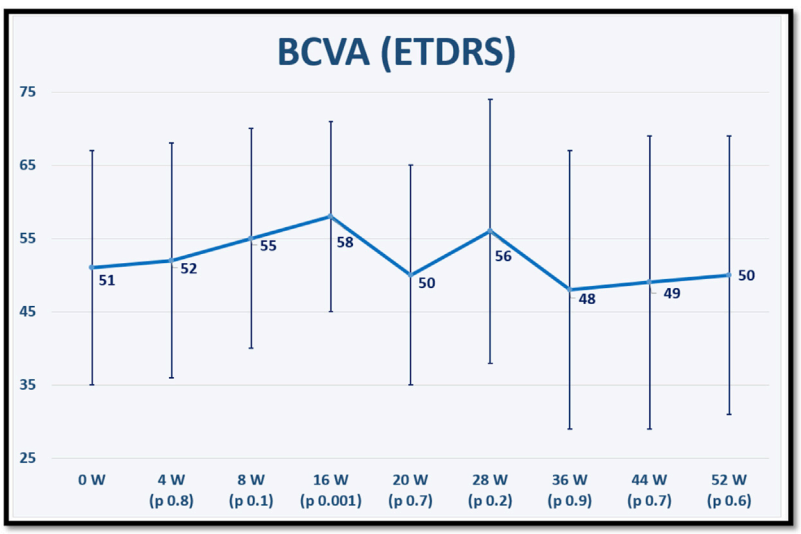

FIGURE 1 | shows the mean best corrected visual acuity (BCVA) in Early Treatment of Diabetic Retinopathy Study (ETDRS) letter score at baseline and for each fellow up visit after switching to brolucizumab. *p* values refer to statistical differences between each time points and baseline.

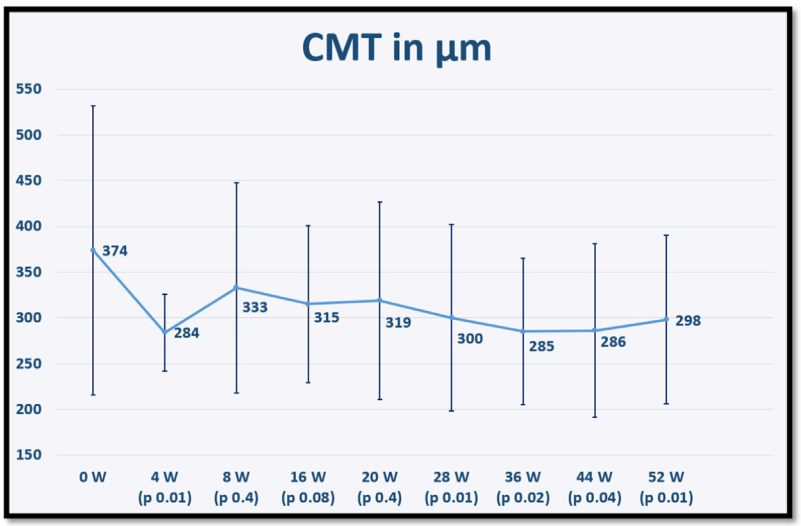

FIGURE 2 | shows the mean central macular thickness (CMT) at baseline and for each fellow up visit after switching to brolucizumab. *p* values refer to statistical differences between each time points and baseline.

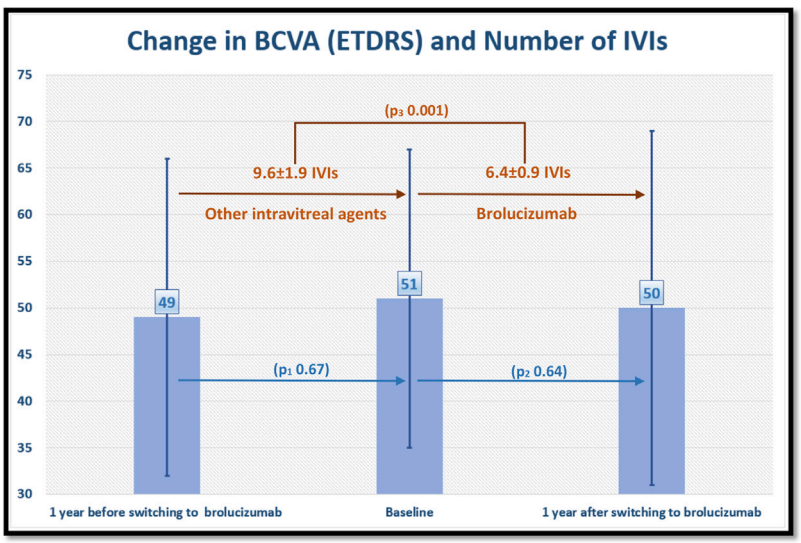

FIGURE 3 | shows the change in best corrected visual acuity (BCVA) in Early Treatment of Diabetic Retinopathy Study (ETDRS) letter score and number of intravitreal injections (IVIs) per eye 1 year before and after switching to brolucizumab. The number of IVIs/eye decreased after switching to brolucizumab, whereas BCVA remained unchanged. $P_{1, 2}$ values refer to statistical differences in BCVA between each time points. P_3 value refers to the statistical difference in the number of IVIs between time points.

A 79-year-old man who had previously received 3 IVIs of aflibercept and 17 IVIs of ranibizumab was switched to brolucizumab due to refractory IRF. He received a total of six IVIs of brolucizumab. 1 week after the sixth brolucizumab-IVI, he developed a CVA that was probably not attributable to brolucizumab, but treatment was terminated as a precaution and later restarted with a previously used anti-VEGF agent.

Intraocular Inflammation (IOI) Was Observed in Two Eyes of Two Patients

Patient 1: A 78-year-old woman who had previously received 15 IVIs of aflibercept and 13 IVIs of ranibizumab was switched to brolucizumab due to refractory PED and SRF. She received a total of 2 IVIs of brolucizumab. 3 days after the second brolucizumab-IVI, she reported worsening visual acuity with ocular pain. Clinical examination revealed a decrease in BCVA from 20/

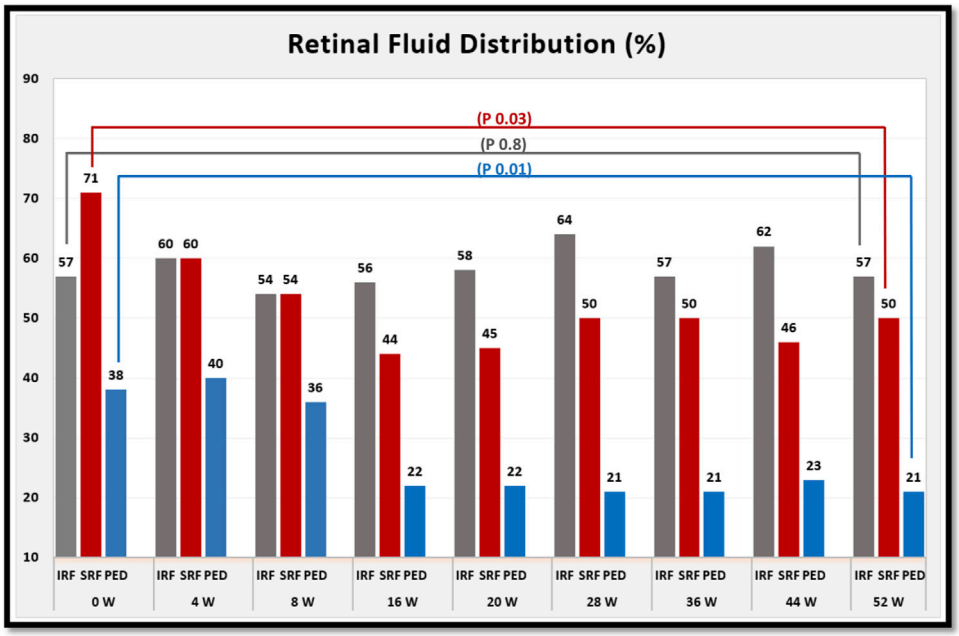

FIGURE 4 | shows the percentage of eyes with each type of retinal fluid at baseline and for each follow-up visit after switching to brolucizumab. At week 52 of treatment, the percentage of eyes with subretinal fluid (SRF) and pigment epithelial detachment (PED) decreased. However, the percentage of eyes with intraretinal fluid (IRF) was comparable between baseline and week 52 of treatment. *p* values refer to the statistical differences between baseline and week 52 of treatment.

TABLE 2 | Observed adverse events.

Adverse event	Gender	Number of patients	Week of incidence
Anterior uveitis	F	1	16
Intermediate uveitis	F	1	8
Ocular hypertension	F	1	16
Cerebrovascular accident	M	1	33

100 to 20/200 with intermediate uveitis (vitreous cells +2, vitreous opacity +2), fundus examination revealed normal retinal findings with no evidence of retinal vasculitis or retinal vascular occlusions. An immediate pars plana vitrectomy with intravitreal antibiotics was performed for suspected endophthalmitis. A vitreous biopsy was performed in order to identify a potentially infectious cause of endophthalmitis. However, a PCR essay was found to be negative. The patient was treated with high-frequency (initially hourly) administration of prednisolone acetate eye drops (1%) and systemic prednisolone (1 mg/kg body weight) for 3 days and then tapered off over a period of 2 weeks. Ocular inflammation improved within 2 weeks, while visual acuity recovered to 20/80 after 1 month. Treatment with brolucizumab was discontinued and the patient was switched back to a previously used anti-VEGF agent with no further adverse events.

Patient 2: A 70-year-old woman who had previously received 3 IVIs of aflibercept and 10 IVIs of ranibizumab was switched to brolucizumab due to refractory IRF. She received a total of 4 IVIs of brolucizumab. 2 days after the fourth brolucizumab-IVI, she reported worsening visual acuity with eye redness. Clinical examination showed worsening of BCVA from 20/40 to 20/80 with keratic precipitation and anterior chamber cells +2. Fundus examination revealed a clear vitreous with normal retinal findings with no evidence of retinal vasculitis or retinal vascular occlusions. Retinal fluid on OCT had completely resolved at that time. A fluorescein angiography (FLA) was performed to rule out any retinal vasculitis or retinal vascular occlusions. The patient was treated with high frequency (initially hourly) administration of prednisolone acetate eye drops (1%) and systemic prednisolone (1 mg/kg body weight) for 3 days and then tapered off over a 2-week period. Further follow-up revealed stable visual acuity (20/60) despite recurrence of IRF. Treatment with brolucizumab was discontinued and the patient was switched back to a previously used anti-VEGF agent with no further adverse events.

Neither patient had a persistent clinically relevant change in visual acuity after IOI was resolved. No occurrence of

retinal vasculitis and/or retinal vessel occlusion was observed.

DISCUSSION

The relatively limited recent experience with intravitreal brolucizumab, as well as the more severe challenge of intraocular treatment-related inflammation, make our data useful and important for further understanding of the role of brolucizumab in the treatment of eyes with refractory nAMD. In this study, we found that brolucizumab appears to be a promising drug for the treatment of such patients, but this must be balanced against a potential risk of adverse events.

Regarding BCVA, our patients achieved statically significant visual improvement at week 16 of treatment, after that, BCVA remained stable compared to baseline until the end of the first year of treatment. This significant visual improvement at week 16 could be due to the intensive therapy with monthly IVIs at the beginning of treatment. This may also suggest that visual outcome could improve by applying IVIs at 4-week intervals after the upload phase. This point was investigated in the MERLIN study. However, upon analysis of the first interpretable data from the MERLIN trial on 28 May 2021, Novartis® discouraged the use of brolucizumab at intervals shorter than 6 weeks after the upload phase because of the disproportionate incidence of treatment-related intraocular inflammation and terminated ongoing studies that allowed for this possibility (Exchange and Koersen, 2021).

On the other hand, the results of our study confirm the efficacy of intravitreal brolucizumab in improving the anatomical outcomes, especially reducing subretinal fluid and pigment epithelial detachment at the end of the first year of treatment and subsequently the mean central macular thickness, which decreased significantly at weeks 4, 28, 36, 44, and 52 of treatment.

There was a statically significant visual improvement at week 16 which was not as evident later, while CMT decreased significantly at the same time point but remained so even afterwards. This phenomenon of discrepancy between anatomy and function has also been reported in other switching studies in patients with nAMD (Gale et al., 2020). This may be explained by the fact that all eyes in our study had a long history of nAMD with chronic IRF, SRF, and PED, resulting in permanent structural changes that could limit the potential of visual improvement despite reduction in CMT.

In addition, our results could be supported by other short-term experiences with brolucizumab (Bulirsch et al., 2021; Montesel et al., 2021; Sharma et al., 2021), which also included previously treated patients and showed significant anatomic improvement on OCT but with no significant visual improvement.

The significant reduction in SRF and PED after 1 year of treatment with brolucizumab in our study are comparable to a similar study from (Book et al., 2021), which also reported similar functional and anatomic results with a significant reduction in SRF and PED after 1 year of treatment.

According to HAWK and HARRIER treatment protocol, the number of injections during the first year of treatment with brolucizumab must range from a minimum of 5 to a maximum of 7 IVIs. Our results showed that patients required 6.4 ± 0.9 IVIs of brolucizumab compared to 9.6 ± 1.9 IVIs of other anti-VEGF agents in the last year before switching. Our results also showed that visual acuity remained stable during this period despite the significantly less number of IVIs, suggesting the conclusion that intravitreal brolucizumab could be able to stabilize visual acuity in patients with refractory nAMD with a significantly less number of IVIs.

This finding be might consistent with the results of the (Haensli et al., 2021) study, which reported that treatment with brolucizumab prolonged the treatment interval in eyes that had responded inadequately to previous anti-VEGF agents. This may play a major role in reducing treatment burdens, which are considered an important cause of non-compliance and undertreatment in many real-world studies (Lad et al., 2014). However, it should be kept in mind that many factors such as the duration of the disease, the type of injury, and most importantly, the prolonged years from therapy with other anti-VEGF agents, may also influence this conclusion.

All of the above benefits of treatment with brolucizumab must be balanced against a potential risk of adverse events, particularly IOI. In this study, we reported two cases of IOI (9.5%) during the first year of treatment. IOI presented as anterior and intermediate uveitis without retinal involvement. However, some papers have reported cases with occlusive vasculitis with severe vision loss after brolucizumab (Haug et al., 2020; Jain et al., 2020).

All anti-VEGF agents may carry the risk of IOI. The overall incidence of sterile IOI after IVI ranges broadly in the literature from 0.005% to 4.4% (Souied et al., 2016; Anderson et al., 2021; Monés et al., 2021). This IOI can range from a mild transient reaction to a potentially sight-threatening outcome. It can manifest as acute onset sterile inflammation or delayed onset inflammatory vasculitis, which has been described with brolucizumab (Anderson et al., 2021; Monés et al., 2021).

A recent study showed a good safety profile as well as lack of inflammatory reactions after multiple bevacizumab IVIs. This was established by evaluating the effects of repeated bevacizumab IVIs on the blood-aqueous barrier (Dolar-Szczasny et al., 2021). A systematic comparison of data from numerous studies of aflibercept, ranibizumab and bevacizumab in nAMD found no difference between their ocular safety profiles, including IOI events (Plyukhova et al., 2020). However, the recent higher incidence of IOI (4.4%) with brolucizumab (Dugel et al., 2021) raises concerns for ophthalmologists treating patients.

The pathogenesis of the IOI events is not yet clear. It may be an auto-immune type IV hypersensitivity reaction (Anderson et al., 2021; Enríquez et al., 2021). It could also be related to the fact that the pharmacological mechanism and pharmacokinetic profile of anti-VEGF agents are different, which in turn affects the risk-benefit ratio (Platania et al., 2015). have already shown that ranibizumab and aflibercept differ significantly both in terms of molecular interactions and stabilizing energy. This calls for further studies on the pharmacokinetic profile of brolucizumab.

Some risk factors have been suggested to play a role in the development of IOI, including:

- Female gender (Witkin et al., 2020; Enríquez et al., 2021), this could be supported by our results, as the 2 IOIs that occurred in our small study were in female patients.
- Patient eyes with history of IOI in the 12 months before the first brolucizumab IVI (Khanani et al., 2021).
- Monthly treatment, most of the IOIs were reported during the first 3 months of treatment (Exchange and Koersen, 2021; Monés et al., 2021).
- Finally, the absence of IOI after 126 brolucizumab IVIs in an Indian study indicates the importance of investigating the potential of race and genetics in predisposing to brolucizumab-related IOI (Chakraborty et al., 2021).

In this study, all patients were followed up 2–5 days after each IVI according to the guidelines of the German Society of Ophthalmology (German Society of Ophthalmology et al., 2021). This is in order to rule out bacterial endophthalmitis, which, unlike noninfectious IOI, typically occurs between days 2 and 5 after IVI. As a consequence, the presence of inflammatory symptoms or signs within the first 5 days after brolucizumab-IVI may be indicative of bacterial endophthalmitis rather than noninfectious IOI. Thus, combined antibiotic and anti-inflammatory therapy might be indicated (Holz et al., 2021). This point was clearly discussed by (Holz et al., 2021). They considered that the prolonged and variable time interval between brolucizumab-IVI and the occurrence of IOI (Baumal et al., 2021) provided no reason to modify the usual approach to IVI follow-up at this point. However, they emphasize the importance of educating patients about the symptoms of IOI and advise them to report immediately if adverse ocular events are noted, even if the IVI was some time ago.

Several experts recommend the suspension of the current brolucizumab treatment with intensive corticosteroid treatment depending on the severity of the inflammation (Baumal et al., 2021; Holz et al., 2021).

Finally, the main potential limitations of our study were the retrospective nature of the work, a relatively small population from a single medical center, and the lack of a control group. Therefore, further studies should be conducted in a much larger population and over a longer follow-up period to provide a more reliable conclusion about the role of brolucizumab in the treatment of eyes with neovascular age-related macular degeneration in our real-world practice.

CONCLUSION

In the first year of treatment, intravitreal brolucizumab was able to stabilize visual acuity with significantly less IVIs in patients with refractory macular edema due to nAMD. It also improved anatomic outcomes in these patients, particularly reducing subretinal fluid and pigment epithelial detachment and subsequently central macular thickness. However, two cases of intraocular inflammation were reported as adverse events.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Association of Saarland, Germany (Nr. 123/20). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AA, WA, and KE. The first draft of the manuscript was written by AA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Vitamin D_3 preserves blood retinal barrier integrity in an *in vitro* model of diabetic retinopathy

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The impairment of the blood retinal barrier (BRB) represents one of the main features of diabetic retinopathy, a secondary microvascular complication of diabetes. Hyperglycemia is a triggering factor of vascular cells damage in diabetic retinopathy. The aim of this study was to assess the effects of vitamin D_3 on BRB protection, and to investigate its regulatory role on inflammatory pathways. We challenged human retinal endothelial cells with high glucose (HG) levels. We found that vitamin D_3 attenuates cell damage elicited by HG, maintaining cell viability and reducing the expression of inflammatory cytokines such as IL-1β and ICAM-1. Furthermore, we showed that vitamin D_3 preserved the BRB integrity as demonstrated by trans-endothelial electrical resistance, permeability assay, and cell junction morphology and quantification (ZO-1 and VE-cadherin). In conclusion this *in vitro* study provided new insights on the retinal protective role of vitamin D_3, particularly as regard as the early phase of diabetic retinopathy, characterized by BRB breakdown and inflammation.

KEYWORDS

vitamin D_3, blood retinal barrier, diabetic retinopathy, inflammation, angiogenesis, P2X7R

Introduction

The etiopathogenesis of diabetic retinopathy is still not fully elucidated and several pathways are involved in the exacerbation of this pathological condition. Oxidative stress, inflammation, and vascular dysfunction affect the integrity of inner blood retinal barrier (iBRB composed, among others, by pericytes, endothelial cells and Müller cells) and the outer blood retinal barrier (oBRB composed by retinal pigment epithelium RPE). Moreover, the upregulation of some proangiogenic factors such as vascular

endothelial growth factor-A (VEGF-A), leads to retinal ischemia and blood retinal barrier (BRB) impairment (Bucolo and Drago, 2004; Tarr et al., 2013; Duh et al., 2017; Lazzara, 2022; Shukla and Tripathy, 2022). The iBRB and oBRB modulate the transport of molecules regulating the permeability across the retinal endothelium and the pigmented epithelial cells, respectively. Tight junctions (TJs) and adherens junctions are multiple junctional protein complexes endowed of regulation of BRB integrity, which is strongly altered by high plasmatic levels of glucose. Hyperglycemia causes retinal micro-vasculopathy, inflammation, and retinal neurodegeneration (Gui et al., 2020). The activation of toll-like receptors 4 (TLR-4), which leads to the over expression of inflammatory markers, such as IL-1β (Cao et al., 2021; Bayan et al., 2022), is one of the diabetes-associated retinal alterations (Wang et al., 2015). It has been demonstrated that the upregulation of IL-1β in retinal endothelial cells is induced by hyperglycemia (Demircan et al., 2006; Liu et al., 2012; Wooff et al., 2019). Moreover, IL-1β is also a stronger inducer of other inflammatory cytokines through the activation of p38MAPK/NF-κB pathway (Liu et al., 2015). High glucose levels represent a strong stimulus that triggers the phosphorylation/activation of ERK proteins, in retinal endothelial cells (Liu et al., 2014; Liu et al., 2015; Lazzara et al., 2019). All these diabetic-related events are correlated to the up-regulation of ICAM-1, induced by both angiogenic (overexpression of VEGF-A) and inflammatory stimuli (up-regulation of inflammatory cytokines). In fact, retinal endothelial cells are the main producers of ICAM-1, which exacerbates the microvascular leukostasis, i.e., the adhesion and transmigration of leukocytes to endothelium, in diabetic retinopathy (DR) (Joussen et al., 2003; van der Wijk et al., 2017). Vitamin D_3 is a fat-soluble steroid hormone, endogenously produced by the human body, acting as a nuclear hormone, it has the highest affinity for the vitamin D receptor (VDR). VDR is ubiquitously expressed in the cells of the whole human body, and it is expressed in retinal cells, including endothelial cells. Vitamin D_3 has genomic (through vitamin D response element, VDRE, on target genes) and nongenomic effects and these last are related to its activity on protein kinases, including MAPKs (Revelli et al., 1998; Hii and Ferrante, 2016; Jamali et al., 2018). Interestingly, it has been demonstrated that VDR is expressed both in vascular endothelial cells and pericytes, and the effects of vitamin D_3 on vascular cells is still object of several studies (Jamali et al., 2018; Jamali et al., 2019).

In the present study we investigated the effects of vitamin D_3 on primary retinal endothelial cells, challenged with high glucose levels. We demonstrated the anti-inflammatory and anti-angiogenic activity of this vitamin in an *in vitro* model of DR, showing its efficacy at reducing DR-related BRB loss of integrity. Our results suggest new insight for potential therapeutic implications of vitamin D_3 for the management of early stages DR.

Methods

Cell culture

Human retinal endothelial cells (HRECs) were purchased from Innoprot® (Derio – Bizkaia, Spain). Cells were cultured at 37°C, in humidified atmosphere (5% CO_2), in Endothelial Cell Medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% ECGS (Endothelial Cell Growth Supplement) and 100 U/ml penicillin 100 µg/ml streptomycin, in flask precoated with fibronectin (1 mg/ml) (Innoprot, Derio – Bizkaia, Spain) for 1 h at 37°C. After reaching confluence (approximately 70%), cells were used for experimental procedures. All the treatments were carried out in medium containing 2.5% FBS. Cells growth in medium containing 5 mM glucose (physiological glucose concentration) served as control group. HRECs were also exposed to medium containing 40 mM glucose (high glucose, HG) (Huang et al., 2016; Lazzara et al., 2019) obtained from the basal glucose concentration of medium (5 mM) with the addition of 35 mM of glucose on basis of used final volume. HRECs were pre-treated for 24 h with vitamin D_3 (1 µM) and then were exposed to HG with or without vitamin D_3 for 24, 48 and 72 h.

MTT

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT; Chemicon, Temecula, CA, United States) was used to assess cell viability after HG (40 mM) challenge and vitamin D_3 (1 µM) treatment. Optimal cell density was obtained by seeding $1,5 \times 10^4$ cells/well in 96-well plates (Costar, Corning, NY, United States). After pretreatment with vitamin D_3 HRECs were subjected to co-treatment in a fresh medium for 24 and 48 h with vitamin D_3 (1 µM) and HG (40 mM). At the end of the treatment, HRECs were incubated at 37°C with MTT (0.5 mg/ml) for 3 h; then DMSO was added, and absorbance was measured at 570 nm in a plate reader (VariosKan, Thermo Fisher Scientific, Waltham, MA, United States). Graphs were built converting absorbance (abs) to viability (% of control) using the following equation $(abs_x \div abs_{ctrl-}) \times 100$, where abs_x is absorbance in the x well, and abs_{ctrl-} is the average absorbance of negative control cells (untreated cells).

Lactate dehydrogenase

Lactate dehydrogenase (LDH) cell release was measured using the Cytotoxicity Detection KitPLUS (LDH) (ROCHE, Mannheim, Germany). HRECs cells were seeded at $1,5 \times 10^4$ cells/well in 96-well plates (Costar, Corning, NY, United States). After pretreatment with vitamin D_3, HRECs were subjected to co-treatment in a fresh medium for 24 and 48 h with vitamin D_3 (1 µM) and HG (40 mM). After these time

points, according to manufacturer's protocol, lysis solution was added to positive control wells (non-treated cells) for 15 min. After transferring 100 µl of medium in a new multi-well plate, 100 µl of working solution was added. After 10–15 min at room temperature, at last, 50 µl of stop solution was added. The absorbance values were measured at 490 nm using a plate reader (VarioSkan, Thermo Fisher Scientific, Waltham, MA, United States). LDH release is reported as LDH (% control) $(abs_x \div abs_{ctrl+}) \times 100$. In the equation, abs_x is absorbance in the x well and abs_{ctrl+} is the average absorbance of positive control cells (untreated lysed cells). Absorbance values were corrected by subtracting medium absorbance.

Blood retinal barrier integrity assessment

The effect vitamin D_3 and HG challenge on BRB integrity was evaluated by measurements of TEER, by using a Millicel-Electrical Resistance System (ERS2) (Merck, Millipore, Burlington, MA, United States) as previously described (Giurdanella et al., 2017; Fresta et al., 2020). To evaluate the modification of paracellular permeability under the above-mentioned conditions, the luminal-to-abluminal movements of Na-F, across endothelial cell monolayers, were measured by using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, United States) as previously described (Fresta et al., 2020).

Immunocytochemistry

ZO-1 immunodetection was carried out as follows. Glass chamber slides were coated with a fibronectin for 1 h at 37°C and washed with sterile water. HRECs (6×10^4 cells/well) were seeded on 24-well fibronectin coated glass chamber slides. Cells were incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. Cell adhesion and confluence was reached within 5 days and the medium was changed every 2 days. Cells were shifted for 24 h with vitamin D_3 and for 48 h to a medium containing 40 mM glucose (HG), with or without vitamin D_3. HRECs growth in medium with physiological glucose concentration (5 mM) served as control. After 48 h, cells were fixed with ice-cold acetone for 15 min and with ice-cold methanol for 20 min. Thereafter, cells were washed with cold phosphate buffered saline (PBS, pH 7.4) and blocked with 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS solution, for 30 min at room temperature. Cells were then incubated overnight at 4°C with primary antibody against ZO-1 (dilution 1:100, rabbit monoclonal; catalog n. 61-7300, Life Technology, Monza, Italy). After overnight incubation and primary antibody washout with PBS, the secondary anti-rabbit Alexa 488-coniugated antibody (dilution 1:200, Life Technology, Monza, Italy) was added for 1 h at room temperature in the dark. VE-cadherin immunodetection was carried out with a different protocol. HRECs (6×10^4 cells/well) were seeded on 24-well fibronectin coated glass chamber slides pre-coated with fibronectin for 1 h at 37°C and then incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed every 2 days. Thereafter, the cells were shifted to different medium, as described for ZO-1 staining. After 48 h of treatment the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with cold PBS and permeabilized with 0.3% Triton X-100 in PBS (pH 7.4) for 5 min at room temperature. After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h, the cells were incubated with the rabbit anti-VE-cadherin antibody (1:100, Catalog n. 2500 Cell signaling, Technology, Danvers, MA, United States) in 1% BSA-PBS solution, overnight at 4°C. Then, the slides were washed three times with PBS and 1 h incubation was carried out with anti-rabbit Alexa 488-coniugated secondary antibody (1:200 dilution, Life Technologies, Monza, Italy), at room temperature in the dark. For p-NFκB p65 immunostaining HRECs were plated at a density of 4×10^4 in 24-well glass chamber slides pre-coated with fibronectin for 1 h at 37°C and then incubated for 3 days at 37°C in a 5% CO_2 humidified atmosphere. Thereafter, the cells were pretreated for 24 h with vitamin D_3 and for 24 h with high glucose. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with cold PBS and permeabilized with 0.2% Triton X-100 in PBS (pH 7.4) for 15 min at room temperature. After blocking with 5% NGS and 0.3% Triton X-100 in PBS solution, for 30 min at room temperature, the cells were incubated with the mouse-anti- phospho-NFκB p65 (Ser536; 1:200, Catalog n. 3036 Cell signaling, Technology, Danvers, MA, United States) in 1% NGS and 0.2% Triton X-100 in PBS solution overnight at 4°C. After overnight incubation, the slides were washed three times with PBS. Then, 1 h incubation was carried out with anti-mouse IgG H + L (Dylight 550) secondary antibody in 0.1% Triton X-100 in PBS (1:300 dilution, Abcam, Cambridge, United Kingdom), at room temperature in the dark. Nuclei staining was carried out for 10 min with 4′,6-diamidino-2-phenylindole (DAPI) (1:10.000; D1306, Life Technologies, Monza, Italy). Finally, the slides were mounted using mounting medium (Life Technologies, Monza, Italy). Images were acquired with a fluorescence microscope Zeiss Observer Z1 equipped with the Apotome.2 acquisition system connected to a digital camera (Carl Zeiss, Oberkochen, Germany). Images were acquired at 40×. Semi-quantitative evaluation of junction protein expression was carried out analyzing images from slides of each condition $n = 4$ (5 mM glucose, 40 mM glucose, 40 mM glucose + 1 µM vitamin D_3). The images ($n = 4$ per group) were analyzed by two investigators unaware of experimental design.

Extraction of total ribonucleic acid and cDNA synthesis

Extraction of total RNA, from HREC cells was performed, after 72 h of treatment, with a TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, United States). The A_{260}/A_{280} ratio of optical density of RNA samples (measured with Multimode Reader Flash di Varioskan™) was 1.95–2.01; this RNA purity was confirmed with the electrophoresis in non-denaturing 1% agarose gel (in TAE). cDNA was synthesized from 2 µg RNA with a reverse transcription kit (SuperScript™ II Reverse transcriptase, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, United States).

qRT-PCR

Real-time PCR was carried out with the Rotor-Gene Q (Qiagen). The amplification reaction mix included the Master Mix Qiagen (10 µl) (Qiagen QuantiNova SYBR Green Real-Time PCR Kit) and cDNA (1 µl, 100 ng). Forty-five amplification cycles were carried out for each sample. Results were analyzed with the $2^{-\Delta\Delta Ct}$ method. Quantitative PCR experiments followed the MIQE guidelines (Bustin et al., 2009). Gene expression levels were normalized with levels of housekeeping gene (18S). Primers were purchased from Eurofins Genomics (Milan, Italy). Forward and reverse primer sequences are herein listed: IL-1β (forward: 5′-AGCTACGAATCTCCGACCAC-3′; reverse: 5′-CGTTAT CCCATGTGTCGAAGAA-3′), VEGF-A (forward 5′-AGG GCAGAATCATCACGAAG-3’; reverse 5′-ATCCGCATAATC TGCATGGT-3′), 18S (forward 5′-AGTCCCTGCCCTTTG-3’; reverse 5′-GATCCGAGGGCCTCACTAAAC-3′), ICAM-1 (forward 5′-ATGCCCAGACATCTGTGTCC-3′; reverse 5′-GGGGTCTCTATGCCCAACAA-3′), TLR-4 forward 5′-ATA TTGACAGGAAACCCCATCCA-3′; reverse 5′-AGAGAGATT GAGTAGGGGCATTT-3′.

Western blot

HRECs were cultured in 60 mm Petri dishes (4×10^5). Proteins of whole cell lysates were extracted with RIPA Buffer, including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, United States). Total protein content, in each cell lysate sample, was determined by means of the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, United States). Extracted proteins (30 µg) were loaded on 4%–12% tris–glycine gel. After electrophoresis, proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA, United States). Membranes were blocked with milk, 5% Trisbuffered saline, and 0.2% Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated overnight (4°C) with appropriate primary phospho-p44/42 MAPK (Rabbit, phospho-Erk1/2, 1: 500 dilution, Catalog n. 9101 Cell Signaling Technology, Danvers, MA, United States), p44/42 MAPK (Rabbit, Erk1/2, 1:500 dilution, Catalog n. 9102 Cell Signaling Technology, Danvers, MA, United States) and anti-GAPDH (Rabbit mAb, 1:500 dilution, Catalog n. 2118 Cell Signaling Technology, Danvers, MA, United States) antibodies. After overnight incubation, the membranes were then incubated with secondary chemiluminescent antibody (ECL anti-rabbit, 1:2000 dilution, NA934) for 1 h at room temperature. After secondary antibody, the membranes were incubated with ECL (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Carlsbad, CA, United States) and were detected through I-Bright™ 1500 (Invitrogen, Life Technologies, Carlsbad, CA, United States) by using chemiluminescence. Densitometry analyses of blots were performed at non-saturating exposures and analyzed using ImageJ software (NIH, Bethesda, MD). Values were normalized to GAPDH, which was also used as loading control.

In vitro tube formation assay

Tube formation assay was performed *in vitro* with Matrigel Basement Membrane Matrix system (BD, Bedford). The experimental protocol was run according to the manufacturer's instructions. Gel solution was thawed at 4°C overnight, then 96-well plates were coated with 50 µl of Matrigel/well and allowed to solidify at 37°C for 2 h. HRECs were seeded at 15,000 cells per well in 50 µl assay medium, with or without HG and/or 1 µM vitamin D_3. Each condition was run in triplicate. After 8 h of incubation, tube-like structures were photographed by using an inverted microscope. The total tube length was quantified with the ImageJ software (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis and graphs design were carried out with GraphPad Prism (GraphPad Software, La Jolla, CA, United States). Data are reported as mean ± SD. One-way ANOVA, followed by Tukey-Kramer post-hoc test, was carried out for multiple comparisons. Post-hoc test was carried out given an F with $p < 0.05$, and no significant variance inhomogeneity was found within groups. Differences between groups were considered significant at $p < 0.05$.

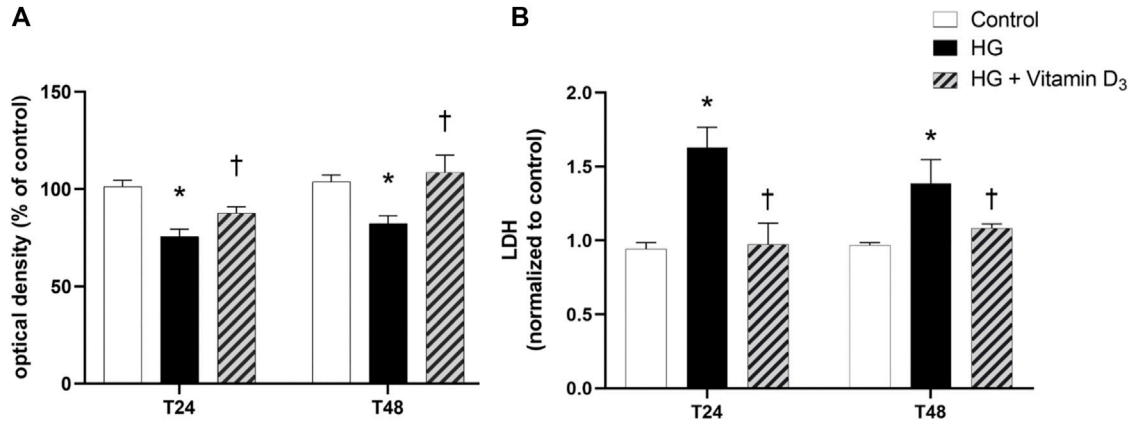

FIGURE 1
Vitamin D_3 shows protective effect in HREC cells against high glucose (HG)-induced damage. Cells were pretreated for 24 h with vitamin D_3 (1 μM) and for 24 and 48 h with HG (40 mM). At the end of treatment MTT **(A)** and LDH **(B)** assay were carried out. Values are reported as mean ± SD (n = 4). Data were analyzed by one-way ANOVA and Tukey's post hoc test for multiple comparisons. *p < 0.05 vs. control; †p < 0.05 vs. HG.

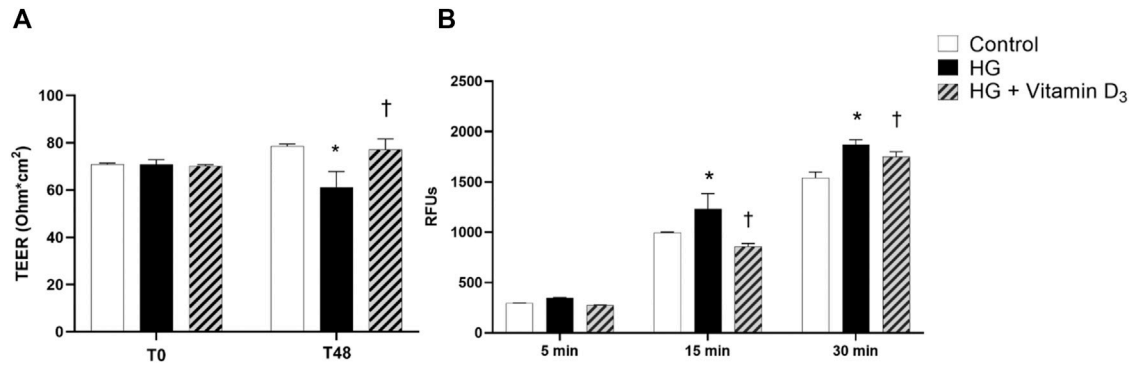

FIGURE 2
Vitamin D_3 protects HREC cell monolayer from HG (high glucose)-induced damage. HRECs were pretreated with vitamin D_3 (1 μM) for 24 h and co-treated with HG (40 mM) for 48 h. **(A)** Vitamin D_3 increased TEER values, which were reduced by HG challenge after 48 h. **(B)** Measurement of apical-to basolateral Na-F permeability after 5, 15 and 30 min. Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *p < 0.05 vs. control; †p < 0.05 vs. HG.

Results

Cell viability and lactate dehydrogenase release

After 24 and 48 h, high glucose induced a significant (p < 0.05) cell toxicity in terms of reduction of cell viability, in comparison to control (roughly 26% and 21% after 24 and 48 h, respectively) (Figure 1A). Pre-treatment with vitamin D_3 (1 μM) significantly (p < 0.05) attenuates cell toxicity after 24 and 48 h, compared to high glucose treated cells (roughly 16% and 32% after 24 and 48 h, respectively). The same profile was observed in terms of LDH release (Figure 1B).

Inner blood retinal barrier integrity

To evaluate vitamin D_3 effects on iBRB integrity, we measured trans endothelial electric resistance (TEER), a parameter of barrier permeability in cell cultures. We found TEER values significantly (p < 0.05) reduced (22%) after 48 h of high glucose damage, compared to control cells (Figure 2A). On the contrary, vitamin D_3 treated cells showed significant (p < 0.05) increased TEER values, superimposable with control group (Figure 2A). These data were supported by the measurement of apical-to-basolateral permeability of sodium fluorescein (Na-F), a spectrophotometric approach for the assessment of cell monolayer permeability. Treatment with vitamin D_3 (1 μM)

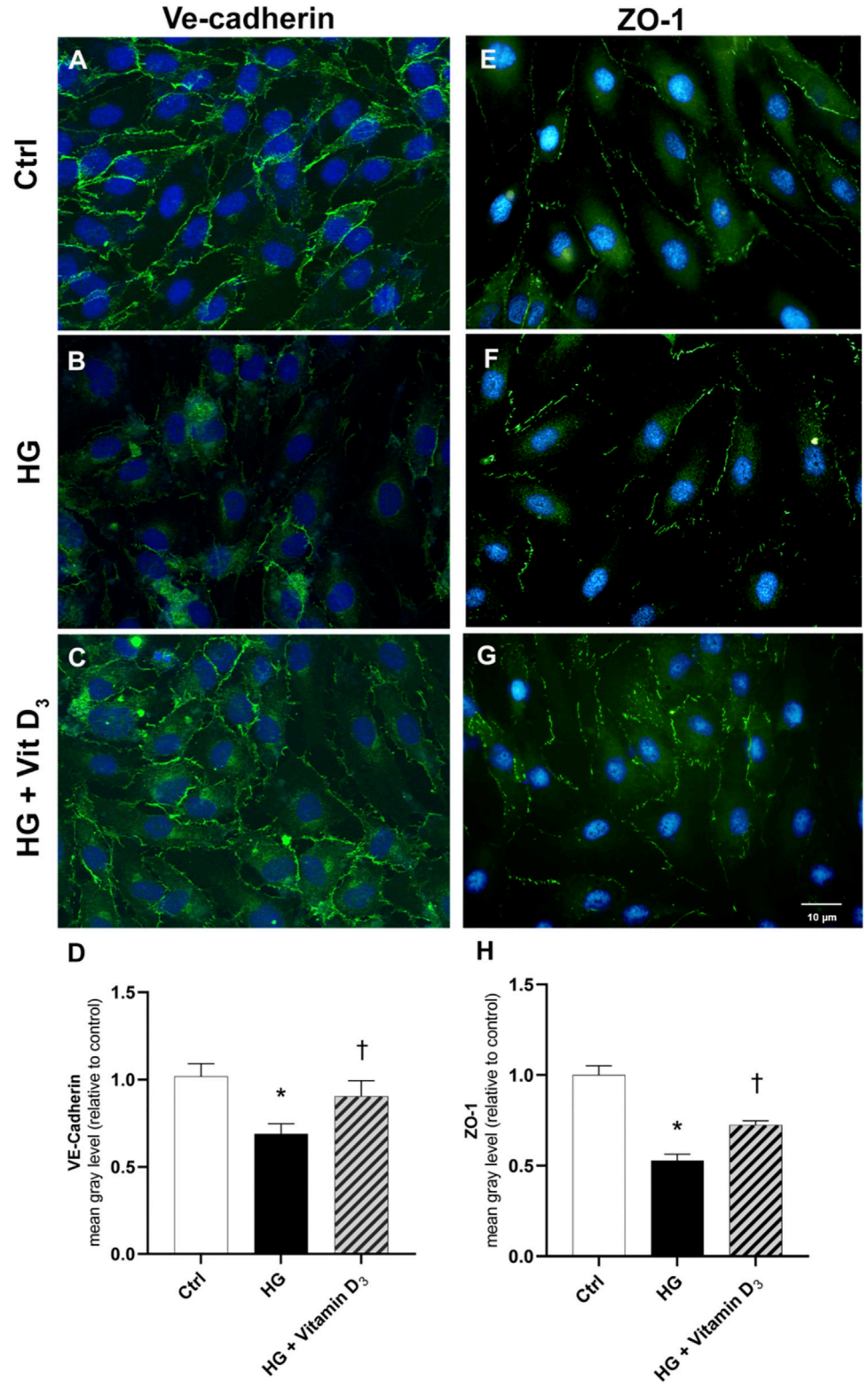

FIGURE 3
Vitamin D_3 re-establishes iBRB integrity through modulation of VE-cadherin and ZO-1. HRECs were pretreated with vitamin D_3 (1 µM) for 24 h and subsequently co-treated with HG (40 mM) for other 48 h. Vitamin D_3 increased the expression of VE-cadherin and ZO-1 proteins, which were significantly reduced by HG. Representative images for VE-cadherin **(A,B,C)** and ZO-1 **(E,F,G)** expression in HRECs after treatment with HG and vitamin D_3. VE-cadherin and ZO-1 were labeled with FITC (green); nuclei were labeled with DAPI (blue). Images were acquired at × 40 magnification. Scale bar: 10 µm. Fluorescence semi-quantification of VE-cadherin **(D)** and ZO-1 **(H)** protein (mean grey levels). Values are reported as mean ± SD; $n = 4$. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *$p < 0.05$ vs. control; †$p < 0.05$ vs. HG.

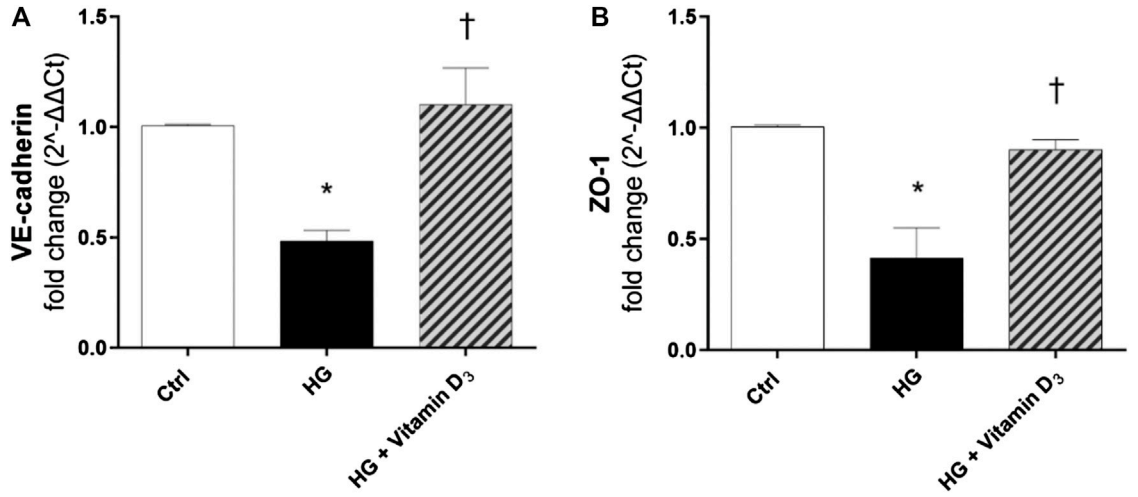

FIGURE 4
Vitamin D_3 induces VE-cadherin and ZO-1 mRNA expression. VE-cadherin **(A)** and ZO-1 **(B)** mRNA levels after treatment with vitamin D_3 and HG for 48 h. Values are reported as mean ± SD; $n = 4$. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *$p <$ 0.05 vs. control; †$p < 0.05$ vs. HG.

was able to significantly ($p < 0.05$) preserve monolayer permeability (15' and 30') elicited by HG (Figure 2B).

Since BRB integrity is related to the expression and cell membrane localization of tight junction (TJ) proteins such as ZO-1 and adherens junction such as VE-cadherin (AJ), the expression of this proteins was analyzed by immunocytochemistry (Figure 3). High glucose damage significantly ($p < 0.05$) decreased the expression of both proteins in HRECs, compared to control cells (roughly 33% and 47% of VE-cadherin and ZO-1, respectively) (Figure 3). On the other hand, pre-treatment with vitamin D_3 protected HRECs from HG-damage preserving the expression of ZO-1 and VE-cadherin after 48 h of exposure to HG and vitamin D_3 (roughly 32% and 37% of VE-cadherin and ZO-1, respectively) (Figure 3).

Further, we analyzed at transcriptional level the modulation of VE-cadherin and ZO-1. After 48 h of HG exposure, ZO-1 and VE-cadherin mRNAs levels were significantly ($p < 0.05$) down-regulated in HRECs (roughly 0.6-fold and 0.5-fold for VE-cadherin and ZO-1, respectively), while pre-treatment with vitamin D_3 reverted this effect (Figures 4A,B), maintaining levels of mRNA expression to control values.

Inflammatory process modulation

After 24 h, HG exposure elicited a significant ($p < 0.05$) increase of phosphorylated ERK protein, in comparison to control cells (Figures 5A,B). As shown in Figures 5 (A,B), vitamin D_3 ($p < 0.05$) led to a significant ($p < 0.05$) reduction of ERK phosphorylation (0.5-fold of reduction compared to HG). Furthermore, we analyzed mRNA expression of inflammatory cytokines after 24 and 48 h of HG challenge, but although the trend was rising, data were not significant (data not shown). Instead, we found that after 72 h, HG challenge induced a significant ($p < 0.05$) up-regulation of ICAM-1 and IL-1β (Figures 5C,D), whose mRNA levels were significantly ($p <$ 0.05) reduced by vitamin D_3 treatment (0.4-fold compared to HG-treated cells). Further, TLR-4 mRNA levels were higher in HRECs challenged with HG, compared to control cells (Figure 5E), and vitamin D_3 treatment restored TLR-4 mRNA to control cell levels (0.5-fold compared to HG) (Figure 5E).

Finally, we evaluated the effects of vitamin D_3 on NFκB activation and nuclear translocation, to confirm the anti-inflammatory activity of vitamin D_3 in HRECs after 24 h of HG exposure. HG induced the nuclear translocation of the phosphorylated p65 subunit of NFκB, as shown in Figure 6A. NFκB activation and translocation was significantly ($p < 0.05$) counteracted by the pre-treatment with vitamin D_3, inhibiting the p65 nuclear translocation (Figures 6A,B).

Anti-angiogenic activity

After 72 h of HG challenge, retinal endothelial cells expressed significant ($p < 0.05$) higher levels of VEGF-A, compared to control cells (Figure 7A). The treatment with vitamin D_3 significantly ($p < 0.05$) reduced VEGF-A mRNA levels, in comparison to cells exposed to HG (Figure 7A). Furthermore, to confirm the anti-angiogenic effect of vitamin D_3, we carried

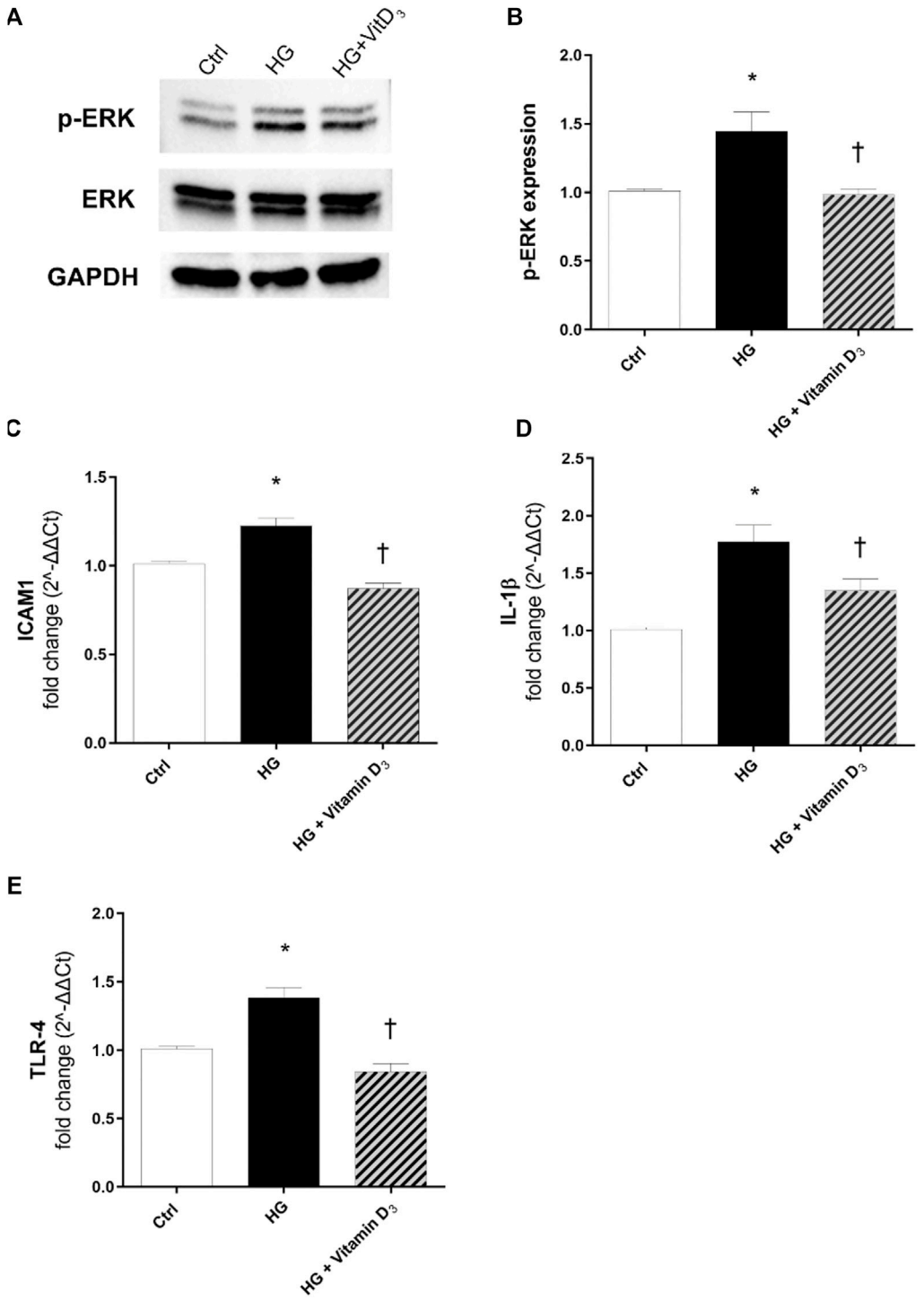

FIGURE 5
Vitamin D_3 counteracts inflammation and angiogenesis in HREC after HG-induced damage. Vitamin D_3 effect on the inflammatory pathway activated by high glucose (HG) in HRECs. **(A)** Immunoblot analysis of ERK1/2 phosphorylation in lysates from HRECs, pre-treated for 24 h with vitamin D_3 (1 μM) and subsequently co-treated with HG (40 mM) for other 24 h. **(B)** Bar graphs show the densitometry analysis of each band, carried out with the Image J program, p-ERK densitometry has been normalized to total ERK values. The effect of HG and vitamin D_3 at mRNA levels was evaluated after 72 h of HG challenge. The treatment with vitamin D_3 reduced ICAM-1 **(C)**, IL-1β **(D)**, TLR4 **(E)** mRNA expression. The mRNA levels were evaluated by qPCR. Each bar represents the means ± SD (n = 4; each run in triplicate). *p < 0.05 vs. control; †p < 0.05 vs. HG.

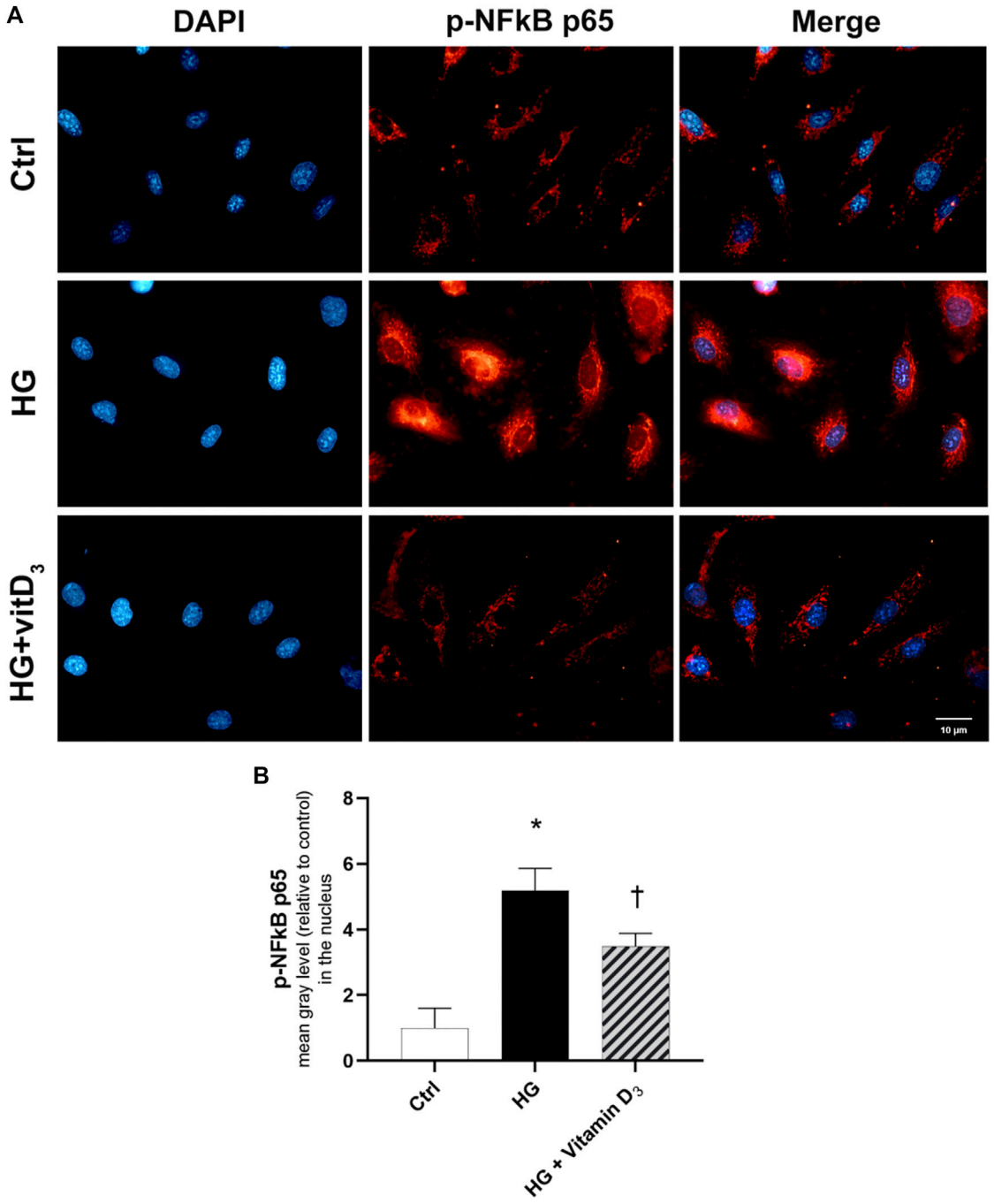

FIGURE 6
Effect of vitamin D_3 on NF-κB activation in HG-challenged HRECs. **(A)** Representative images of phosphorylated p-NFκB p65 (red) translocation into the nuclei (blue) stained with DAPI. HRECs were pre-treated with vitamin D_3 (1 μM) for 24 h and then with or without high glucose for 24 h. **(B)** Fluorescence semi-quantification of p-NFκB p65 protein (mean grey levels) into the nucleus. Nuclei were labeled with DAPI (blue). Images were acquired at × 40 magnification. Scale bar: 10 μm. Values are reported as mean ± SD; $n = 4$. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *$p < 0.05$ vs. control; †$p < 0.05$ vs. HG.

out the tube-formation Matrigel assay (Figures 7B–H), as previously used for the evaluation of angiogenic potential of HRECs (Yadav et al., 2012; Giurdanella et al., 2017; Platania et al., 2020). Vitamin D_3 exerted a significant ($p < 0.05$) anti-angiogenic activity on HRECs treated with 80 ng/ml VEGF-A (Figures 7B–H). In particular, vitamin D_3 significantly ($p < 0.05$)

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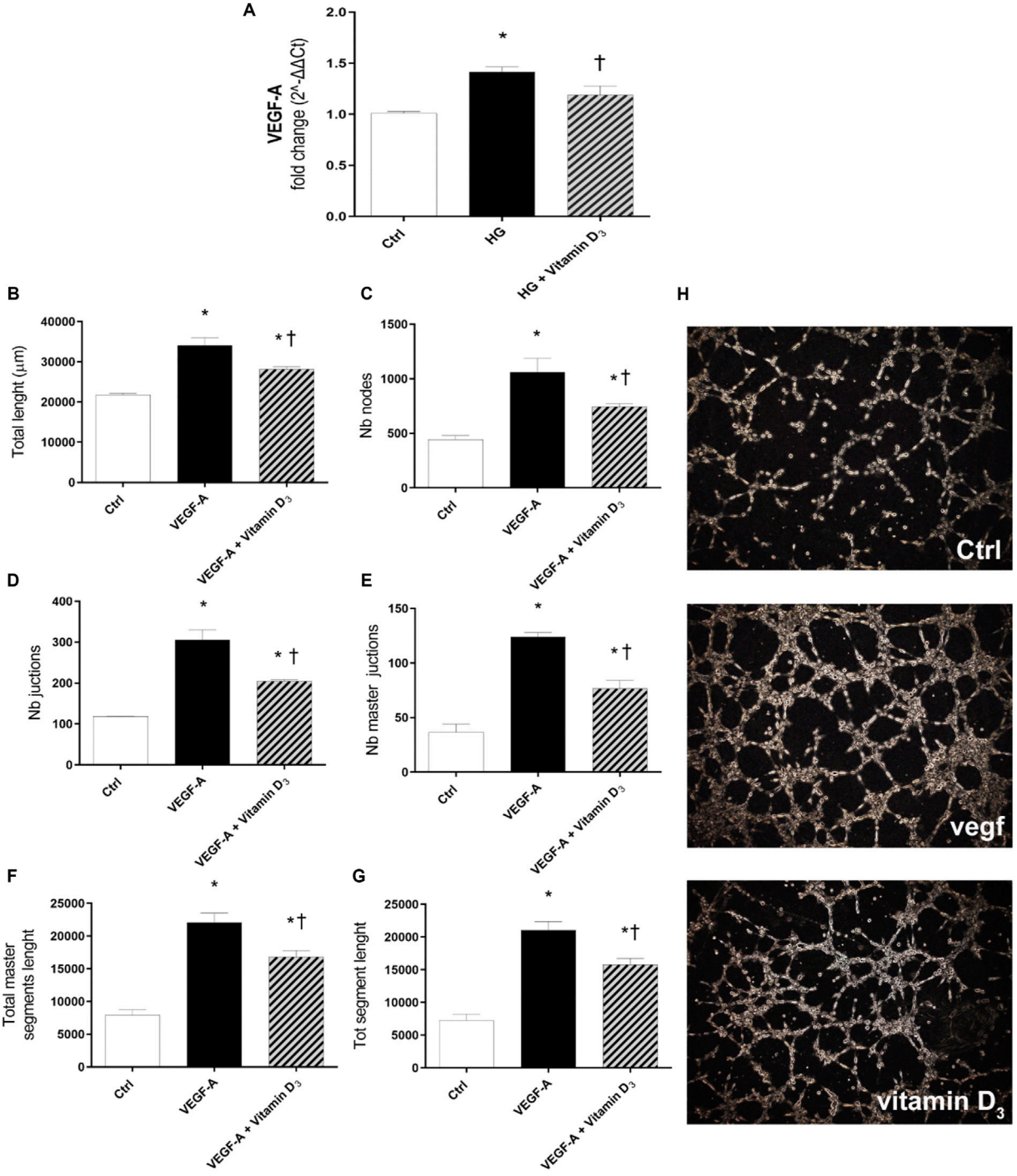

FIGURE 7
Effect of vitamin D_3 on angiogenesis. **(A)** Real-time PCR; VEGF-A mRNA expression. HRECs were pre-treated with vitamin D_3 (1 µM) for 24 h and then with or without HG (40 mM) for 72 h. Vitamin D_3 decreased mRNA levels of VEGF-A and exerted antiangiogenic activity. **(B–G)** Quantification of total tube length, nb nodes, nb junctions, Nb master junctions, total master segments length and total segment length, was carried out using the Angiogenesis Analyzer tool for ImageJ software. HRECs were treated with 80 ng/ml VEGF-A in presence or absence of vitamin D_3 (1 µM). **(H)** Representative optical phase-contrast micrographs of tubelike structures (× 40 magnification) observed in the tube formation assays (Matrigel) after 8 h. Values are reported as mean ± SD; $n = 4$. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *$p <$ 0.05 vs. control; †$p < 0.05$ vs. HG or VEGF-A.

decreased the number of branches point of new vessels and the tube length of new vessels in comparison to cells treated with exogenous VEGF-A (Figures 7B–H).

Discussion

Blood retinal barrier breakdown is a hallmark of diabetic retinopathy. The BRB is a tight and limitative barrier that manages the flux of ions, proteins, metabolic waste compounds, and water flow through the retina, and consists of two distinct regions, the inner BRB (iBRB) and outer BRB (oBRB). The iBRB is established by tight junctions between retinal capillary endothelial cells, surrounded by pericytes and supported by glial cells (Cunha-Vaz et al., 2011; Frey and Antonetti, 2011). The outer BRB (oBRB) is formed by retinal pigmented epithelial cells connected by tight junction proteins, which regulate transport between the choriocapillaris and the retina. Both iBRB and oBRB include tight junction proteins (TJs) (i.e., occludin, claudin family ,and zonula occludens proteins) and adherens junction proteins (i.e., VE-cadherin) (Cunha-Vaz et al., 2011; Frey and Antonetti, 2011). Hyperglycemia, oxidative stress and inflammation are detrimental events that compromise the stability and the expression of those proteins (Tien et al., 2013; Yuan et al., 2014; Platania et al., 2019). The protective effects of vitamin D_3 have been studied in different pathological systems, including eye diseases (Jia et al., 2019; Gouni-Berthold and Berthold, 2021; Johansson et al., 2021; Plesa et al., 2021; Bakhshaee et al., 2022). Beyond the role of vitamin D_3 in calcium and bone homeostasis, several evidence highlight the anti-inflammatory, antioxidant and anti-angiogenic activity of this natural compound (Saad El-Din et al., 2020; Ghanavatinejad et al., 2021). Recently, the attention has been focused on the correlation between vitamin D_3 deficiency and diabetic retinopathy progression (Aksoy et al., 2000; Kaur et al., 2011; Lu et al., 2018), although the mechanism behind its effect on DR pathogenesis is not so clear. It has been hypothesized that vitamin D_3 deficiency has a role in type 1 and type 2 diabetes pathogenesis; in particular, different studies highlighted the leading role of vitamin D receptor (VDR) in maintenance normoglycemia, and the alteration of VDR function has been linked to insulin resistance (Zeitz et al., 2003; Oh et al., 2015; Ni et al., 2016). Moreover, different allelic variations in vitamin D_3 metabolism-related genes have been proposed as predictive markers of insulin imbalance and glucose intolerance (Ren et al., 2012; Yu et al., 2018; Shaat et al., 2020). Furthermore, vitamin D_3 showed promising implications for diabetic retinopathy treatment, preventing inflammatory-related complications. Indeed, Lu et al. (2018) demonstrated that vitamin D_3 inhibits the activation of inflammasome both in an *in-vitro* and *in-vivo* model of DR reducing the detrimental effects induced by high concentration of glucose. Interestingly, vitamin D_3 also showed a relevant anti angiogenic activity in a mouse oxygen-induced ischemic retinopathy model (Albert et al., 2007). The mechanism underlying the protective effect of vitamin D_3 in hyperglycemia-stimulated endothelial cells has not been fully elucidated. Different cytoplasmic and nuclear pathways are involved following VDR activation (Ryan et al., 2015). Incidentally, the anti-inflammatory effect of vitamin D_3 could be related to calcium homeostasis and purinergic receptors (P2X7R) activation (Uekawa et al., 2018). On this regard, some studies demonstrated that vitamin D_3 was able to reduce the calcium influx through P2X7R in resting human mononuclear cells and, as consequence, to down-regulate the expression of this receptor strongly linked to the exacerbation of inflammation in several diseases (Lajdova et al., 2008; Adinolfi et al., 2018). Based on this evidence the binding of vitamin D_3 on P2X7 receptor, acting as allosteric modulator, cannot be rule out, and it is worthy of further investigations. Moreover, long-term vitamin D_3 supplementation was shown to normalized intracellular Ca^{2+} levels in early-stage chronic kidney disease patients without any changes in intracellular calcium storage or cellular intake (Lajdova et al., 2009).

In the present study, vitamin D_3 was able to counteract the effects mediated by inflammatory processes induced by high concentrations of glucose. In fact, we found a relevant rescue in the BRB integrity of retinal endothelial cells mediated by vitamin D_3 in HG conditions with restored levels of junction proteins. As expected, the stimulation with HG significantly reduced TEER values after 48 h compared to control cells (Figure 2). The Na-F permeability test confirmed the BRB integrity impairment after HG treatment, thus mimicking the clinical features of DR patients (Fresta et al., 2020; Nian et al., 2021). Data reported in Figures 3, 4 indicate that vitamin D_3 treatment reduced paracellular permeability in presence of hyperglycemia by preventing the HG-induced decrease of junction protein levels, ZO-1 and VE-cadherin, restoring their central role regarding the tight and the adherens junctions, respectively. Similarly, Won S. et al. demonstrated that vitamin D_3 treatment was able to prevent hypoxia/reoxygenation-induced blood-brain barrier disruption through VDR-mediated NF-κB signaling pathways, in an *in vitro* model of blood brain barrier (Won et al., 2015). In our model, the protective effect of vitamin D_3 against HG can be ascribed to its capability to block inflammatory processes that underlie the pathogenesis of diabetic retinopathy (Forrester et al., 2020). We evaluated the effects of vitamin D_3 regarding the mRNA levels of inflammatory cytokines, ICAM-1, IL-1β, and TLR- 4 in endothelial cells treated with HG. As previously reported, HG treatment led to a significant increase in the pro-inflammatory cytokines mRNA levels, as well as TLR-4 (Xie et al., 2014; Wang et al., 2018; Zhou et al., 2019; Giurdanella et al., 2021). It has been demonstrated that high glucose promotes the activation of TLR (2/4) and, through myeloid differentiation proteins (MyD88)-dependent and -independent signaling pathway, it stimulates the release of inflammatory mediators (Devaraj et al., 2008; Dasu and Jialal,

2011), which are also significantly increased in the vitreous fluid of DR patients (Boss et al., 2017; Iyer et al., 2021; Wu et al., 2021). Our data (Figure 5) are in line with other evidence about the stimulation of TLR-4 pathway exerted by HG (Pahwa, Nallasamy and Jialal, 2016). Moreover, we have previously demonstrated that HG mediate cell damage through the activation of MAPK/NFκB axis through the phosphorylation of both these proteins (Giurdanella et al., 2017; Giurdanella et al., 2020; Lazzara et al., 2019). On these bases, here we tested the HG-induced cytokines mRNA up-regulation, as direct consequence of the activation of ERK/NFκB pathway; in our model, vitamin D_3 clearly reduced the phosphorylation of ERK protein and counteracted the nuclear translocation of phosphorylated p65 NFκB subunit and the cognate increase in cytokine mRNA levels (Figures 5, 6). Vitamin D_3 could exert a pleiotropic anti-inflammatory activity considering its capability to counteract different pathways; this point would certainly need further investigation. We cannot rule out the hypothesis that vitamin D_3 could interfere with the activation of ROS-related HMGB1-TLR4 signaling, described to induce endothelial dysfunction in presence of HG (Rao et al., 2017; Zhang et al., 2018; Fernandez-Robredo et al., 2020; Huang et al., 2020). Moreover, our results could be consistent with a putative contribution of vitamin D_3 in calcium homeostasis through the involvement of the purinergic system (P2X7R) that we found involved in high glucose-induced retinal endothelial damage (Platania et al., 2017). Our *in-vitro* findings confirm the effect of vitamin D_3 as inhibitor of retinal neo-angiogenesis. It has well demonstrated, that vitamin D_3 hampered VEGF-induced endothelial cell sprouting and elongation (Mantell et al., 2000; Albert et al., 2007; Jamali et al., 2019). It has also been shown that vitamin D_3 treatment inhibited VEGF-induced activation of VEGFR-2, ERK and Akt pathway (Kim et al., 2017). Indeed, in our study we found that vitamin D_3 affects the pro-angiogenic activity of VEGF-A on HRECs, and significantly reduced VEGF-A mRNA levels elicited by high levels of glucose (Figure 7).

In conclusion, we provided new evidence on the role of vitamin D_3 in an *in vitro* model of DR using human retinal endothelial cells. The BRB integrity, significantly compromised by high glucose exposure, was restored by vitamin D_3 treatment. These data suggest that vitamin D_3 could be a good candidate to counteract inflammation in several retinal conditions and warranting further clinical evaluation of the efficacy profile.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

FL, AL, and CB made substantial contributions to conception, design, and interpretation of data. FL, AL, GG, carried out formal analysis of data. FL, AL, GG, GL, CA, and CB wrote initial draft of the manuscript. FL, AL, GG, GL, CA, CBMP, SR, FD, and CB reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Topical nerve growth factor prevents neurodegenerative and vascular stages of diabetic retinopathy

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Specific and effective preventive treatment for diabetic retinopathy (DR) is presently unavailable, mostly because the early stages of the complication have been, until recently, poorly understood. The recent demonstration that the vascular phase of DR is preceded and possibly caused by the neurodegeneration of retinal ganglion cells suggests that DR could, at least theoretically, be prevented through an early neuroprotective approach. The aims of our study were to clarify the natural history of diabetes-driven retinal neurodegeneration and to verify the possibility to prevent DR using topical nerve growth factor (NGF). The results of the study show that retinal neurodegeneration, characterized by the loss of retinal ganglion cells represents a relatively early phenomenon of diabetes (between 5 and 16 weeks of age), which tends to be self-limiting in the long run. Neurodegeneration is followed by the development of DR-related vascular dysfunctions, as confirmed by the development of acellular capillaries and the loss of retinal pericytes. Both retinal neurodegeneration and subsequent vascular dysfunction can be successfully prevented by topical NGF administration. These findings suggest that: 1) The first stage of DR consists in a self-limiting retinal neurodegeneration 2) The demonstrated effectiveness of topical NGF in the prevention of DR could be rapidly translated into clinical practice.

KEYWORDS

diabetic retinopathy, nerve growth factor, prevention, neurodegeneration, topical treatment

Introduction

It takes several years to move from the onset of diabetes (both type-1 and type-2) to the development of retinal microaneurysms, the first clinical sign of diabetic retinopathy (DR) (Antonetti et al., 2012). Once these abnormalities have appeared, controlling their evolution becomes difficult and only laser photocoagulation and/or intravitreal anti-VEGF treatment are effective during the final stage of the complication (Antonetti et al., 2012; Ting and Wong, 2017).

It is reasonable to assume that the "silent" interval, spanning between onset of diabetes and development of microaneurysms, might represent the ideal time window to start a successful strategy to prevent DR (Lorenzi, 2006). Attempts carried out to reach this aim have been so far unsuccessful mainly because, until recently, the initial dysfunctional mechanisms leading to the development of DR have been poorly understood.

A number of studies over the last few years (Lieth et al., 2000; Fletcher et al., 2005; Antonetti et al., 2006; Santos et al., 2017) have suggested that the vascular phase of DR could be preceded by a diabetes-driven neurodegenerative process affecting, in particular, the retinal ganglion cells (RGC). Natural history and clinical relevance of this phenomenon remain however unknown and whether early neurodegeneration may or may not represent an indispensable and pharmacologically targetable step in the pathogenesis of DR is still under debate (Hammes et al., 1995; Simó et al., 2018).

To clarify these issues, we: 1) investigated and characterized in a mouse model of spontaneous diabetes (Ins2akita) (Barber et al., 2005) the morphological and functional evolution of hyperglycemia-driven retinal neurodegeneration;

2) verified the possibility to prevent both retinal neurodegeneration and subsequent vascular phase of DR through early treatment with recombinant human nerve growth factor (rhNGF, here called NGF) eye drops (topically applied NGF has been shown to reach retina, optic nerve and brain in rodents (Lambiase et al., 2005; Lambiase et al., 2007) and to specifically protect RGC (Guo et al., 2020)).

Materials and methods

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the San Raffaele Scientific Institute in Milan, in accordance with National Legislation (D.L. 116/1992) and the European Directive (2010/63/EU) concerning the use of laboratory animals, and with the license of the Italian Board of Health.

Design of the study

Four groups of seven animals were studied for 21 weeks (between 3 and 24 weeks of age). At the beginning of the study (3 weeks of age), blood glucose levels of all the animals (both wild type and akita) were similar (as shown in Figure 1A). The animals were therefore randomly assigned to the treatment with placebo (vehicle) or NGF. The groups were topically treated (two drops per eye per day starting at 3 weeks of age) either with placebo (vehicle) or with NGF (180 mg/ml) as follows: 1) Placebo-treated wild type (C57BL/6J) mice; 2) Placebo-treated akita mice; 3) NGF-treated wild type mice; 4) NGF-treated akita mice. Eye drops recombinant human NGF (rhNGF, here called NGF) was provided by Dompé S.p.A. L'Aquila, Italy. Only male animals were studied, as akita females show significantly lower blood glucose levels (Barber et al., 2005).

Thickness of retinal neuron layers was sequentially (3, 5, 8, 16, and 24 weeks of age) evaluated by optical coherence tomography (OCT). Neuroretinal function was evaluated (8, 16, and 24 weeks of age) by electroretinogram (ERG). The animals were sacrificed at the end of the study. The number of RGC was evaluated in the left eye after Brn3a staining (Nadal-Nicolás et al., 2009) (goat polyclonal anti-Brn3a, Santa Cruz, Santa Cruz, CA, United States) while the number of acellular capillaries and pericytes was evaluated in the right eye after performing a trypsin digest (Dietrich and Hammes, 2012). An extra set of seven animals per group was included, treated as previously described and sacrificed at 8 weeks of age to evaluate the number of RGC.

Optical coherence tomography

In vivo analysis of the retina was carried out using Micron IV together with Image-Guided 830 nm OCT (Phoenix Research Laboratories, Pleasanton, CA, United States). The animals were anesthetized with intraperitoneal injection of 80 mg/kg Ketamine, 10 mg/kg Xylazine (Sigma-Aldrich, Munich, Germany). OCT scans were acquired in mydriatic animals through a bidimensional scan (B-scan), performing a 550 μm diameter circular scan around the optic nerve head. Both eyes were examined and results were averaged. Retinal layer segmentation and quantification was performed using Insight software (Phoenix Research Laboratories). Results shown here concern the sum of the thicknesses of Retinal Nerve Fiber Layer (RNFL, that contains RGC axons) plus Ganglion Cell Layer (GCL, that contains RGC bodies). The measurement of RNFL/GCL complex in the mouse is preferable to the measurement of RNFL alone that is usually too thin to be correctly quantified (Jagodzinska et al., 2017).

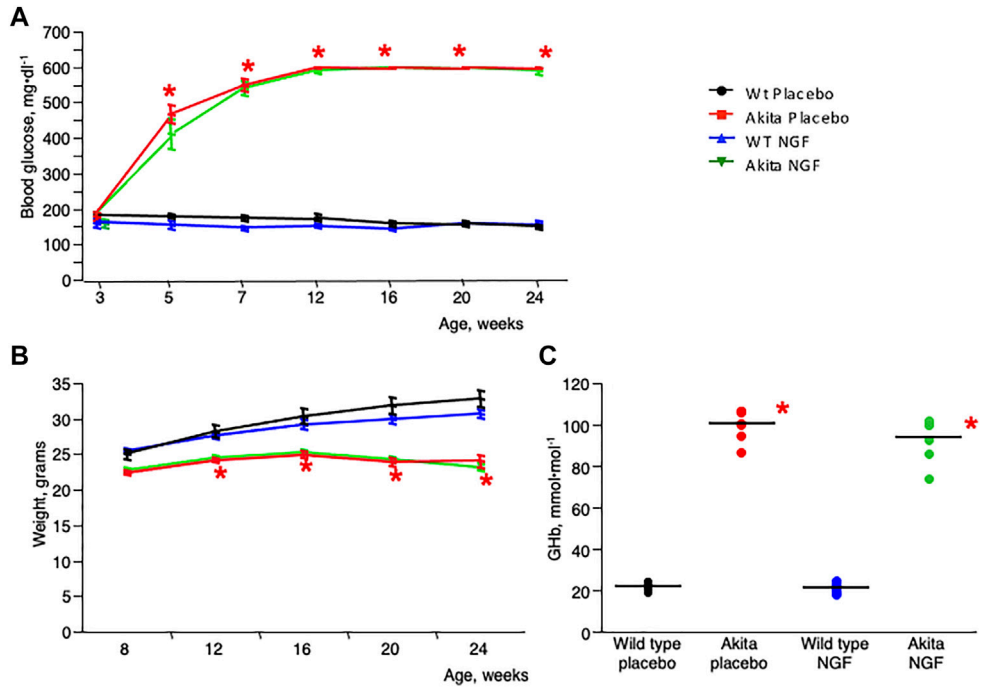

FIGURE 1
Clinical characteristics. **(A)**. Change of blood glucose levels with time in the four animal groups included in the study. Blood glucose level was similar in the four groups at the beginning of the study (3 weeks of age). In the subsequent time points the two akita groups showed a significantly increased blood glucose level when compared to the two wild type groups. *$p < 0.05$. **(B)**. Change of body weight with time in the four groups of animals included in the study. The two akita groups show a significantly decreased body weight when compared to the two wild type groups. *$p < 0.05$. **(C)**. Glycated hemoglobin (GHb) levels at the end of the study (24 weeks) were significantly higher in the akita mice (independent of treatment) compared to wild type animals *$p < 0.05$.

Electroretinogram

Mice were dark-adapted for 2 h before the recordings and all procedures were conducted under dim-red light (Pinto et al., 2004). Briefly, mice were anesthetized as above. Body temperature was maintained with a homeothermic pad at 37°C (Harvard Apparatus, Holliston MA, United States). ERG was concurrently recorded from left and right mydriatic eyes using two corneal ERG electrodes connected to a Micromed amplifier (SystemPlus Evolution-Micromed s.p.a., Mogliano Veneto, Italy). Data were acquired at a sampling frequency of 4096 Hz, coded with 16 bits and filtered between 5–70 Hz. Flash stimuli, with intensity of 231 mJ and duration of 10 μs, were delivered to both eyes at a frequency of 0.5 Hz with a Flash10s photo stimulator (Micromed) (Giannelli et al., 2018). For each session, six series (3 for each eye) of 10 flash stimuli were mediated and used for measuring the amplitude of a-wave (baseline to negative a-wave peak) and b-wave (negative a-wave peak to positive b-wave peak).

Trypsin digest

Trypsin digest was performed as described by Dietrich and Hammes (2012). After hematoxylin and eosin staining the slides were scanned via Aperio® ePathology digital scanner and images were analyzed with ImageScope™ software (both from Leica Biosystems, Nussloch, Germany). The total number of acellular capillaries and pericytes was counted in ten randomly chosen fields for each retina and corrected for the capillary density (number of acellular capillaries/pericytes per mm2 of capillary area).

Glycated hemoglobin

For the quantification of glycated hemoglobin (GHb), an automated HPLC analyzer, based on boronate affinity chromatography, was used (Premier Hb9210, Trinity Biotech, Menarini, Firenze, IT, United States).

Blood samples were studied as haemolysates in specific racks. An internal quality control process was performed per each

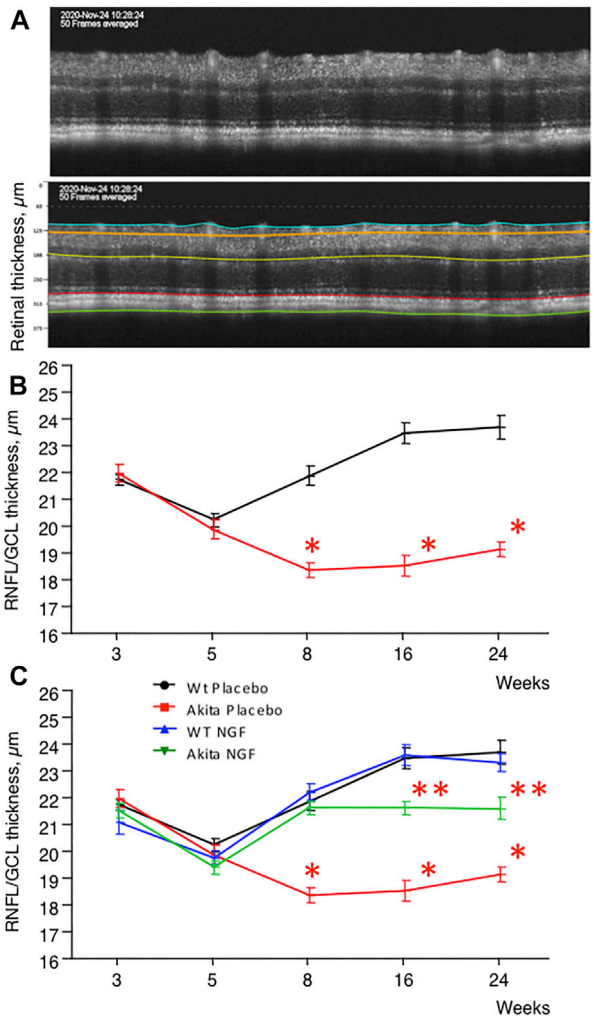

FIGURE 2
RNFL/GCL complex, time course. **(A)**. Upper panel: retina of a control (C57BL6J) mouse as seen by OCT. Lower panel: Segmentation of the retina. The upper layer is the one specifically considered in this study (RNFL/GCL complex). **(B)**. Time course (3–24 weeks) measurement of RNFL/GCL thickness in placebo-treated akita mice (red line) compared to placebo-treated wild type mice (black line). Placebo-treated akita mice shows significant thickness reduction (*$p < 0.05$) at 8, 16, and 24 weeks of age. **(C)**. RNFL/GCL thickness increased significantly (*$p < 0.05$) at 8, 16, and 24 weeks of age in NGF-treated akita mice (green line) when compared to placebo-treated akita mice (red line) even though it remained significantly thinner (**$p < 0.05$) than in placebo-treated (black line) and NGF-treated (blue line) wild type mice.

analytical run by assaying two control materials with low and high HbA1c level, supplied by the manufacturer.

Statistics

Data are shown as arithmetical means ± SE. Comparisons between groups were addressed by ANOVA, and multiple comparisons were performed with the Tukey-Kramer test (JMP software for the Apple Macintosh; SAS Institute, Cary, NC). The null hypothesis was rejected at the 5% level (two tailed).

Results

During the study, blood glucose levels became rapidly and significantly higher in akita groups (Figure 1A) compared to controls. Weight was progressively lower in akita mice (Figure 1B), as expected in animals with heavy glycosuria. Hyperglycemic stability in akita groups was confirmed after measurement of glycated hemoglobin (GHb) at the end of the study (Figure 1C). In particular GHb concentrations were significantly higher in placebo-treated akita mice (100.4 ± 2.5 mmol/mol, mean ± SE) and in NGF-treated akita mice (93.7 ± 2.5) when compared to placebo-treated wild type mice (21.8 ± 2.4) and to NGF-treated wild type mice (21.2 ± 2.5).

As shown in Figures 2A,B, the RNFL/GCL complex, became progressively thinner in akita mice compared to control animals during the first weeks of diabetes and then substantially stabilized after the eighth week of age. Topical treatment with NGF resulted in a significant maintenance of RNFL/GCL thickness in akita mice (green line, Figure 2C). NGF treatment of control animals did not affect RNFL/GCL thickness (blue line, Figure 2C).

To clarify whether RNFL/GCL thickness reduction was paralleled by functional abnormalities, ERG was performed on each animal involved in the study. As shown in Figures 3A,B, placebo-treated akita mice were characterized by reduced activity of both A and B waves, a dysfunction that was substantially prevented by NGF treatment. No effect on ERG came from NGF treatment of control animals.

Final confirmation that RNFL/GCL thickness reduction and ERG abnormalities were the consequence of RGC loss was obtained by counting RGC in the retina of sacrificed animals after staining for Brn3a (Figure 3C), a specific nuclear antigen of RGC (Nadal-Nicolás et al., 2009). As shown in Figure 3D, at 8 weeks of age RGC count was similar in all the groups considered, but at 24 weeks of age RGC count was significantly lower in placebo-treated akita mice compared to controls. NGF treatment fully prevented RGC loss in akita mice and, once again, NGF had no effect on RGC count in control animals.

To clarify whether early retinal neurodegeneration plays a relevant role in the pathogenesis of DR and whether NGF treatment can also prevent the vascular stage of DR, trypsin digestion was carried out in the retinas of sacrificed animals to search for acellular capillaries and pericyte dropouts (Figures 4A,B), two features of the vascular phase of DR that are shared by humans and mice (Hammes et al., 1995). As shown in Figure 4C, the number of acellular capillaries was significantly higher in placebo-treated akita mice (74.3 ± 6.5 number/mm^2 of capillary area, mean ± SE) when compared to placebo-treated wild type mice (25.4 ± 4.4) and to NGF-treated wild type mice (22.5 ± 4.0).

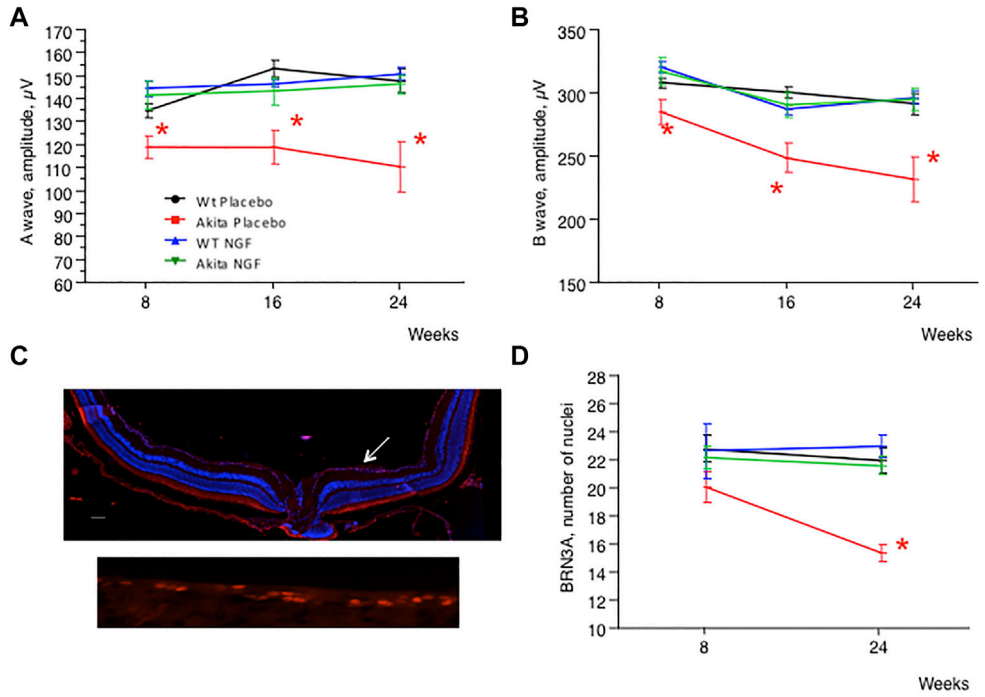

FIGURE 3
ERG and number of RGC cells, time course. **(A)**. Time course (8–16-24 weeks) measurement of ERG A wave amplitude in the four animal groups considered. The placebo-treated akita group (red line) shows significant amplitude reduction (*$p < 0.05$) at 8, 16, and 24 weeks of age when compared to placebo-treated wild type mice (black line). The dysfunction improves significantly (*$p < 0.05$) when akita mice are treated with NGF (green line) and there are no differences between NGF-treated akita mice, placebo-treated wild type mice and NGF-treated wild type mice (blue line). **(B)**. Time course (8–16-24 weeks) measurement of ERG B wave amplitude in the four animal groups considered. The placebo-treated akita group (red line) shows significant amplitude reduction (*$p < 0.05$) at 8, 16 and 24 weeks of age when compared to placebo-treated wild type mice (black line). The dysfunction improves significantly (*$p < 0.05$) when akita mice are treated with NGF (green line) and there are no differences between NGF-treated akita mice, placebo-treated wild type mice and NGF-treated wild type mice (blue line). **(C)**. Upper panel: immunofluorescence for the RGC nuclear antigen Brn3a in a retinal section of a control (C57BL/6J) mouse. The arrow indicates the layer formed by the nuclei of RGC (red staining). Lower panel: detail of the nuclei of RGC stained for Brn3a. **(D)**. Time course (8–24 weeks) measurement of the number of RGC cells in the four animal groups considered. No difference between the groups could be demonstrated at 8 weeks of age. The placebo-treated akita group (red line) shows significant numerical reduction (*$p < 0.05$) at 24 weeks of age when compared to the other three groups. This dysfunction improves significantly when akita mice are treated with NGF (green line) to the point that there are no differences between NGF-treated akita mice, placebo-treated wild type mice (black line) and NGF-treated wild type mice (blue line).

NGF treatment resulted in a significant reduction and consequent "normalization" of acellular capillaries number in akita mice (34.7 ± 5.5).

In parallel (Figure 4D), the number of retinal pericytes was significantly reduced in placebo-treated akita mice (1021.8 ± 38.1 number/mm^2 of capillary area, mean ± SE) when compared to placebo-treated wild type mice (1360.3 ± 55.4) and to NGF-treated wild type mice (1316.0 ± 54.0). As above, NGF treatment substantially "normalized" the number of pericytes in akita mice (1237.4 ± 57.5).

Discussion

The results of the study demonstrate that the natural history of diabetes-driven retinal neurodegeneration, as characterized by OCT (Figure 2B), consists of a relatively early (between 5 and 16 weeks of age), self-limiting phenomenon that shows no tendency to worsen in the final period of observation (between 16 and 24 weeks of age). Only approximately 20–25% of RGC were actually lost during the entire 21-weeks period of observation.

Another important point is that topical NGF treatment is able not only to prevent neurodegeneration, as confirmed from morphologic (OCT), functional (ERG) and histologic (Brn3a) analyses, but also to avoid the development of the vascular stage of DR, which indicates at the same time neurodegeneration as an essential step in the pathogenesis of DR and topical NGF as an effective preventive treatment.

The discovery that, in our mouse model of diabetes, retinal neurodegeneration develops early and tends not to progress with time reproduces and reasonably explains the clinical finding that, even though neurodegeneration represents an early retinal dysfunction (Simó and Hernández, 2012) in at least a subset

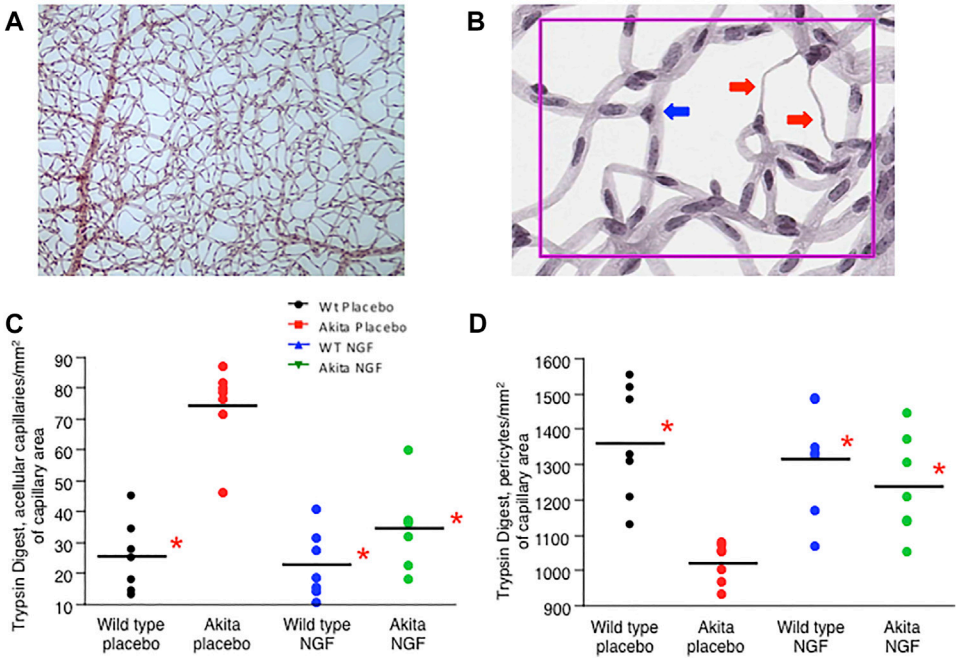

FIGURE 4
Trypsin digest. **(A)**. Trypsin digestion of a murine retina. **(B)**. Details of trypsin digestion of a murine retina. The presence of acellular capillaries can be appreciated (red arrows). Triangular nuclei are the hallmark of pericytes (blue arrow). **(C)**. The number of retinal acellular capillaries at the end of the study (24 weeks) was significantly higher (*$p < 0.05$) in the placebo-treated akita mice (red dots) when compared to placebo-treated wild type mice (black dots), to NGF-treated wild type mice (blue dots) and to NGF-treated akita mice (green dots). The last three groups were similar to each other (P=NS) confirming that NGF treatment normalizes the number of retinal acellular capillaries in akita mice. **(D)**. The number of retinal pericytes at the end of the study (24 weeks) was significantly lower (*$p < 0.05$) in the placebo-treated akita mice (red dots) when compared to placebo-treated wild type mice (black dots), to NGF-treated wild type mice (blue dots) and to NGF-treated akita mice (green dots). The last three groups were similar to each other (P=NS) confirming that NGF treatment normalizes the number of retinal pericytes in akita mice.

of diabetic patients (Santos et al., 2017), there is no indication that the dysfunction may worsen with time (Sacconi et al., 2020).

A major question on this regard concerns the reason why, as described in Figure 2B, the diabetes-driven neurodegenerative process tends to stabilize with time while, as shown in Figure 1A, hyperglycemia is not treated or corrected in any way. One possibility is linked to the evidence that RGC are not a homogeneous cellular population as at least 40 subtypes have been identified (Laboissonniere et al., 2019). Although the different subsets have not yet been characterized, a different response to glucose toxicity in different type of cells cannot be excluded, in particular when considering that retinal neurons, at difference with the ones of the brain, rely mostly on aerobic glycolysis (Hurley et al., 2015), thus possibly justifying the hypothesis that a subgroup of glucose "resistant" RGC may survive the death of the "sensitive" ones.

Another possible and in some way fascinating hypothesis is that RGC might progressively become tolerant to hyperglycemia, something similar to what happen to endothelial cells chronically exposed to high ambient glucose (de Zeeuw et al., 2015).

This is not the first time that topical administration of neuroprotective agents such as GLP-1 Receptor Agonists (Hernández et al., 2017), DPP-IV inhibitors (Hernández et al., 2016) and dual endothelin receptor antagonist Bosentan (Bogdanov et al., 2018) were shown to be useful in experimental models of diabetic retinopathy DR. NGF in particular was found to be protective after both systemic (Hammes et al., 1995) and topical administration (Mantelli et al., 2014). If topical administration of NGF will be confirmed to be effective also on the human retina, as suggested by a first study (Lambiase et al., 2009), prevention of DR could become a feasible and realistic task also when the treatment is started at a very young age.

The finding that RNFL-GCL between 3–5 weeks of age tends to become thinner in all the groups of animals considered in the study (Figure 2B) is for sure intriguing but, at the very end, not so surprising. A previous study (Brais-Brunet et al., 2021) in control mice shows that, when measured consecutively by OCT between postnatal days 7 and 21, the thicknesses of four retinal layers (RNFL, IPL, INL and ORL) are progressively and significantly changing, suggesting a temporal "plasticity" of the neural retina. Unfortunately, the above-described study was stopped at 21 days of age and cannot therefore be of use to quantify the change of RNFL thickness during the period of interest (3–5 weeks of age).

Finally, our results also show that in diabetic animals the thinning of RNFL/GCL complex precedes and predicts the loss of

RGC (at 8 weeks of age thinning of RNFL/GCL is already significant in placebo-treated akita mice (Figure 2B) while the number of RGC is still similar among the groups considered (Figure 3D), hence qualifying as a new biomarker for both neurodegenerative and vascular stages of DR.

Limitations of the study: 1) To monitor the development of the vascular phase of diabetic retinopathy we counted pericytes + acellular capillaries after the demonstration that trypsin digest presently represents the gold standard method to analyze the retinal vasculature (Chou et al., 2013) and because this technique has been previously used to identify retinal dysfunctions common to humans and animal models (Mizutani et al., 1996). Evaluation of vascular leakage by means of the Evans blue method (Xu et al., 2001), or staining of the retina looking for markers specific for endothelial cells or pericytes would have for sure added important functional and morphological results but could not be done in our case because of the limited number of animals (and consequently of retinas) included in the study. 2) Diabetes-driven changes of RNFL/GCL and microvasculature cannot be directly compared in our study because pericytes and acellular capillaries were quantified only at the end (24 weeks) of the study. A time course study aimed to clarify the natural histories of retinal neurodegeneration and microvascular dysfunction induced by diabetes would have been very interesting but unfortunately it goes beyond the aims of the present study.

In conclusion this study shows, in an animal model of diabetes, that DR is characterized by two consecutive stages (neurodegenerative and vascular) and that, by preventing neurodegeneration through NGF topical treatment, it is possible to avoid the development of the microvascular one, known to be particularly aggressive in humans.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the San Raffaele Scientific Institute in Milan, Italy.

Author contributions

GZ, SM, DG, and PR contributed to the design of the study, LL and VC carried out the electroretinogram study, AM and RP performed the glycated hemoglobin analysis, SM, IV, MG, AB, and VT carried out the longitudinal study, GZ, AL, PT, SG, and PR.analyzed the results of the study, wrote the draft of the paper and had full access to the data of the study.

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Conflict of interest

AL Consultant/advisor and Licensed intellectual property Dompé Farmaceutici SpA (Milan, Italy). PR Licensed intellectual property Dompé Farmaceutici SpA (Milan, Italy).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Increased efficacy of dietary supplement containing wax ester-rich marine oil and xanthophylls in a mouse model of dry macular degeneration

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Age-related macular degeneration (AMD) is nowadays considered among the retinal diseases whose clinical management lacks established treatment approaches, mainly for its atrophic (dry) form. In this respect, the use of dietary patterns enriched in omega-3 and antioxidant xanthophylls has emerged as a promising approach to counteract dry AMD progression although the prophylactic potential of omega-3 of fish origin has been discussed. Whether enriched availability of omega-3 and xanthophylls may increase the effectiveness of diet supplementation in preventing dry AMD remains to be fully established. The present study aims at comparing the efficacy of an existing orally administered formulation based on lutein and fish oil, as a source of omega-3, with a novel formulation providing the combination of lutein and astaxanthin with Calanus oil (COil), which contains omega-3 together with their precursors policosanols. Using a mouse model of dry AMD based on subretinal injection of polyethylene glycol (PEG)-400, we assessed the comparative efficacy of both formulations on PEG-induced major hallmarks including oxidative stress, inflammation, glial reactivity and outer retinal thickness. Dietary supplementation with both mixtures has been found to exert a significant antioxidant and anti-inflammatory activity as reflected by the overall amelioration of the PEG-induced pathological hallmarks. Noteworthy, the formulation based on COil appeared to be more protective than the one based on fish oil, presumably because of the higher bioavailability of omega-3 in COil. These results support the use of dietary supplements combining omega-3 and xanthophylls in the prevention and treatment of AMD and suggest that the source of omega-3 might contribute to treatment efficacy.

KEYWORDS

calanus oil, omega-3 fatty acids, carotenoids, oxidative stress, inflammation, gliosis, retinal thickness

1 Introduction

Age-related macular degeneration (AMD), nowadays considered as a leading cause of severe and irreversible vision impairment, includes an early phase of atrophic or dry AMD (Cabral de Guimaraes et al., 2022), which is caused by sub-retinal pigment epithelium (RPE) waste products, called drusen, that are deposited underneath the macula. Drusen accumulate over time leading to RPE dysfunction and retinal cell death, which causes early blurring with limited compromission of central vision.

Dry AMD, accounting for most of the AMD cases, is eventually followed in a minority of patients by wet (or neovascular) AMD, that is responsible of severe vision loss as weak new vessels grow behind the retina. Neovessels leak fluid and blood, thus resulting in macular edema and retinal cell death, finally leading to a rapid central vision loss (Campochiaro, 2021). In this respect, early treatment of dry AMD may potentially prevent its progression to the neovascular form, that is usually treated by intravitreal injections of anti-vascular endothelial growth factor (VEGF) molecules.

As no treatments are currently available for dry AMD, many studies are being conducted to find effective drugs to prevent the progressive growth of drusen, leading to atrophic areas within the RPE. In particular, investigations on the impact of nutrients and specific dietary patterns on the incidence and progression of AMD revealed that regular consumption of foods that contain omega-3 is associated with a lower risk of developing wet AMD (Jiang et al., 2021). In addition to omega-3 supplementation, randomized controlled trials demonstrated that treatment with antioxidants such as carotenoids may be effective against AMD progression (Cao et al., 2022). In particular, carotenoids including the xanthophylls lutein, astaxanthin and zeaxanthin accumulate in the macula and their protective efficacy is mainly related to their antioxidant, anti-inflammatory and antiapoptotic activities (Giannaccare et al., 2020). In this respect, the only option currently recommended by the American Academy of Ophthalmology is the use of antioxidant vitamins, considering their potential in slowing the progression from earlier to later stages of dry AMD (Flaxel et al., 2020).

Despite preclinical studies are indicative of the benefit of diet supplementation including fatty acids and antioxidant vitamins, evidence for their benefit to limit AMD progression remains to be fully established (de Almeida Torres et al., 2022). In this respect, diets with enriched availability of fatty acids and antioxidant xanthophylls may increase the effectiveness of diet supplementation in preventing dry AMD and therefore its progression to wet AMD.

In the present study, the polyethylene glycol (PEG)-400 model mimicking the drusen-like insult leading to dry AMD (Lyzogubov et al., 2011, 2014; Cammalleri et al., 2017; Rudolf et al., 2018) was used to assess the efficacy of an orally-administered formulation based on xanthophylls (lutein, zeaxanthin) in addition to omega-3 derived from fish oil versus a novel formulation also including astaxanthin in addition to lutein and zeaxanthin *plus* omega-3 derived from Calanus oil (COil), extracted from the marine zooplankton *Calanus finmarchicus,* mostly present in cold waters of the North. In COil, omega-3 bioavailability is increased by the presence of stearidonic acid (SDA), a precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) Sea (Swanson et al., 2012; Cook et al., 2016). In addition, COil contains policosanols, fatty alcohols with antioxidant potential, the esterification of which may give rise to additional omega-3 including EPA and DHA (Schots et al., 2020). We tested the hypothesis that this latter enriched formulation administered before and after the PEG-400 insult, could indeed provide an increased protective efficacy in counteracting the major hallmarks of dry AMD by evaluating markers of oxidative stress, inflammation, glial activation and outer retina damage.

2 Materials and methods

2.1 Animals

Two-month-old C57BL/6J male mice were purchased from Charles River Laboratories Italia (Calco, Italy) and housed in a regulated environment (23 ± 1°C, 50 ± 5% humidity; 12 h light/dark cycles), fed with a standard diet and water *ad libitum*. A total of 48 mice were randomly assigned to 8 different groups (N = 6 each) as follows: control, PEG-injected untreated, PEG-injected treated with vehicle, PEG-injected treated with i. low or high-dose of mixture 1 (M1) or ii. low or high dose of mixture 2 (M2). For M1 and M2 composition see below. Animals used in this study were managed in agreement with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. The present study was carried out following the European Communities Council Directive (2010/63/UE) and the Italian guidelines for animal care (DL 26/14). The experimental protocol was authorized by the Commission for Animal Wellbeing of the University of Pisa (protocol n°656/2018-PR). In accordance with the 3Rs principles for ethical use of animals in scientific research, the experimental plan was designed in order to reduce both the number and suffering of the animals.

2.2 Subretinal injection of polyethylene glycol

Subretinal injections of PEG-400 were performed in agreement with the protocol by Lyzogubov et al. (2014). Forty-two mice were anesthetized by intraperitoneal injection of avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water, 0.02 ml/g body weight: Sigma-Aldrich) before

undergoing a sub-retinal injection of 0.5 mg of PEG-400 in 2 µl phosphate buffer saline (PBS) by mean of a Hamilton syringe equipped with 36-gauge needle. The formation of blebs at level of RPE/choroid complex was considered as successful injection. In order to minimize the number of the animals involved in the present study, the experimental plan did not provide a vehicle-only subretinal injection group since PBS itself does not alter cell viability or retinal structure (Lyzogubov et al., 2014).

2.3 Dietary supplementations

Mice were treated by oral gavage once daily 15 days before and 15 days after the sub-retinal injection of PEG-400. Twelve mice were treated with 2 different doses of M1 dissolved in sunflower oil (vehicle M1) to the final concentration of 43.8 mg/ml and administered at low dose (100 µl; 87,6 mg/kg) or high dose (200 µl; 175.2 mg/kg). M1 included fatty acids from fish oil (35.18 mg/ml of which 60% omega-3), lutein (0.88 mg/L), zeaxanthin (0.18 mg/ml), vitamin E (0.88 mg/ml), vitamin C (5.3 mg/ml), zinc (1.3 mg/ml) and copper (0.09 mg/ml). Twelve mice were treated with 2 different doses of M2 dissolved in sunflower oil *plus* tween 20 at 0.2% (vehicle M2) to the final concentration of 64 mg/ml and administered at low dose (100 µl; 128 mg/kg) or high dose (200 µl; 256 mg/kg). The M2 solution included fatty acids from COil (55.3 mg/ml of which 12% omega-3 + 33% polycosanols), astaxanthin (0.37 mg/ml), lutein (1.35 mg/ml), zeaxanthin (0.18 mg/g), vitamin E (2.7 mg/ml), vitamin C (5.3 mg/ml), zinc (1.3 mg/ml) and copper (0.09 mg/ml). In respect to fatty acids administration, despite the two mixtures display different amounts of fishery oil and Calanus oil, the actual concentration of omega 3 fatty acids delivered with the fish oil (21.5 mg/ml) is similar to that of omega 3 + polycosanols (24.5 mg/ml) delivered with Calanus oil, thus keeping the composition in fatty acids and xanthophylls as discriminant. The low doses calculated for both M1 and M2 correspond to those recommended in humans normalized by the body surface area method for interspecies' drug dosage translation (Nair and Jacob, 2016).

2.4 Western blot

Fifteen days after sub-retinal PEG-400 injection, mice were sacrificed by cervical dislocation and eyes were enucleated for Western blot analysis on explanted retinas or immunofluorescence/morphometrical analysis on eye sections. Isolated retinas were lysed by sonication in RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, United States) containing phosphatase and proteinase inhibitors (Roche Applied Science, Indianapolis, IN, United States). Protein content was quantified by Micro BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, United States). Equal amounts of each protein sample (30 µg) were separated by SDS-PAGE (4–20%; Bio-Rad Laboratories, Inc., Hercules, CA, United States) and gels were trans-blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% non-fat diet milk for 1 h at room temperature and then incubated overnight at 4°C with the solutions of primary antibodies listed in Table 1. Then, membranes were incubated for 2 h at room temperature with appropriate HRP-conjugated secondary rabbit anti-mouse (sc-2768, Santa Cruz Biotechnology) or goat anti-rabbit (170–6515, Bio-Rad Laboratories, Inc.) antibodies (1:5000). Blots were developed by the Clarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc.) and the images were acquired using the ChemiDoc XRS+ (Bio-Rad Laboratories, Inc.). The optical density (OD) relative to the target bands (Image Lab 3.0 software; Bio-Rad Laboratories, Inc.) was normalized to the corresponding OD of β-actin as loading control or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) p65 as appropriate.

2.5 Immunofluorescence and histological analysis

Enucleated eyes were fixed by immersion in 4% w/v paraformaldehyde in 0.1 M PBS for 2 h at room temperature and then stored at 4°C in 25% w/v sucrose in 0.1 M PBS. After being embedded in a cryo-gel medium, fixed eyes were cut into 10 µm thick coronal sections and mounted onto glass slides. Retinal sections corresponding to the site of lesion were selected for further immunofluorescence or histological processing.

For immunofluorescence analysis, mounted sections were incubated with the solution of primary rabbit anti-GFAP (1:400) or rabbit anti-Iba-1 (1:200) antibodies diluted in 0.1% *v/v* Triton X-100 in 0.1 M PBS overnight at 4°C. Subsequently, they were incubated with appropriate secondary goat anti-rabbit antibodies conjugated with Alexa-Fluor 488 (ab150077, Abcam; dilution: 1:200) diluted in 0.1% *v/v* Triton X-100 in 0.1 M PBS for 2 h at room temperature. Finally, retinal sections were coverslipped with Fluoroshield mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Abcam) and stored upon image acquisition. Images were acquired using an epifluorescence microscope (Ni-E; Nikon-Europe, Amsterdam, Netherlands) equipped with a digital camera (DS-Fi1 c; Nikon-Europe) and a ×20 plan apochromat objective. Glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule (Iba)-1 immunostaining was quantified by averaging the fluorescence intensity of 5 sections corresponding to the site of the lesion in each retina (6 retinas per group). Fluorescence intensity was retrieved from grayscale

TABLE 1 Western Blot primary antibodies.

Antibody	Dilution	Source	Catalogue
Rabbit monoclonal anti-GFAP	1:5000	Abcam	ab207165
Rabbit monoclonal anti-Iba-1	1:1000	Abcam	ab178846
Rabbit polyclonal anti-HO-1	1:2000	Abcam	ab13243
Rabbit monoclonal anti-NQO-1	1:10000	Abcam	ab80588
Rabbit monoclonal anti-pNF-kB p65 (Ser 536)	1:1000	Abcam	ab76302
Rabbit polyclonal anti-NF-kB p65	1:1000	Abcam	ab16502
Mouse monoclonal anti-IL-6	1:500	Santa Cruz Biotechnology	sc-57315
Rabbit monoclonal anti-TNF-α	1:1000	Abcam	ab205587
Mou se monoclonal anti-β-actin	1:2500	Sigma-Aldrich	A2228

images, after normalizing for the background, by measuring the mean gray level using the analysis tool of Image J (Version 1.47, NIH freeware, Bethesda, MD, United States).

For histological analysis, mounted sections were stained with hematoxylin and eosin (H&E) and images were acquired using a microscope (Ni-E; Nikon-Europe) equipped with a ×20 objective. The outer nuclear layer (ONL) thickness was considered as the interface between the outer plexiform layer and photoreceptor inner segment and quantified by averaging the values from 5 consecutive sections per retina (n = 6 retinas per group).

2.6 Statistical analysis

Statistical analysis was performed using the Graph Pad Prism 8.0.2 software (GraphPad Software, Inc., San Diego, CA, United States). Differences among groups were tested using one-way ANOVA followed by Tukey multiple comparison post hoc test. Differences with $p < 0.05$ were considered significant. All data are expressed as mean ± SEM of the indicated n values.

3 Results

3.1 Comparative efficacy of the dietary supplementations: Analysis of oxidative stress and inflammatory markers

The antioxidant efficacy of the two mixtures (M1 and M2) was evaluated by analyzing the protein levels of NAD(P)H quinone dehydrogenase-1 (NQO-1) and heme oxygenase-1 (HO-1) as major antioxidant enzymes involved in the adaptive response to altered redox balance in retinal cells (Kaspar and Jaiswal., 2009; Turpaev, 2013; Rossino et al., 2021). As shown in Figure 1, PEG-400 injection induced increased protein levels of NQO-1 and HO-1 by 115% and 103% ($p < 0.001$) respectively, as compared to control values. The oral administration of M1 and M2 vehicles did not affect the PEG-induced increase in NQO-1 and HO-1. Low doses of M1 and M2 did not prevent NQO-1 increase, whose levels were comparable to those of vehicle treated mice, while they significantly prevented the PEG-induced HO-1 increase with a 16% reduction in respect to the relative vehicles ($p < 0.05$). Treatment with high doses of both M1 and M2 significantly prevented the PEG-induced increase in NQO-1 protein levels, with M2 displaying a 23% higher efficacy as compared to the M1-treated mice. HO-1 levels in retinas of mice treated with the high dose of M1 and M2 were further decreased as compared to the respective low dose groups bringing them down to control values.

The effect of the oral administration of the two compounds on PEG-induced retinal inflammation was evaluated by analyzing the phosphorylation levels of the p65 subunit of NF-kB, a key transcriptional factor regulating the proinflammatory response, and interleukin (IL)-6, a related proinflammatory cytokine (Liu et al., 2017). As shown in Figure 2, PEG injection increased NF-kB phosphorylation by 120% ($p < 0.001$) with a consequent increased production of IL-6 (+ 85%, $p < 0.001$) in respect to the control. The PEG-induced increase in pNF-kB and IL-6 was not influenced by the oral administration of either M1 or M2 vehicles. The administration of M1 and M2 at low dose significantly prevented the PEG-induced increase of both pNF-kB (−30% $p < 0.01$) and IL-6 (−25%, $p < 0.01$) in respect to vehicles, without overt differences between the two compounds. The protein levels of pNF-kB and IL-6 following the administration of M1 at high dose resulted slightly lower than those obtained in the low dose group, with a significant difference recorded exclusively for the pNF-kB levels. Conversely, the M2 high dose group displayed a more pronounced effect as compared to M1, with significantly lower levels of both pNF-kB (−22%, $p < 0.01$) and IL-6 (−17%, $p < 0.5$) compared to high dose M1 group, resulting statistically comparable to controls.

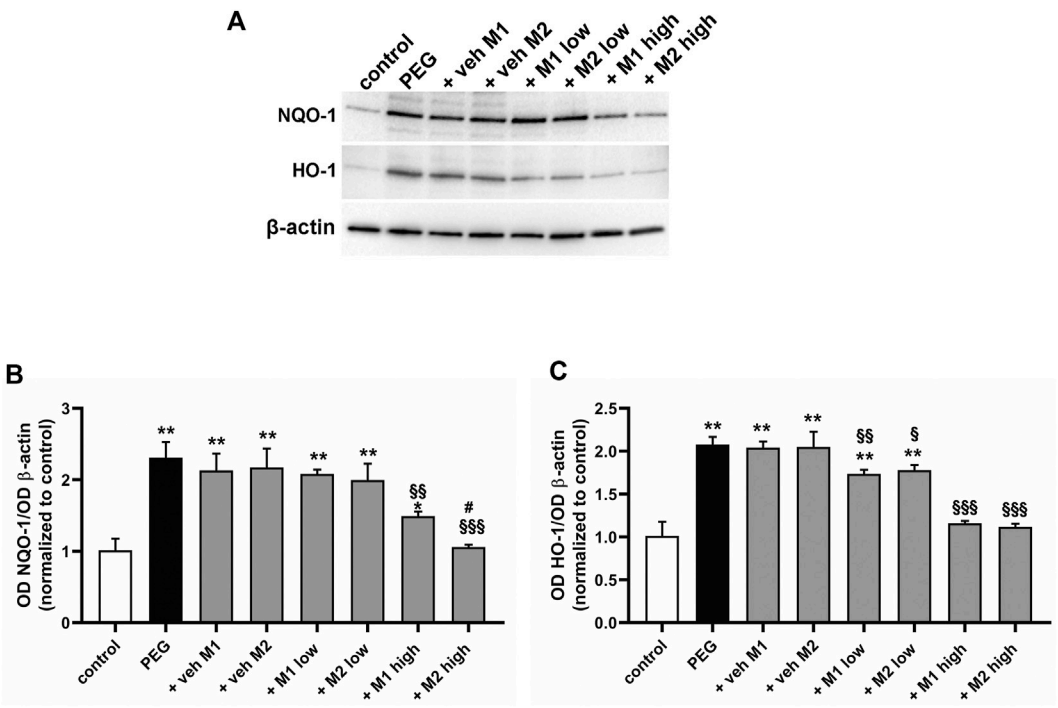

FIGURE 1
Effect of M1 and M2 on oxidative stress markers. Representative Western blots **(A)** and densitometric analysis of NQO-1 **(B)** and HO-1 **(C)** in controls, PEG-injected untreated, PEG-injected treated with M1 or M2 vehicles, PEG-injected treated with low or high dose of M1 as well as with low or high-dose of M2. β-actin was used as loading control. Data are plotted as mean ± SEM. Differences among groups were tested using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). *$p < 0.01$ and **$p < 0.001$ vs. control; §$p < 0.05$, §§p < 0.01 and §§§p < 0.001 vs. respective vehicle; #$p < 0.05$ vs. M1 high.

3.2 Comparative efficacy of the dietary supplementations: Analysis of müller cells gliosis and microglial reactivity

Among the relevant outcomes directly linked to the inflammatory activity in the retina, the glial and microglial cells reactivity is one of the main phenomena driving the retinal cell damage in AMD (Telegina et al., 2018; Rashid et al., 2019). Therefore, we analyzed the effect of the oral administration of either M1 or M2 on the transition of both Müller cells, as the glial cells mainly involved in AMD-like damage, and microglial cells to their reactive phenotypes. In particular, we analyzed the protein levels of GFAP and Iba-1 as well-established markers for reactive Müller cells and microglia, respectively, together with the immunolabeling patterns of the two markers in the PEG-induced lesion site.

As shown in Figure 3, PEG injection significantly increased the protein levels of GFAP (+85%, $p < 0.001$) and Iba-1 (+356%, $p < 0.001$) as compared to control values. Vehicle administration did not affect GFAP and Iba-1 protein levels while low doses of both M1 and M2 comparably prevented the PEG-induced increase of GFAP (-17%, $p < 0.05$) and Iba-1 (−53%, $p < 0.001$) as compared to vehicle. Both compounds at high dose displayed a more prominent effect as compared to the low doses, with M2 resulting significantly more effective than M1 in inhibiting the PEG-induced GFAP and Iba-1 increase (−18% e −25%, respectively, $p < 0.05$).

As shown in Figure 4A, in control retinas, GFAP staining was limited to astrocytes localized to the ganglion cell layer (GCL), while Iba-1 immunolabeling was weakly represented in the inner retinal layers. In retinas of PEG-untreated or vehicle-treated mice, Müller cells showed extensive GFAP immunolabeling along their processes spreading across retinal layers and Iba-1 immunoreactivity was more prominent with enlarged amoeboid-shaped immune-positive cells localized in all retinal layers. Treatment with either M1 or M2 at low dose partially prevented Müller cell gliosis and microglial activation as demonstrated by decreased GFAP-positive processes and Iba-1 immunolabeled cells. In retinas of mice treated with M1 at high dose, GFAP and Iba-1 immunoreactivity was less evident, but still detectable. On the contrary, in retinas of mice treated with M2 at high dose, GFAP and Iba-1 immunostaining was not different from control immunostaining. Quantitative analysis of fluorescence intensity showed that in PEG-untreated or vehicle-treated groups, GFAP and Iba-1 immunoreactivity was increased (+440% and +256%, $p < 0.0001$, respectively) as compared to

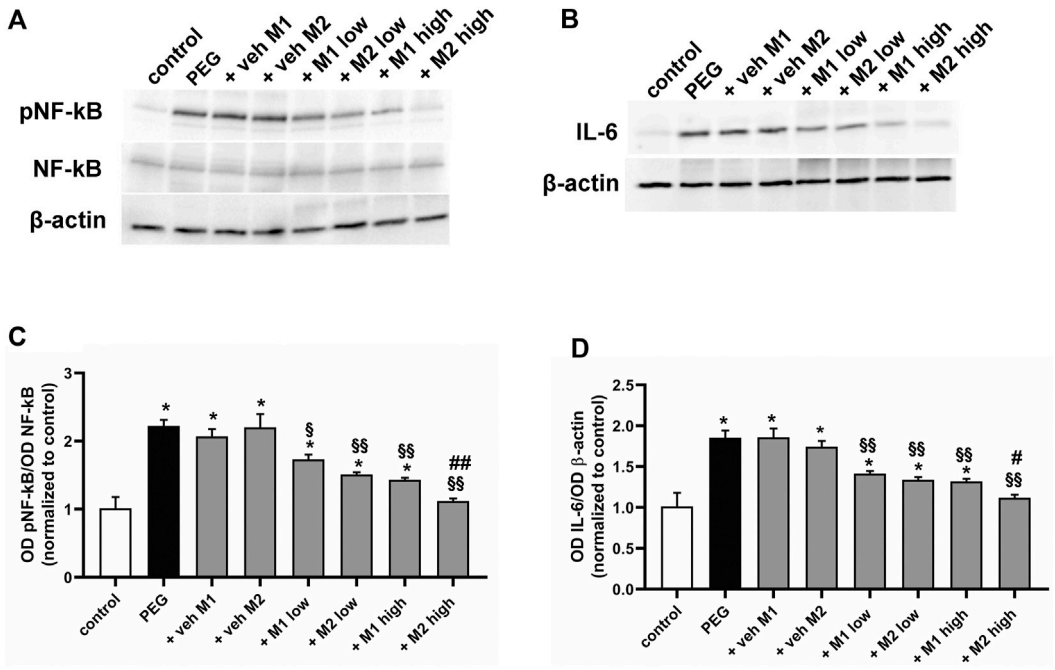

FIGURE 2
Effect of M1 and M2 on inflammatory markers. Representative Western blots **(A,B)** and densitometric analysis of pNF-kB/NF-kB ratio **(C)** and IL-6 **(D)** in controls, PEG-injected untreated, PEG-injected treated with M1 or M2 vehicles, PEG-injected treated with low or high dose of M1 as well as with low or high-dose of M2. β-actin was used as loading control. Data are plotted as mean ± SEM. Differences among groups were assessed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). *$p < 0.001$ vs. control; §$p < 0.01$ and §§$p < 0.001$ vs. respective vehicle; #$p < 0.05$, ##$p < 0.01$ vs. M1 high.

control values. Low dose of M1 or M2 reduced GFAP (-22%, $p < 0.01$) and Iba-1 intensity (−30%, $p < 0.001$) with no difference between the two compounds. Treatment with high dose resulted in a further decrease of GFAP and Iba-1 immunofluorescence, with the high dose of M2 resulting more effective than M1 and preserving GFAP and Iba-1 immunoreactivity to control levels.

3.3 Comparative efficacy of the dietary supplementations: Analysis of the proangiogenic marker vascular endothelial growth factor

Pro-inflammatory cytokines are known to increase VEGF expression, which in turn induces a number of pro-inflammatory genes (Schweighofer et al., 2009), indicating that inflammation can promote angiogenesis and new vessel growth thus enhancing tissue inflammation. As shown in Figure 5, PEG-400 injection was found to increase VEGF levels by 131% in respect to the control ($p < 0.001$). Vehicles did not affect VEGF levels while M1 and M2 dose-dependently prevented the PEG-induced VEGF increase with a 26% reduction by both M1 and M2 at low dose. At high dose, M2 was more effective than M1 (−25%, $p < 0.05$ vs. M1) until reaching control levels.

3.4 Comparative efficacy of the dietary supplementations: Morphometric analysis of the outer retina

Representative images of H/E-stained retinas are shown in Figure 6 in which histological analysis of the outer retina revealed an evident thinning of the ONL accompanied by the appearance of severe dystrophic alterations of RPE cells upon PEG-400 damage. Dietary supplementation with high doses of both M1 and M2 recovered the control thickness of the ONL including a noticeable recovery of the RPE layer. Quantitative analysis of the ONL thickness revealed that PEG-400 injection leads to a significant reduction of the ONL thickness of about 40% in respect to the control value ($p < 0.05$). High doses of both M1 and M2 were found to lead to ONL thickness almost comparable to that of controls.

4 Discussion

Our goal in this piece of translational research was dual: on the one hand, to compare two different, however similar, formulations beyond a direct comparison of the different components in each mixture since their individual role in

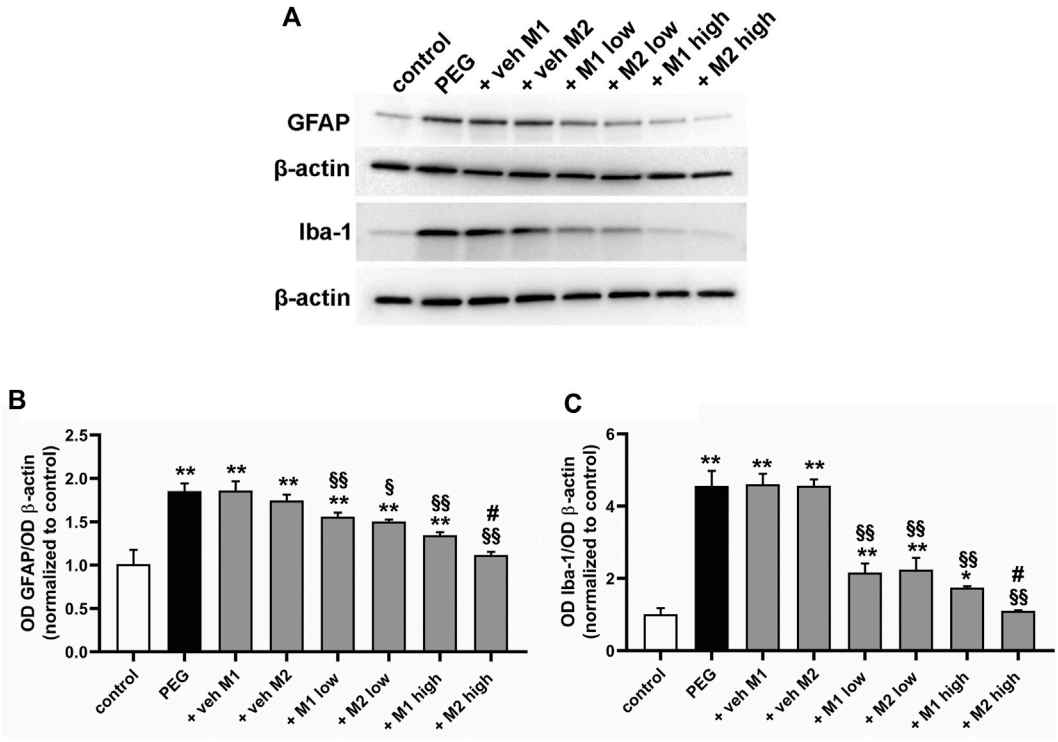

FIGURE 3
Effect of M1 and M2 on Müller cells gliosis and microglial reactivity. Representative Western blots **(A)** and densitometric analysis of GFAP **(B)** and Iba-1 **(C)** in controls, PEG-injected untreated, PEG-injected treated with M1 or M2 vehicles, PEG-injected treated with low or high dose of M1 as well as with low or high-dose of M2. β-actin was used as loading control. Data are plotted as mean ± SEM. Differences among groups were assessed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). *$p < 0.01$ and **$p < 0.001$ vs. control; $^{§}p < 0.01$ and $^{§§}p < 0.001$ vs. respective vehicle; #$p < 0.05$ vs. M1 high.

the protection of the retina is already clear and widely shown, on the other hand, to demonstrate that experimental AMD could be prevented and attenuated by food supplements at doses normally given to humans, and that a significant improvement on the existing formulation is possible by using similar kinds of ingredients, however from different origins. Having in mind the potential antioxidant/anti-inflammatory properties of multicomponent mixtures including xanthophylls and fatty acids, we decided to run a study directly assessing the efficacy of one formulation using fish oil enriched in omega-3 plus lutein and zeaxanthin versus a novel formulation theoretically with a higher efficacy because of a different source of omega-3 and a higher content in xanthophylls. In particular, the novel formulation includes the COil, as an alternative source of omega-3, plus astaxanthin, in addition to lutein and zeaxanthin, whose antioxidant properties in ocular pathologies have been previously demonstrated (Giannaccare et al., 2020; Lombardo et al., 2022). As pharmacological therapies against dry AMD are still lacking, treatments based on potentially innovative dietary supplementations remain as possible tools to solve unmet clinical needs. In an animal model of dry AMD, we established the relative efficacy of both compounds against the PEG-400-induced insult and we found that the COil-based formulation, despite a lower content of omega-3 in respect to that of fish oil origin, had a better protective effect, likely because of the presence of policosanols and the higher amount of additional xanthophylls, now also including astaxanthin beside lutein and zeaxanthin.

In respect to the first Age-Related Eye Disease Study (AREDS) trial demonstrating the efficacy of dietary supplements with antioxidant compounds in reducing the progression to advanced AMD in patients with intermediate AMD (Age-Related Eye Disease Study Research Group, 2001), in the AREDS 2 trial, the addition of lutein and zeaxanthin to the AREDS formula was shown to further decrease the long-term risk of AMD progression (Chew et al., 2022). Conversely, the beneficial effect of the omega-3 fatty acids remained controversial since their addition did not appear to significantly improve the efficacy of the AREDS2 formula (Age-Related Eye Disease Study 2 Research Group, 2013) despite a large background of preclinical and epidemiological evidence favoring the implication of omega-3 in the prevention of AMD progression (Chong et al., 2008). The negative results

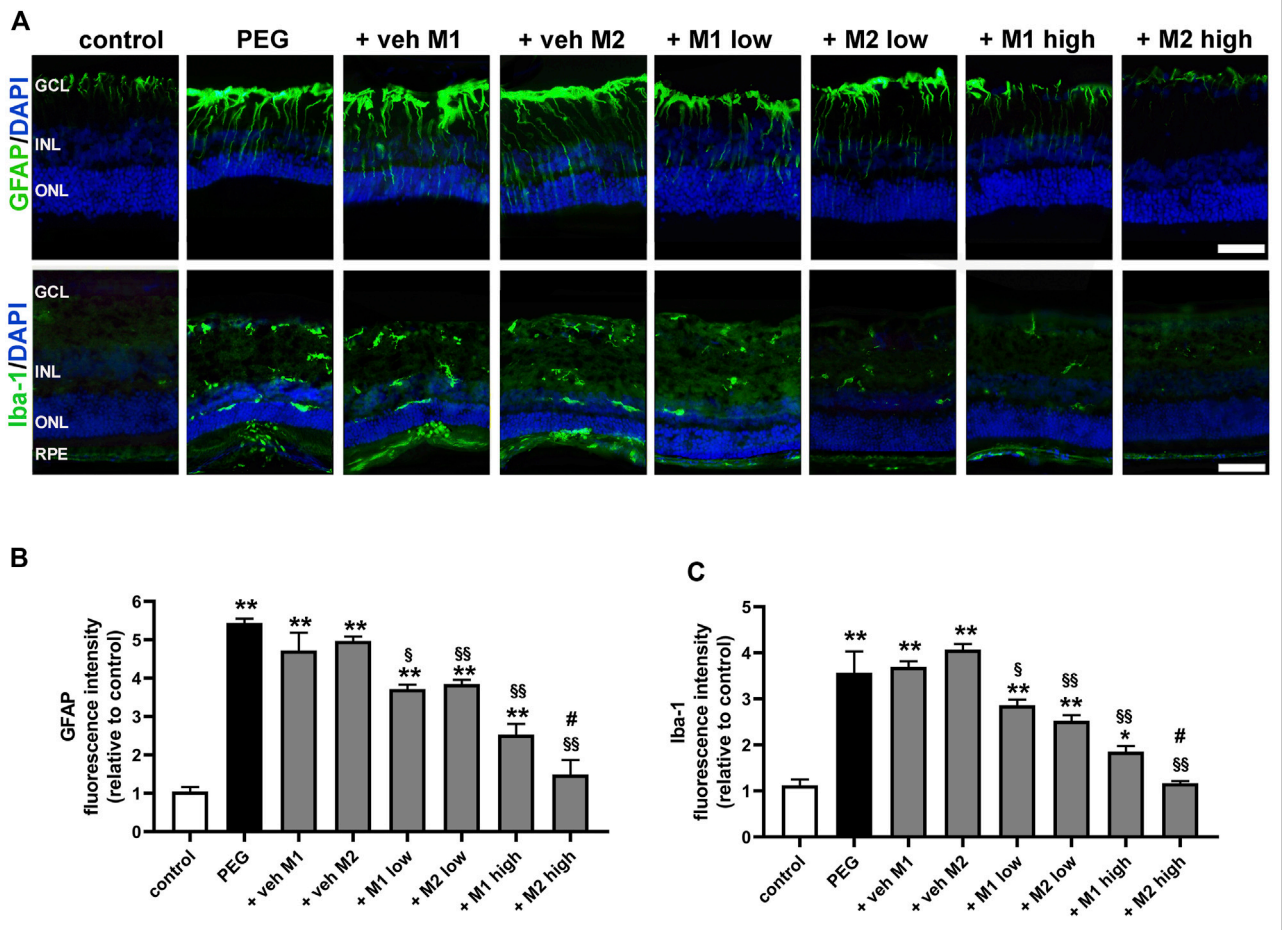

FIGURE 4
Effect of M1 and M2 on Müller cells gliosis and microglial reactivity in the site of lesion. **(A)** Representative images of retinal cross sections immunolabeled for GFAP or Iba1 in controls, PEG-injected untreated, PEG-injected treated with M1 or M2 vehicles, PEG-injected treated with low or high dose of M1 as well as with low or high-dose of M2. Scale bar, 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Quantitative analysis of GFAP **(B)** and Iba1 **(C)** immunofluorescence intensity. Data are plotted as mean ± SEM. Differences among groups were assessed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). $*p < 0.01$ and $**p < 0.001$ vs. control; $^{§}p < 0.01$, $^{§§}p < 0.001$ vs. respective vehice; $\#p < 0.05$ vs. M1 high.

of the AREDS2 study were then debated in light of the possibility that the prophylactic potential of omega-3 was not adequately demonstrated (Souied et al., 2015). For instance, the need of an "ideal" ratio of omega-3 to omega-6 fatty acids to be assumed in the diet in respect to omega-3 alone, was also recognized in terms of prophylactic benefits as an adequate intake of omega 6 was reported to lower the risk of AMD (Seddon et al., 2003). In this respect, preclinical evidence from the PEG-400 model demonstrated that in addition to omega-3, a correct balance of omega-6/omega-3 is mostly important for preventing dry AMD occurrence and that the efficacy of fatty acids in reducing retinal degeneration may be enhanced by their combination with xanthophylls (Cammalleri et al., 2017). In addition, increasing the amount of fatty acids in the diet would achieve the desired increase in their levels in the body's tissues with increased effectiveness.

Although the role of omega-3 in AMD still remains controversial, COil, a fatty acid source of copepod origin, raised some interest as a novel source of omega-3 that not only responds to the increasing demand of fatty acids by the market, but also differs from other commercial marine oils in terms of chemical properties and, possibly, bioactivity. In fact, COil contains wax esters, whereas classic omega-3 fatty acids come from triglycerides, ethyl esters and phospholipids. Wax esters are fatty acids that are esterified with alcohols including the long-chain fatty alcohols eicosanol and docosanol that are contained in COil (Schots et al., 2020).

Since early studies in which COil efficacy has been demonstrated in models of human diseases, results of beneficial effects of COil supplementation are rapidly increasing. In this respect, long term intake of COil has been shown to increase the blood level of omega-3 fatty acids despite the low levels of EPA and

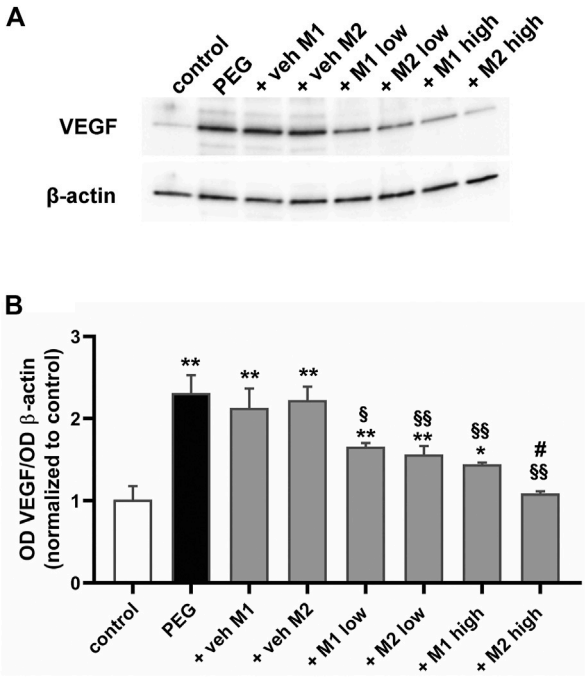

FIGURE 5
Effect of M1 and M2 on VEGF levels. Representative Western blots **(A)** and densitometric analysis of VEGF **(B)** in controls, PEG-injected untreated, PEG-injected treated with M1 or M2 vehicles, PEG-injected treated with low or high dose of M1 as well as with low or high-dose of M2. β-actin was used as loading control. Data are plotted as mean ± SEM. Differences among groups were assessed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). $*p < 0.01$ and $**p < 0.001$ vs. control; $^{§}p < 0.01$, $^{§§}p < 0.001$ vs. respective vehicle; $\#p < 0.05$ vs. M1 high.

DHA indicating that fatty acids are mostly of wax ester origin (Wasserfurth et al., 2021). In addition, preclinical findings have indicated antioxidant and anti-inflammatory efficacy of this copepod oil beyond that provided by EPA and DHA (Schots et al., 2020), which are presumably due to fatty acid conversion from their corresponding fatty alcohols (Gasmi et al., 2020). In this respect, notable is the discovery of major effectiveness of COil on glucose metabolism and insulin resistance in obese patients in which the different lipid components of COil may have potential as nutraceuticals for reducing obesity and obesity-related metabolic disorders (Burhop et al., 2022). In mouse models of obesity, COil supplementation in addition to efficiently counteract obesity-induced alterations, has been also demonstrated to exert anti-hypertensive effects and to improve post-ischemic cardiac recovery (Salma et al., 2016; Jansen et al., 2019). In addition, COil supplementation in combination with exercise training has been demonstrated to display additional efficacy on cardiorespiratory function in respect to exercise training alone although its clinical relevance remains to be verified (Štěpán et al., 2021).

The present study comparing the efficacy of two mixtures that differ in their fatty acid source and in their xanthophyll content was performed in the polyethylene glycol- (PEG)-400 model. Although no ideal animal model has been established that fully recapitulates the human features of dry AMD, both the PEG-400 model (Lygozubov et al., 2014) and the chemokine ligand 2 (CCL2) knock out (KO) model (Ambati et al., 2003) share certain common characteristics with dry AMD. Both models contain drusen-like bodies and other features of AMD including progressive outer retinal degeneration and geographic/RPE atrophy although in the CCL2 KO, drusen accumulation consequent to the CCL2 deletion has been also interpreted as a result of normal aging (Luhmann et al., 2009). In both models, dietary supplementation with fatty acids-based mixtures has been found to counteract pro-inflammatory and angiogenic responses, thus hampering the retinal degeneration associated with advanced AMD (Cammalleri et al., 2017; Prokopiou et al., 2017).

The PEG-400 model shows drusen-like deposits that are located between the RPE and the Bruch's membrane triggering oxidative stress, inflammation and angiogenesis, as key pathways that co-dependently participate in AMD progression. In particular, high levels of reactive oxygen species (ROS) have been associated with drusen formation leading to RPE atrophy in both human and animal eyes (Nowak, 2013; Rabin et al., 2013; Shaw et al., 2016). In this respect, a recent study using a mouse model of AMD has demonstrated an early increase in the expression of oxidative stress and inflammatory markers into the subretinal space and the neural retina (Kim et al., 2021). Chronic inflammation further promotes ROS generation, thus initiating a vicious cycle that promotes the amplification of the pathological events (Datta et al., 2017). The accumulation of ROS triggers the endogenous antioxidant response inducing the activity of nuclear factor erythroid 2-related factor 2 (Nrf2) as the primary redox sensitive transcriptional factor. Nrf2, in turn, mediates the increment in antioxidant enzymes including NQO-1 and HO-1 that are involved in the adaptive response to the altered redox balance (Kang and Yang, 2020). However, the chronic accumulation of ROS overwhelms the antioxidant potential of the endogenous defenses, thus leading to oxidative damage of retinal cells (Kowluru and Chan, 2007). In addition, ROS increase further stimulates the inflammatory response by triggering the activation of NF-kB, a transcription factor primarily involved in the proinflammatory response mediating the expression of cytokines such as IL-6 (Liu et al., 2017). Pro-inflammatory stimuli result in the activation of macroglia, whose switch to reactive phenotype is classically manifested by the overexpression of GFAP. In effect, GFAP expression is widely used as a reliable marker of retinal pathological injury since its localization in the healthy retina is limited to astrocytes, whereas in the diseased retina, GFAP appears densely localized also to Müller cells (Vecino et al., 2016). In this respect, effects of PEG-400 administration are further confirmed by GFAP staining that demonstrates an increase in Müller cell reactivity, as also confirmed by the molecular analysis highlighting the increment in

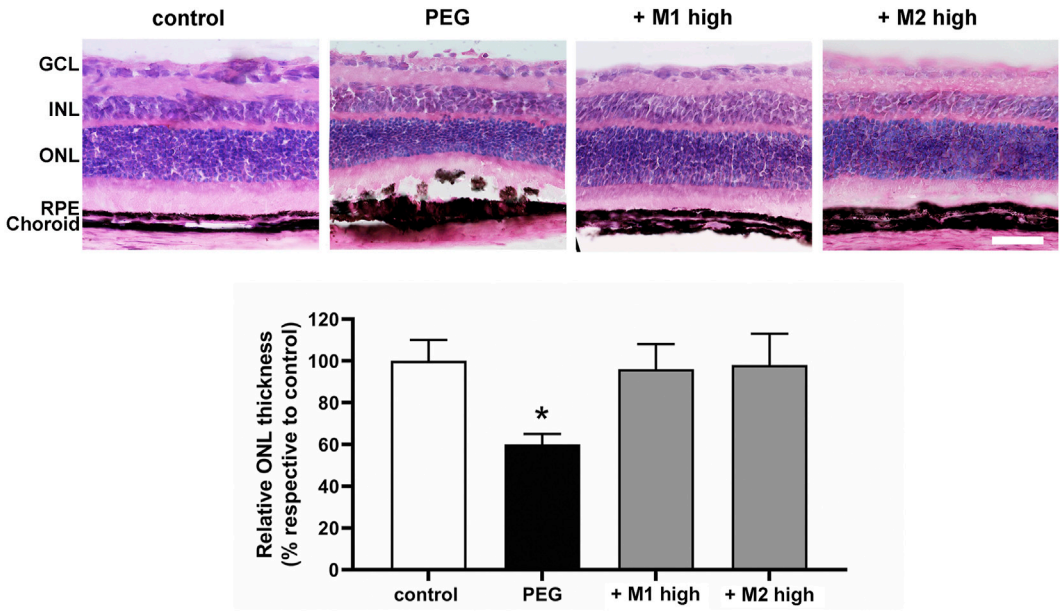

FIGURE 6
Effect of M1 and M2 on PEG-induced retinal damage. Representative images of H/E-stained retinal cross sections of controls, PEG-injected mice untreated, PEG-injected mice treated with the high doses of either M1 or M2. Morphometric analysis of the ONL thickness normalized to the total retinal thickness and expressed as percentage of controls. Data are expressed as mean ± SEM. Differences among groups were assessed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test (N = 6). *$p < 0.05$ vs. control. Scale bar, 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

GFAP protein levels. In addition to Müller cell gliosis, over-activated microglial cells participate to the release of pro-inflammatory mediators and further increase oxidative damage (Block and Hong, 2005). Gliotic Müller cells are recognized as the main secretion source of inflammatory cytokines (Eastlake et al., 2016) among which VEGF induces angiogenesis and vascular hyper-permeability thus playing as a major trigger of retinal and choroidal neovascularization leading to wet AMD (Tan et al., 2022). Among the first indications of VEGF role in patients with neovascular AMD, Kliffen et al. (1997) reported its increased expression in human specimens of the RPE and the outer retina at the macular level. The substantial role of VEGF in AMD is sustained by the fact that new therapies for wet AMD are mainly focused on VEGF inhibition although anti-VEGF therapies display significant limitations due to their limited half-life and subsequent treatment costs (Chakravarthy et al., 2022; Sarkar et al., 2022), Therefore, alternative strategies aiming at preventing or delaying the disease progression represents one of the current goals for the management of AMD. In this respect, preclinical studies have demonstrated that treatments with functional nutrients able to inhibit oxidative stress and inflammation may represent a promising approach for non-invasive prevention and/or treatment of AMD. For instance, dietary supplementation based on fatty acids intake has been shown to counteract the production of pro-inflammatory and angiogenic markers thus preventing degenerative events in the outer retinal layers (Cammalleri et al., 2017; Prokopiou et al., 2017). This is confirmed by the present results demonstrating that both mixtures supplemented here exert a multi-target role by interfering with major pathological hallmarks after the PEG-400 insult. In particular, dietary supplementation with fish oil- or COil-based mixtures leads to a consequent reduction of oxidative stress and inflammatory markers thus preventing Müller cell gliosis as determined by the drastically reduced GFAP staining. Microglial reactivity is also prevented as demonstrated by reduced Iba-1 at the immunohistochemical and protein level. Preventing oxidative stress and inflammatory processes can be reflected in counteracting VEGF upregulation typical of AMD-like conditions, thus suggesting a possible inhibition of the switch from dry to wet AMD. At the structural level, dietary supplementation with both mixtures promotes the recovery of the ONL thickness, with clear benefits to the RPE cells.

Most importantly, the present findings confirm the efficacy of an oral delivery of lutein and omega-3 fatty acids in the prevention of dry AMD, and likely its progression towards the wet form. Such efficacy can be further improved by a novel formulation based on a different source of omega-3 fatty acids (the COil) also containing policosanols, and an increased amount of xanthophylls, namely astaxanthin in addition to lutein. A contribution to this higher efficacy might be ascribed to the increased bioavailability of omega-3 in the COil formulation (Cook et al., 2016) and its content in astaxanthin with higher

antioxidant activity as compared to lutein and zeaxanthin (Naguib, 2000). Increased availability of omega-3 fatty acids might depend on their partial origin from policosanols, which could restore with higher efficacy the deficiency of bioactive lipids as those derived from polyunsaturated fatty acids that are known to play a protective role against AMD development (Kishan et al., 2011; Elmasry et al., 2019). Moreover, despite COil is relatively low in EPA and DHA as compared to fish oil, its high content in the monounsaturated SDA, a precursor of EPA and DHA, may further contributes to increase omega-3 fatty acid content (Pedersen et al., 2014; Cook et al., 2016).

5 Conclusion

Nowadays, there is no cure for AMD, much less for its atrophic form. The neovascular form can be treated by intravitreal anti-inflammatory and/or anti-angiogenic drugs. Antioxidant xanthophylls and carotenoids are used to delay progression of the atrophic form, which may degenerate into the neovascular disease. Experimental therapies, mainly based on the control of oxidative stress, are under study including clinical trials using compounds that reduce mitochondria-derived oxidants.

Among dietary supplementations eventually counteracting dry AMD, the AREDS studies have clearly indicated the role of diets containing antioxidants and omega-3 in the prevention and control of the progression of AMD. Among structural components of retinal cell membranes, cholesterol mostly of Muller glia origin is the second most abundant lipid in the neuroretina behind phospholipids. Cholesterol plays a key role in retinal function and its downregulation can contribute to the progression of several multifactorial diseases like glaucoma, diabetic retinopathy and macular edema. A lipid shuttle has been identified from Müller glia to retinal ganglion cells to sustain their projections and synaptic formation. Whether fatty acids supplementation would cover the increasing demand of fatty acids by retinal neurons, then nutritional supplementation with omega-3 may represent an important coadjuvant in the therapeutic management of AMD.

Among antioxidants, carotenoids accumulate in the macula that naturally contains antioxidants and photo-bleachers such as lutein and zeaxanthin. Astaxanthin, which is even more potent than lutein, is not naturally present at the macular level. Therefore, the main, if not the only, treatment for the prevention and the treatment of the early forms of AMD is the supply of nutraceuticals to replenish the antioxidant defense of the retina and to give anabolic support to photoreceptors. In this respect, individuals with high risk of developing AMD, as predictable with an available genetic test, might be advised to use more antioxidants and omega-3 in their diet.

Results presented in this paper show the relevance of the combination of dietary omega-3 and xanthophylls in the prevention and treatment of AMD and indicate that the source of these components might be also critical, because the formulation based on COil appears to be more protective than the one based on fish oil. In this respect, innovative dietary treatments including fatty acids of COil origin and xanthophylls may potentially represent AMD modifying therapies with higher efficacy than currently used therapies including fatty acids of fish oil origin.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Commission for Animal Wellbeing of the University of Pisa.

Author contributions

Conceptualization, DR, PB, and MC; methodology, AM, RA, and DL; validation, AM and RA; formal analysis, AM and RA; investigation, AM, RA, and DL, resources, MC; data curation, RA and MC; writing—original draft preparation, RA, PB, and MC; writing—review and editing, RA, MD, DR, PB, and MC; supervision, MC; project administration, DR, PB, and MC; funding acquisition, MC All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

DR was an employee of Fidia Farmaceutici SpA. Fidia Farmaceutici SpA had no direct role in the collection, analyses or intepretation of the data or in the decision to publish the results.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Noncoding RNAs as a novel approach to target retinopathy of prematurity

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Retinopathy of prematurity (ROP), a vascular disease characterized by abnormal vessel development in the retina, has become a primary cause of blindness in children around the world. ROP can be developed during two different phases: vessel loss and vessel proliferation. Once preterm infants with immature retinal vessel growth are exposed to high level of oxygen inside the incubator, vessel loss can occur. When infants are exposed to room air, they may experience the proliferation of vessels in the retina. Although multiple factors are reported to be involved in the pathogenesis of ROP, including vaso-endothelial growth factors (VEGFs) and hypoxia-inducible factors, the pathogenesis of ROP is not completely understood. Although laser therapy and pharmacologic agents, such as anti-VEGF agents, have been commonly used to treat ROP, the incidence of ROP is rapidly rising. Given that current therapies can be invasive and long-term effects are not fully known, the search for novel therapeutic targets with less destructive properties needs to be considered. Within the last decade, the field of noncoding RNA therapy has shown potential as next-generation therapy to treat diverse diseases. In this review, we introduce various noncoding RNAs regulating ROP and discuss their role as potential therapeutic targets in ROP.

KEYWORDS

microRNA, long noncoding RNA, circular RNA, retinopathy of prematurity, retinal vascular disease, noncoding RNA

Introduction

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a progressive retinal vascular disease that occurs in preterm infants. It is characterized by abnormal vessel growth in the retina. The incidence of ROP has gradually increased due to the development of neonatal care and has become a leading cause of childhood blindness (Freitas et al., 2018; Filippi et al., 2022). Various factors including oxygen supplementation, low birthweight or gestational age, blood transfusion, and sepsis can be risk factors for ROP (Alajbegovic-Halimic et al., 2015). The progression of the disease can be divided into two phases (Hartnett and Penn, 2012). During phase 1, retinal vessel development is delayed due to various factors, such as

hyperoxia caused by oxygen supplementation, reactive oxygen stress, or low maternal-derived factors (Graziosi et al., 2020). As a result, a peripheral avascular region may occur in preterm infants. During phase 2, when the preterm infants are returned to room air, the proliferation of retinal vessels can occur in the avascular region of the retina causing vision loss (Hartnett and Penn, 2012).

Diverse molecular factors and signaling cascades are involved in the development of ROP (Hartnett, 2015; Ryu, 2022). There is evidence of downregulation of hypoxia-inducible factor 1-alpha (HIF1α), vascular endothelial growth factor (VEGF), and erythropoietin during phase 1, whereas these factors are upregulated during phase 2 (Ryu, 2022). Various signaling pathways, including HIF, VEGF, Wnt, Notch-Sox, and Semaphorin, are activated in ROP (Chen et al., 2011; Lee et al., 2014; Hartnett, 2015; Yang et al., 2015; Kim et al., 2016; Ramshekar and Hartnett, 2021).

Currently, laser photocoagulation has been used as a first-line therapy to treat ROP (Shulman and Hartnett, 2018; Linghu et al., 2022). Although laser therapy is a widely used method to treat ROP, it has been shown to increase the risk of myopia and other unfavorable ocular outcomes, such as macular dragging (Mintz-Hittner et al., 2011; Geloneck et al., 2014; Stahl et al., 2019). Anti-VEGF agents are the alternative therapy to treat ROP. Several anti-VEGF drugs including bevacizumab, ranibizumab, pegaptanib, and aflibercept have been investigated in the context of ROP, but only ranibizumab has received the indication for the treatment of ROP in Europe and Japan (Lee and Shirley, 2021). Although, long-term systemic effects of VEGF inhibition on other organs have been reported, there is no consensus on the optimal dosage and timing of anti-VEGF agent administration (Sato et al., 2012; Harder et al., 2014; Han et al., 2018; Hamad et al., 2020; Seery et al., 2020). Thus, it is worthwhile to investigate novel therapeutic targets to treat ROP and reveal more detailed mechanisms regulating ROP.

Noncoding RNAs

Noncoding RNAs (ncRNAs) are RNAs that do not generally translate proteins. Various types of ncRNAs are reported to play a critical role in regulating heart, vascular, and neuronal diseases (Poller et al., 2018; Jae and Dimmeler, 2020; Ryu et al., 2021; Yoon et al., 2021; Ryu et al., 2022). Compared to messenger RNA (mRNA), the function of ncRNAs has not been thoroughly investigated. In this article, we will focus on the role of three different types of ncRNAs: microRNA (miRNA), long noncoding RNA (lncRNA), and circular RNA (circRNA).

miRNAs, relatively short ncRNAs with length around 22 nucleotides, have been widely studied in diverse diseases including ocular diseases. Various miRNAs were differentially expressed in vitreous humor of patients with ocular diseases (Ragusa et al., 2013). In addition, expression of miRNAs in vitreous humor of patients with uveal melanoma was analyzed and several miRNAs such as miR-146a were significantly upregulated suggesting their potential use as diagnostic biomarkers (Ragusa et al., 2015). miRNAs are highly conserved in mammals and function as modulators during post-transcription. They can bind to mRNA and inhibit protein translation or completely break down mRNA (Esteller, 2011). Single miRNAs can target multiple mRNAs and may involve in various biological processes (Hashimoto et al., 2013).

lncRNAs, longer-length ncRNAs, are typically longer than 200 nucleotides. Although lncRNAs have 5′ capped ends and are spliced showing similar characteristics as those of mRNAs, they lack open reading frames (ORF) resulting in the absence of protein-coding potential. Compared with mRNAs, the expression of lncRNAs is relatively weak and may differ depending on the types of species, tissue, or cell (Derrien et al., 2012). Moreover, the roles of lncRNAs may differ based on their localization. lncRNAs localized in the nucleus can modulate gene expression, whereas lncRNAs localized in the cytoplasm may function as miRNA sponges (Fatica and Bozzoni, 2014).

circRNAs, circular form RNA without 5′ and 3′ ends, are variable in length and are most frequently created by back-splicing events. Previously, circRNAs were considered as byproducts of mRNA processing; however, the diverse regulatory roles of circRNAs have been reported in various diseases. The most extensively studied function of circRNAs is their possible role as miRNA sponges (Hansen et al., 2013; Lasda and Parker, 2014). Additionally, circRNAs have been reported to act as protein sequesters or are involved in translation in cases where an ORF is included within the circRNAs. circRNAs are stable because they are not readily degraded by exonuclease due to their circular structure (Ryu et al., 2021). Thus, circRNAs may be a promising approach to treat various diseases. Recently, ncRNAs have also been investigated in various retinal vascular diseases. Compared to other retinal vascular diseases, the pharmacologic treatment options for ROP are limited. Thus, in this article, we review studies of ncRNAs in ROP and discuss their potential as novel therapeutic targets for the treatment of ROP.

Noncoding RNA studies in retinopathy of prematurity

We searched Pubmed and Embase databases for ncRNAs studies in the context of ROP, using the ncRNA-relevant search terms such as miRNA, lncRNA, and circRNA, and disease-related terms, such as retinopathy of prematurity, retinopathy, preterm infants, oxygen-induced retinopathy, retinal neovascularization, vaso-proliferation, vessel loss, and vaso-obliteration. We then manually reviewed the title and abstract of the articles to verify their relevance to our topic and selected

TABLE 1 microRNAs regulating ROP.

miRNA	Expression	Study phase	Effects on ROP	Study model	Key findings	Reference
18a-5p	Up	Phase 2	Inhibits RNV	*in vivo*: OIR mice	Inhibits FGF1 and HIF1α	Guan et al. (2020)
34a	Down	Phase 2	Inhibits RNV	*in vitro*: VEGF-treated HRMECs *in vivo*: OIR rats	Inhibits Notch1	Shi et al. (2019)
96	Down	Phase 1	Inhibits vessel loss	*in vitro*: hyperoxia-induced HRMECs *in vivo*: OIR rats, vaso-obliteration model (80% O_2) *ex vivo*: choroid isolated from rats at vaso-obliteration phase	Regulates VEGF and Angiopoietin-2	Desjarlais et al. (2020)
145	Up	Phase 2	Promotes RNV	*in vitro*: hypoxia-treated HRMECs *in vivo*: OIR mice	Inhibits TMOD3	Liu et al. (2019)
150	Down	Phase 2	Inhibits RNV	*in vitro*: VEGF-induced HRMECs *in vivo*: miR-150 KO mice *ex vivo*: aortic rings and choroidal explants from miR-150 KO mice	Inhibits CXCR4, DLL4, or FZD4	Liu et al. (2015)
181a-5p	Down	Phase 2	Inhibits RNV	*in vitro*: VEGF-induced HRECs *in vivo*: OIR mice	Inhibits Endocan	Chen et al. (2020)
182-5p	Down	Phase 2	Inhibits RNV	*in vitro*: hypoxia-induced HRMECs *in vivo*: OIR mice	Inhibits ANG and BDNF	Li et al. (2022)
299	Down	Phase 2	Inhibits RNV	*in vitro*: $COCl_2$-induced HRECs *in vivo*: OIR mice	Inhibits VEGF-A	Wang et al. (2022)

ANG, angiogenin; BDNF, brain-derived neurotrophic factor; CXCR4, C-X-C chemokine receptor type 4; DLL4, delta like ligand 4; FGF1, fibroblast growth factor 1; FZD4, frizzled class receptor 4; HIF1a, hypoxia-inducible factor 1-alpha; HREC, human retinal endothelial cell; HRMEC, human retinal microvascular endothelial cells; KO, knockout; miRNA, microRNA; Notch1, neurogenic locus notch homolog protein 1; OIR, oxygen-induced retinopathy; RNV, retinal neovascularization; TMOD3, tropomodulin3; VEGF, vascular endothelial growth factor.

TABLE 2 long noncoding RNAs and circular RNAs regulating retinopathy of prematurity (ROP).

lncRNA	Expression	Study phase	Effects on ROP	Study model	Key findings	Reference
MALAT1	Up (P12)	Phase 2	Promotes RNV	*in vivo*: OIR mice	Inhibition of MALAT1 reduced RNV	Wang et al. (2020)
	Up	Phase 2	Promotes RNV	*in vitro*: hypoxia-induced HUVECs *in vivo*: OIR mice	Functions as miR-124-3p sponge and regulates EGR1 Inhibition of MALAT1 reduced RNV	Xia et al. (2021)
MEG3	Down (P12)	Phase 2	Inhibits RNV	*in vivo*: OIR mice	Overexpression of MEG3 inhibited RNV through PI3K/AKT/VEGF signaling pathway and reduced the expression of inflammatory factors	Di et al. (2022)
MIAT	Not applicable	Phase 2	Promotes RNV	*in vivo*: OIR mice	Inhibition of MIAT1 reduced RNV by regulating PI3K/AKT/VEGF signaling pathway	Di et al. (2021)
TUG1	Up	Phase 2	Promotes RNV	*in vitro*: $CoCl_2$-treated HRECs *in vivo*: OIR mice	Acts as miR-299 sponge and regulates VEGF-A	Wang et al. (2022)
circRNA						
circPDE4B	Down	Phase 2	Inhibits RNV	*in vitro*: hypoxia-induced HRECs *in vivo*: OIR mice	Acts as miR-181c sponge and regulates VHL	Deng et al. (2020)
circZNF609	Up (P17)	Phase 2	Promotes RNV	*in vitro*: H_2O_2 or $CoCl_2$-treated HUVECs *in vivo*: OIR mice	Functions as miR-615-5p sponge and modulates MEF2A Inhibition of circZNF609 reduced vessel loss and pathological RNV	Liu et al. (2017)
OIR retinal circRNAs	Up/down	Phase 2	Not applicable	*in vivo*: OIR mice	May work as ceRNAs in ROP	Zhou et al. (2019)

AKT, protein kinase B; ceRNA, competing endogenous RNA; circRNA, circular RNA; EGR1, early growth reponse 1; HREC, human retinal endothelial cell; HUVEC, human umbilical endothelial cells; lncRNA, long noncoding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MEF2A, myocyte Enhancer Factor 2A; MEG3, maternally expressed gene 3; MIAT, myocardial infarction-associated transcript; OIR, oxygen-induced retinopathy; PI3K, phosphoinositide 3-kinase; RNV, retinal neovascularization; ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

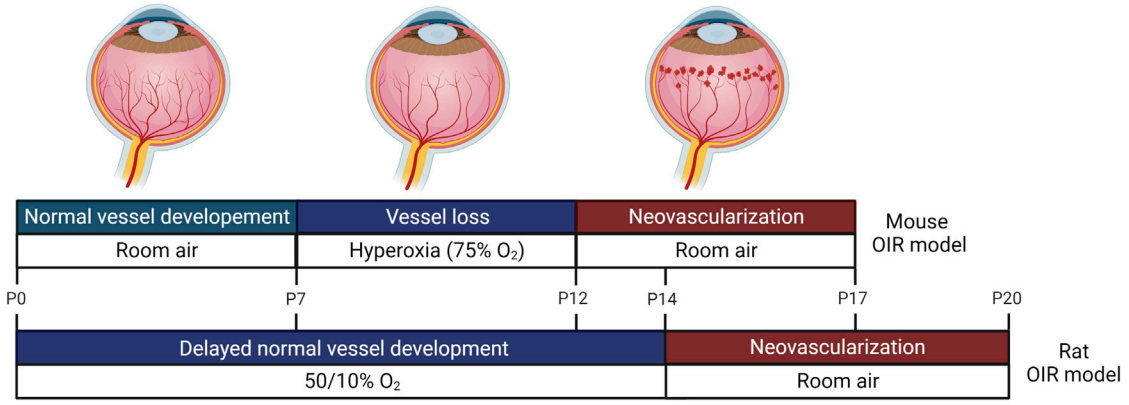

FIGURE 1
Oxygen-induced retinopathy (OIR) animal models. Animal models commonly used to investigate retinopathy of prematurity (ROP) are shown. In an OIR mice model, mouse pups and their mothers are placed in hyperoxia 7 days after birth for 5 days, which mimics phase 1 of ROP in humans. During hyperoxia, shrinkage of newly developed vessels occurs. The animals are then returned to room air (relative hypoxia) for 5 days, which resembles phase 2 of human ROP. During hypoxia, retinal neovascularization (RNV) begins and maximizes at 17 days after birth. In an OIR rat model, rat pups and their mothers are exposed to 50% oxygen on the first day and 10% oxygen on the next day, and this cycle is repeated for 14 days. During this fluctuating oxygen level period, normal vessel development is delayed. From 14 to 20 days, the rat pups are moved to room air, and RNV is induced.

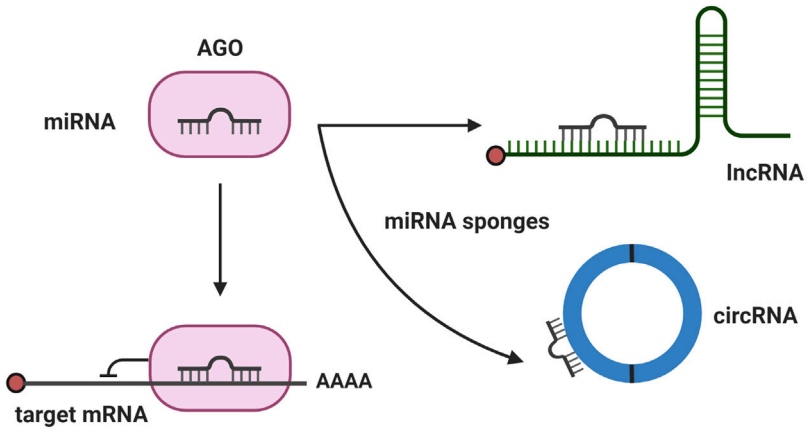

FIGURE 2
Major mechanism of noncoding RNAs (ncRNA). The major mechanisms of ncRNAs regulating ROP are depicted. microRNAs (miRNAs) bind with their target mRNA and degrade mRNA or inhibit translation. When long noncoding RNA or circular RNA act as a miRNA sponge, it can prevent miRNA binding with its target mRNA and recover mRNA processing.

articles for review (Tables 1, 2). By analyzing ncRNA studies in the context of ROP, we found that most studies focused on phase 2 of human ROP, in which retinal neovascularization (RNV) is maximized and the anti-angiogenic function of ncRNAs was investigated. Additionally, we found that the majority of studies were conducted in oxygen-induced retinopathy (OIR) *in vitro* or *in vivo* models. In the OIR *in vitro* model, human retinal microvascular endothelial cells (HRMECs), human retinal endothelial cells (HRECs), or human umbilical endothelial cells (HUVECs) were commonly used. Introducing cells to

hyperoxia induced vaso-obliteration, whereas exposing cells to hypoxia promoted RNV. In addition, VEGF, H_2O_2, or $CoCl_2$ was applied to cells to induce ROP. In the case of OIR *in vivo* models, mice or rats were commonly used, and the oxygen level was altered to mimic human ROP (Figure 1). miRNAs were the most commonly studied ncRNAs in ROP. Many miRNAs were found to target angiogenesis-related genes, such as VEGF, HIF1α, and Angiopoietin-2 (Figure 2). Compared to published research on miRNAs, there are comparatively few studies of lncRNAs and circRNAs in the context of ncRNAs in ROP. Most lncRNA and circRNAs were reported to function as miRNA sponges and indirectly regulate the target of miRNA (Figure 2).

MicroRNAs regulating retinopathy of prematurity

Various miRNAs have been investigated for their effects on ROP (Table 1). The role of miR-18a-5p was studied in both *in vivo* and *in vitro* OIR model (Guan et al., 2020). miR-18a-5p was found to be upregulated in the OIR retina. Invitreal administration of the miR-18a-5p mimic, agomiR-18a-5p, significantly reduced the neovascular area in OIR mice retina. Similarly, agomiR-18a-5p-treated HRMECs showed reduced proliferation, migration, and tube formation. In addition, direct targets of miR-18a-5p, fibroblast growth factor 1 (FGF1) and HIF1α, were found. The mRNA expressions of FGF1 and HIF1α were significantly reduced after agomiR-18a-5p treatment in HRMECs. This study revealed that miR-18a-5p regulates pathological angiogenesis by targeting HIF1α and FGF1 in the OIR model.

The effect of miR-34a on retinal angiogenesis was studied in OIR *in vitro* and *in vivo* model (Shi et al., 2019). Previously, miR-34a was reported to inhibit tumor angiogenesis in endothelial cells, and the neurogenic locus notch homolog protein 1 (Notch1) pathway has been reported to play an important role in vascular endothelial growth factor (VEGF)-treated angiogenesis (Liu et al., 2003; Kumar et al., 2012). Therefore, the potential relationship of miR-34a and Notch1 in retinal angiogenesis was also investigated. miR-34a was downregulated, whereas Notch1 was upregulated in the *in vivo* OIR rat models. Similarly, administration of miR-34a mimic significantly reduced the expression of Notch1 in VEGF-induced HRMECs. Additionally, silencing of Notch1 significantly suppressed proliferation, migration, and tube formation in VEGF-induced HRMECs. Thus, it was shown that miR-34a reduces retinal neovascularization through inhibition of Notch1.

The role of miR-96 was investigated by Desjarlais et al. (2022). Expression of miR-96 was found to be downregulated in OIR rats and hyperoxia-induced HRMECs. Moreover, miR-96 mimics upregulated pro-angiogenic factors, such as VEGF, Angiopoietin-2, and FGF2, whereas antagomiR-96 inhibited these factors. Additionally, intravitreal injection of miR-96 mimic before hyperoxia significantly suppressed the vessel loss, which suggests that miR-96 has vaso-protective properties.

miR-145 was found to play a significant role in the regulation of endothelial cells during pathological angiogenesis (Liu et al., 2019). The investigators revealed that miR-145 directly targets tropomodulin3 (TMOD3), an actin-capping protein. The expression of miR-145 was upregulated, whereas the expression of TMOD3 was downregulated in the retinas of the OIR mice model and hypoxia-treated HRMECs. The function of miR-145 was investigated in an *in vitro* and *in vivo* OIR model. miR-145 inhibitor reduced RNV, whereas miR-145 mimic promoted retinal angiogenesis in HRMECs. Administration of miR-145 mimic reduced expression of TMOD3 and altered the structure of actin and endothelial cells. Additionally, either miR-145 inhibitor or miR-145 inhibitor along with small interfering RNA (siRNA) of TMOD3 was administered intravitreally in the eyes of OIR mice. Compared to the control group, the mice group injected with miR-145 inhibitor showed a significant reduction in neovascular area. However, when TMOD3 was suppressed along with miR-145, the neovascular region increased significantly. These results demonstrated that miR-145 regulates TMOD3 and revealed the role of the miR-145/TMOD3 axis in pathological RNV of the OIR model.

miR-150 was reported to involve in the pathological RNV (Liu et al., 2015). The authors found that expression of miR-150 was reduced in the retinal vessels of OIR mice. miR-150 mimic significantly inhibited proliferation, migration, and tube formation in HRMECs and the neovascular region in an OIR *in vivo* model. Targets of miR-150, C-X-C chemokine receptor type 4 (CXCR4), delta-like ligand 4 (DLL4), and frizzled class receptor 4 (FZD4) were identified by using the seed region sequence of miR-150. The miR-150 mimics were shown to inhibit the expression of CXCR4, DLL4, and FZD4 in HRMECs. Moreover, compared to the control, significant enlargement of the sprouting area of the aortic ring and choroid was detected in *ex vivo* experiments of miR-150 knockout mice. This study revealed the anti-angiogenic role of miR-150 in retinal neovascularization and downstream target genes of miR-150.

The role of miR-181a-5p in RNV was investigated in an OIR model (Chen et al., 2020). Previously, endocan was found to regulate the expression of proangiogenic factors, such as VEGF-A and VEGF-C, and be involved in cell activation and angiogenesis of endothelial cells. Endocan was upregulated in an *in vivo* experiment using OIR mouse model retinas. Suppression of endocan reduced survival, proliferation and tube formation in VEGF-treated HRECs and the neovascular area in OIR mice retinas. Additionally, the target of endocan, miR-181a-5p, was predicted using bioinformatics analysis and verified through the use of a luciferase assay. miR-181a-5p mimic inhibited proliferation, tube formation, survival in VEGF-treated

HRECs. Moreover, overexpression of miR-181a-5p reduced the neovascular area in OIR mice retinas. Although the authors found the anti-angiogenic function of miR-181a-5p and miR-181a-5p/endocan regulatory axis, the effects of miR-181a-5p on other angiogenic pathways remain to be discovered in further research.

In a study by Li et al. (2022) the target of angiogenin (ANG) and brain-derived neurotrophic factor (BDNF) was revealed to be miR-182-5p. ANG was shown to be a pro-angiogenic factor that accelerates cell growth and endothelial tube formation (Miyake et al., 2015). Additionally, BDNF was found to promote migration and angiogenesis in endothelial cells (Matsuda et al., 2012). Through bioinformatics, the authors predicted that miR-182-5p was a potential target of ANG and BDNF. The expression level of miR-182-5p was downregulated, whereas the expressions of ANG and BDNF mRNA were upregulated in the retinas of OIR mice and hypoxia-induced HRMECs. In addition, when miR-182-5p mimic was introduced, the expression of ANG and BDNF was reduced in hypoxia-induced HRMECs. Compared to the scramble group, the miR-182-5p mimic group showed decreased cell migration and increased cell viability and tube formation in hypoxia-induced HRMECs (Li et al., 2022). Thus, the authors discovered that miR-182-5p, ANG, and BDNF can be potential targets to treat RNV.

Long noncoding RNAs regulating retinopathy of prematurity

Several lncRNAs have been investigated in terms of their role in ROP (Table 2). The pro-angiogenic role of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was revealed in two studies (Wang et al., 2020; Xia et al., 2021). Wang et al. reported that lncRNA MALAT1 expression was upregulated in OIR mice. Compared to the control, inhibition of MALAT1 reduced RNV and suppressed CCN1/Akt/VEGF pathway and inflammatory cytokines, including IL-1β, IL-6, and TNF-α, during hyperoxia (Wang et al., 2020). These results suggest that the inhibition of lncRNA MALAT1 may reduce the progression of ROP. In addition, Xia et al. revealed that lncRNA MALAT1 can act as an miR-124-3p sponge and modulate early growth response 1 (EGR1) (Xia et al., 2021). The expression levels of miRNAs, lncRNAs, and mRNAs in an OIR mice model were evaluated in the microarray. miR-124-3p, a significantly downregulated miRNA in microarray, was selected for further study. As shown in the microarray, expression of miR-124-3p expression was significantly reduced in a hypoxia-induced *in vitro* model. The addition of miR-124-3p inhibited proliferation and migration, whereas suppression of miR-124-3p promoted proliferation and migration of hypoxia-treated HUVECs. Through bioinformatics analysis, the interacting partners of miR-124-3p, lncRNA MALAT1, and EGR1 were predicted. The expression of EGR1 and lncRNA MALAT1 was upregulated in hypoxia-treated HUVECs and retinas of OIR mice. Overexpression of miR-124-3p or inhibition of MALAT1 also suppressed EGR1 in hypoxia-induced HUVECs. Thus, the results of the study revealed the novel regulatory axis of lncRNA MALAT1/miR-124-3p/EGR1 in OIR *in vitro* and *in vivo* models.

The role of the maternally expressed gene 3 (MEG3) in ROP was revealed by Di et al. (2022). Intravitreal injection of MEG3 overexpressing lentivirus reduced retinal angiogenesis *via* VEGF/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway and suppressed inflammatory markers, such as IL-1β and IL-6 in OIR mice. Additionally, this research group also investigated the effect of lncRNA myocardial infarction-associated transcript (MIAT) in the OIR mice model (Di et al., 2021). The investigators found that silencing lncRNA MIAT by administering intravitreal injection suppressed retinal angiogenesis through downregulation of the VEGF/PI3K/Akt pathway. Thus, lncRNA MEG3 or MIAT may be a promising therapeutic target to treat ROP.

Lastly, Wang et al. explored the function of lncRNA TUG1 in retinal angiogenesis (Wang et al., 2022). Previously, lncRNA TUG1 was studied in various cancer; however, its role in retinal angiogenesis was not investigated. The authors found that the expression of TUG1 was upregulated in the retinas of OIR mice, whereas the expression of miR-299-3p was downregulated. They showed that knockdown of lncRNA TUG1 reduced RNV, apoptosis, inflammation, and the level of angiogenesis markers such as VEGF-A in OIR mice retinas. Moreover, lncRNA TUG1 was found to act as a miR-299-3p sponge and modulate VEGF-A. Overexpression of miR-299 inhibited VEGF and TUG1 and reduced tube formation, migration, and apoptosis in $CoCl_2$-treated HRECs.

Circular RNAs regulating retinopathy of prematurity

There are a few studies in which the role of circRNA in ROP has been investigated (Table 2) (Liu et al., 2017; Zhou et al., 2019; Deng et al., 2020). Deng et al. reported that the expression of circPDE4B was decreased in hypoxia-treated HRECs and retinas of OIR mice (Deng et al., 2020). Overexpression of circPDE4B inhibited the expression of angiogenic factors, such as HIF1α and VEGF-A, cell proliferation, and vascular tube formation *in vitro*. Moreover, the authors found that circPDE4B works as a miR-181c sponge and modulates von Hippel-Lindau (VHL). This study revealed the anti-angiogenic function of circPDE4B and found that circPDE4B/miR-181c/VHL regulatory axis regulates ROP.

Liu et al. investigated the function of circZNF609 (Liu et al., 2017). The expression of circZNF609 was upregulated during

hypoxia. Inhibition of circZNF609 promoted cell viability, migration, and tube formation and suppressed RNV in OIR mice model. Using bioinformatics databases, the investigators predicted miR-615-5p would interact with circZNF609. CircZNF609 was verified to act as a miR-615-5p sponge in H_2O_2-treated HUVECs. Subsequently, the downstream target of miR-615-5p, Myocyte Enhancer Factor 2A (MEF2A), was predicted using a bioinformatic database. Overexpression of MEF2A reduced cell migration and tube formation promoted by inhibition of circZNF609. Therefore, circZNF609/miR-615-5p/MEF2A axis was revealed to regulate vascular endothelial cell function.

CircRNA profiles of retinas from OIR and normal mice were analyzed by Zhou et al., 2019. They revealed differentially expressed circRNA, miRNA, and mRNA in the OIR mice model. Based on gene ontology analysis, angiogenesis was found to be one of the more prevalent biological processes. The potential of circRNA acting as competing endogenous RNA (ceRNA) was predicted using a bioinformatics database, miRanda. The levels of expression of selected circRNA, miRNA, and mRNAs were verified using RT-qPCR, suggesting that various circRNA-miRNA-mRNA regulatory axes may be involved in the progression of ROP.

Strategies to modulate ncRNAs

By overexpressing or inhibiting ncRNAs, the progression and severity of ROP can be modulated. miRNA can be overexpressed by using miRNA mimics or microRNA expression vectors. miRNA mimic, a synthetic miRNA with the identical sequence as an endogenous miRNA, can be used to upregulate expression of miRNA. Double-stranded miRNA mimic is processed to single-stranded miRNA inside RNA-induced silencing complex (RISC) and subsequently inhibit target mRNA (van Rooij and Kauppinen, 2014). Several miRNA mimics have been investigated in clinical trials. For example, MRX34, the miR-34a mimic, was investigated in phase 1 clinical trials with liver cancer (Beg et al., 2017; Hong et al., 2020). miRNA expression vectors are promoter-containing vectors designed to express miRNAs of interest (Ling et al., 2013). For instance, miR-26a expression vector was used to inhibit the progression of cancer in the *in vitro* and *in vivo* hepatocellular carcinoma model (Kota et al., 2009). On the other hand, miRNAs can be suppressed by anti-miR or miRNA sponge (Ling et al., 2013). Anti-miR is an antisense oligonucleotide that inhibit target miRNAs and has a partially or fully complementary sequence to its target endogenous miRNA. Miravirsen, miR-122 anti-miR, was tested for the treatment of hepatitis C virus infection in phase 2 clinical trial (Janssen et al., 2013). miRNA sponge vectors are designed to contain multiple complementary sequence sites of single or multiple miRNAs of interest (Ebert et al., 2007; Chang, 2018). For instance, the miR-122 sponge vector reduced miR-122 in liver and effectively inhibited cholesterol level for 25 weeks in miR-122 sponge vector-injected mice (Xie et al., 2012). Furthermore, backbone or sugars of miRNA mimics or miRNA inhibitors can be modified to enhance stability (Baumann and Winkler, 2014).

LncRNAs can be overexpressed by constructing lncRNA overexpression plasmids. For instance, promoter region of lncRNAs can be combined with CRISPR activator complex to upregulate transcription of lncRNA (Dominguez et al., 2016; Lim et al., 2020). Strategies to inhibit lncRNA vary depending on localization. Nuclear lncRNAs can be suppressed by antisense oligonucleotides *via* degradation through RNase H. On the other hand, cytoplasmic lncRNAs can be suppressed by siRNAs *via* RNA interference (Lennox and Behlke, 2016). In addition, CRISPR-Cas 13 system can be used to inhibit or degrade lncRNA (Cox et al., 2017; Zhang et al., 2020). CircRNA can be upregulated using the circRNA overexpression vector through induction of backsplicing (Kramer et al., 2015). On the contrary, circRNAs can be downregulated by siRNAs, given that most circRNAs are enriched in the cytoplasm (Jeck et al., 2013).

Future perspective on ncRNA therapy

Recently, RNA therapies have received attention, and several ncRNA therapeutics are under clinical trials (Huang et al., 2020; Winkle et al., 2021). Various ncRNAs have been investigated for their uses in diagnosis and treatment. To date, miRNA therapeutics have been explored in various diseases, including hepatitis C virus infection and cancer, and have undergone clinical trials (Janssen et al., 2013; Beg et al., 2017; van der Ree et al., 2017). Currently, there are few pharmacological options for the treatment of ROP, and laser therapy can be invasive and increase the risk of myopia and other undesirable ocular outcomes; thus, it is worth finding novel therapeutic targets (Ryu, 2022). Compared to conventional therapy, ncRNA therapy can be effective against targets that have been unresponsive to the currently available drugs and the stability of RNA therapy can be enhanced by using carriers, such as liposomes (Ozpolat et al., 2014; Damase et al., 2021). Additionally, RNA therapy does not cause gene alteration. Because ROP occurs in preterm infants, the incidence is low compared to other retinal diseases such as diabetic retinopathy, it may not be a promising research and development target for pharmaceutical companies. Thus, RNA therapy is a reasonable treatment option in ROP, especially given that it is less expensive to develop than conventional therapy.

Conclusion

Although laser therapy and anti-VEGF agents have been used to treat ROP, the ROP incidence has increased and current therapies pose risks due to invasive methods and lack of data on long-term safety issues and dosage. Recently, RNA therapy has been extensively investigated in diverse diseases and has shown potential as a novel therapy. Among the different types of RNAs, ncRNAs have been investigated as emerging therapeutics in many diseases. In this article, we discussed the role of ncRNAs, including miRNAs, lncRNAs, and circRNAs, that have been investigated in the context of ROP. Because ROP can be regulated through overexpression or inhibition of ncRNAs, modulation of ncRNA can be a novel therapeutic approach to treat ROP.

Author contributions

HK and JK. collected the available literature. HK, JK, and JR. analyzed and interpreted the literature. HK, JK, and JR. wrote the first draft. JR revised the manuscript. JR acquired funding.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparison of seven cyclosporine A formulations for dry eye disease: A systematic review and network meta-analysis

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Background: Dry eye disease is a common ocular surface disease affecting tens of millions of people worldwide. It is characterized by an unstable tear film and increasing prevalence. Different commercial formulations of cyclosporine A for dry eye have been approved, however, it is still unclear whether the differences in formulations of these products will make a difference in clinical efficacy and safety.

Methods: Randomized controlled trials of commercial cyclosporine A formulation for dry eye disease were searched in Pubmed, EMBASE, Scopus, and Cochrane controlled trials registries and Web of Science from inception till 1 December 2021. Independent literature screening, data extraction, quality evaluation, and the study in line with quality standards were analyzed by using Stata16.0 software. The study is registered with PROSPERO under the number CRD42022301423. Code and data for this study is publicly available (https://github.com/DongYangGao/Dongyang.github.io.git).

Results: 21 randomized clinical trials with a total of 4,107 participants were included in this study. Restasis® (OR-4.82, 95% CI-6.18 to 3.45, SUCRA 77.2%) was the most effective commercial formulation for reducing OSDI, Zirun® (SUCRA 73.9%) performed better in improving Schirmer's test. TJ Cyporin® (SUCRA 65.3%) ranked first in terms of improving tear film break-up time. For treatment-emergent adverse events incidence, Clacier® was close to placebo. The risk of reporting bias is considered low.

Conclusion: In the comparison of outcomes included in this study, the optimal order of various commercial cyclosporine A formulations is different, so it is difficult to select the optimal formula. Appropriate commercial formulations should be selected according to patients' conditions in clinical practice.

KEYWORDS

dry eye disease, cyclosporine, commercial formulae, network meta-analysis, meta-analysis

Introduction

Dry eye disease (DED), also known as keratoconjunctivitis sicca, is one of the common ocular surface diseases affecting tens of millions of people worldwide (Craig et al., 2017a; Stapleton et al., 2017; Agarwal et al., 2021). Globally, the prevalence of DED in adults is 5%–50% (Stapleton et al., 2017). Changes in the function of the lipid layer on the surface of the eyeball and the quality and/or quantity of tears lead to instability of the tear film, which is an important sign of DED and is often accompanied by ocular irritation, visual impairment, pain or burning (Aragona et al., 2021; Chennakesavalu et al., 2021). Hormonal changes, gender, age, lifestyle, surgical procedures and wearing of contact lenses are related to the onset and deterioration of dry eye (Willcox et al., 2017; Clayton, 2018). DED affects patients' visual function and quality of life, resulting in increased medical costs and reduced work efficiency, with significant social and economic impacts (Mcdonald et al., 2016; Craig et al., 2017b; Wolffsohn et al., 2017). TFOS DEWS II Pathophysiology Subcommittee proposed that the main mechanism of DED pathophysiology is the vicious inflammatory cycle (Bron et al., 2017). Evaporation and water loss lead to hyperosmolar tissue damage, decreased moisture and humidity on the surface of the eye lead to tear film break up, the instability and hyperosmolar then cause inflammation, malignant inflammatory cycle drives the interaction between the local immune system of the eye and intraocular sensory nerve, causing nerve paresthesia, and the homeostasis of the eye is destroyed and continued circulation (Chen et al., 2010; Belmonte et al., 2017; Yamaguchi, 2018).

Blocking the chronic malignant inflammatory cycle and rebuilding and maintaining the homeostasis of the ocular surface should be the ultimate goal of DED treatment (Baudouin et al., 2016). Topical corticosteroids and cyclosporine should be used for patients with the inefficacy of artificial tears or moderate and severe DED (Beckman et al., 2020; Gupta et al., 2020). Dozens of studies showed that long-term external use of corticosteroids may lead to the risk of ocular hypertension, glaucoma, and cataract (Utine et al., 2010; Agarwal and Rupenthal, 2016; Jones et al., 2017), while preferred immune modulator local cyclosporine A (CsA) could target the chronic inflammatory cycle (Periman et al., 2020) and deal with different underlying pathologic conditions with almost no systemic effect (Pflugfelder, 2004; Baudouin et al., 2016) (Figure 1). CsA is recommended for long-term management of dry eye syndrome.

Restasis® (Allergan, Inc., Irvine, CA, United States), the first commercial topical cyclosporine A ophthalmic emulsion, was approved by the US Food and Drug Administration (FDA) for the treatment of DED in 2002 and has achieved convincing efficacy (Tatlipinar and Akpek, 2005; Bataoel, 2007; Wan et al., 2015). Even so, cyclosporine A is lipophilic and castor oil is used as a solvent, resulting in poor tolerance and low bioavailability

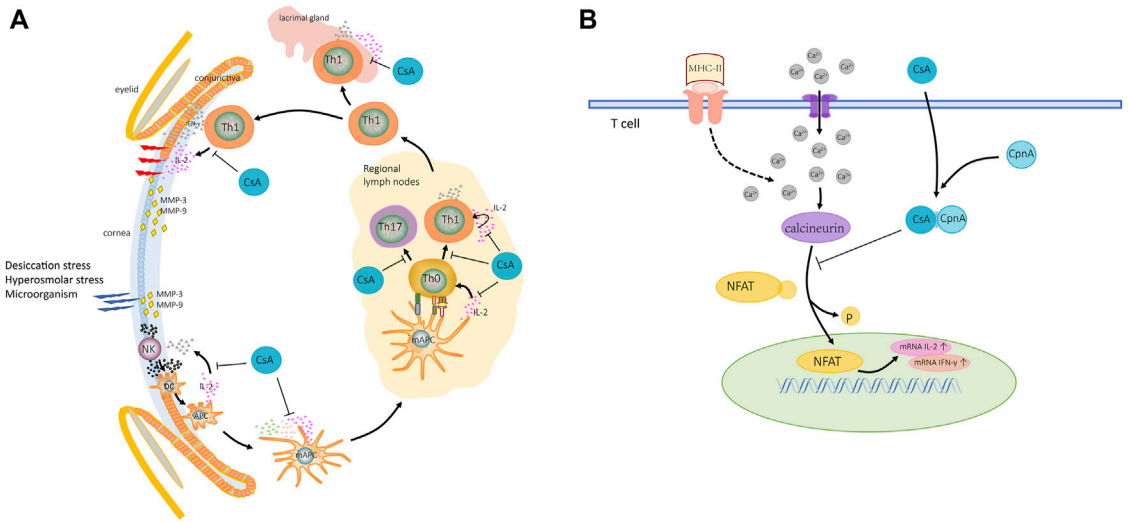

FIGURE 1
Mechanism of action of CsA for DED. **(A)** The pathogenic factor triggers the ocular surface immune response. In innate immune response, CsA inhibits maturation of DCs and activation and differentiation of T-cell. In adaptive immunity, CsA inhibits secretion of IL-2 from T-cells, reduces the proliferation and infiltration of immune cells in tissues, and the production of MMPs. **(B)** Activated T-cells increased calcium ions in cytoplasm, calcineurin activation, NFAT dephosphorylation, and increased IL-2 and IFN-γ expression. CsA binds CpnA to form complexes, which in combination with calcineurin inhibits the expression of inflammatory factors. Abbreviations: DCs, dendritic cells; NK, natural killer cell; APC, antigen presenting cell; mAPC, mature antigen presenting cell; Th0, naive T-cell; IL-2, interleukin 2; INF-γ, interferon gamma; MMPs, matrix metalloproteinases; TCR, T-cell receptor; NFAT, nuclear factor of activated T-cells; P, phosphorylated; CpnA, cyclophilin A.

TABLE 1 Basic information of seven commercial cyclosporine A products.

Trade name	The company	Approval time	Approval agency	Formula features	Cyclosporine content (%)
Restasis® Tuan et al. (2020)	Allergan Inc., Irvine, CA, United States	2002	FDA	Anionic turbid oil-in-water emulsion	0.05
TJ Cyporin® Park et al. (2019)	Taejoon Pharmaceutical Co., Seoul, Korea	2003	MFDS	Nanoemulsion	0.05
Ikervis® Leonardi et al. (2016)	Santen Pharmaceuticals Co., Ltd., Osaka, Japan	2015	EMA	Cationic emulsion	0.1
Clacier® Kim et al. (2017)	Huons Co., Seongnam, Korea	2016	MFDS	Transparent nanoemulsion with uniform particle size not more than 50 nm	0.05
Cequa® Tauber et al. (2018)	Sun Pharmaceutical Industries, Cranbury, NJ, United States	2018	FDA	Nanomicellar, clear aqueous solution	0.09
Zirun® Chen et al. (2019)	Sinqi Pharmaceutical, Shenyang, China	2020	NMPA	Emulsion	0.05
CyclASol® Zhou and WEI (2014)	Novaliq GmbH, Heidelberg, Germany	2022	NDA	SFA-based nonaqueous preservative-free solution	0.1

FDA, Food and Drug Administration; MFDS, Ministry of Food and Drug Safety; EMA, European Medicines Agency; NMPA, National Medical Products Administration; NDA, New Drug Application; SFA, semifluorinated alkanes.

(Lallemand et al., 2017; Periman et al., 2020). The need to improve CsA delivery systems has increased in recent years due to the low bioavailability of Restasis®, thus, new commercialized registrations apply new technologies and formulations (Periman et al., 2020) such as TJ Cyporin® (which uses nanoemulsion technology to improve bioavailability) (Park et al., 2019; Kang et al., 2020), Ikervis® (which is a cationic nanoemulsion formulation) (Leonardi et al., 2016; Baudouin et al., 2017; Lallemand et al., 2017; Agarwal et al., 2018), Clacier® (which is a transparent nanoemulsion with particle size not exceeding 50 nm) (Kim et al., 2017), Cequa® (which is a transparent aqueous nanomicelle preparation) (Vaishya et al., 2014; Tauber et al., 2018; Goldberg et al., 2019), Zirun® (Chen et al., 2019) and CyclASol® (Gehlsen et al., 2017; Sheppard et al., 2021). The basic information of the seven commercial CsA products is shown in Table 1. Previous studies or reviews (Zhou and WEI, 2014; Wan et al., 2015; Tuan et al., 2020) using paired (head-to-head) comparisons to compare two different formulations of commercial dosage forms, however it is not clear whether the discrepancy in the formulations of these seven products makes a difference in clinical efficacy and safety.

The aim of the study was to compare and rank the effectiveness and safety of different cyclosporine A formulations for the treatment of dry eye using existing datasets (Rochwerg et al., 2018). We then designed and conducted a network meta-analysis (Huang et al., 2016), which combines direct and indirect evidence to compare multiple interventions at the same time in the presence of high-quality placebo-controlled trials (Gao et al., 2021) to increase the accuracy of results to guide clinical practice (Cipriani et al., 2018).

Methods

Search strategy

Pubmed, EMBASE, Scopus, and Cochrane Controlled Trials Registries and Web of Science for all potential RCTs were searched. Additionally, ClinicalTrials.gov was searched for unpublished trials. The search period is from inception of these libraries up till 1 December 2021 with no restrictions on source or language. Keywords (MeSH in PubMed and Emtree in Embase) and free words are used for retrieval: 1) Dry Eye Syndrome, Dry Eye Disease, Dry Eye, Evaporative Dry Eye Disease, Evaporative Dry Eye Syndrome; 2) Cyclosporine, Cyclosporine A, Cyclosporin A, Ciclosporin, Restasis, Ikervis, Clacier, Cequa, OTX-101, Zirun, TJ Cyporin, Cyporin N, Cyclosporine Nanoemulsion, CyclASol, Cyclosporine A cationic emulsion, 0.1% Cyclosporine, 0.05% Cyclosporine, 0.09% Cyclosporine; 3) Randomized controlled trial, randomized, placebo. Heading terms AND free words in each group are linked by "OR", AND three groups are combined by "AND". The complete search strings for all databases retrieved are provided in Supplementary Table S2.

Inclusion and exclusion criteria

According to our objective, retrieved articles that meet the following criteria will be included in the meta-analysis: 1) Study design: all randomized controlled studies (RCTs) that compare commercial CsA with placebo or vehicle for the treatment of dry eye, and have access to complete data. 2) Participants: All patients clinically diagnosed with DED were

not limited by age, region, gender, race, or other factors. 3) Type of intervention: The intervention in the experimental group was topical with different types of commercial CsA with or without artificial tears and placebo. 4) Type of comparison: The control group could be treated with artificial tears, excipients, or placebo in addition to CsA. 5) Outcome of dry eye intervention, such as OSDI score, Schirmer's test (ST) with or without anesthesia on, tear film break-up time (BUT), and Treatment-emergent adverse events (TEAEs).

Studies were excluded if they met one of the following criteria: 1) observational studies, non-randomized controlled trials, and real-world studies. 2) All animal studies and cadaver studies. 3) All reviews, letters, case reports, conference summaries or records, systematic reviews, and meta-analyses. 4) Low-quality studies were assessed according to the Cochrane Manual. 5) The outcome data could not be extracted, nor could they be calculated according to the graphs in the article, or the studies obtained by contacting the authors.

Data extraction

Two reviewers (GDY and DZL) extracted independently from the full text of the studies that met the screening criteria. After re-checking with Endnote X9 for Windows (Thomson Reuters, United States) literature management software, the preliminary screening was completed by reading the titles and abstracts, and the full text of potential studies was read to determine whether to include them. If necessary, the authors of the original study can be contacted by email or phone to obtain information of critical importance. All information was independently extracted into a Microsoft Excel spreadsheet, including, if any, country of origin, first author, year of publication, study type, a sample size of patients included, diagnostic criteria, interventions, outcome measures, and baseline information and outcome data were extracted into a standardized form. Results are checked back-to-back and any discrepancies can be resolved by referring to the original study or consulting a third reviewer (SYY).

Risk of bias assessment

Two reviewers (GDY and DZL) performed independent quality evaluations of the included studies, and the Cochrane Collaboration Risk of Bias tool (Higgins et al., 2011) was used to assess: Random sequence generation, allocation hiding, blinding of participants and personnel, blinding of outcome evaluation, incomplete outcome data, selective reporting, and other biases. Each study is assessed as low risk, high risk, or unclear risk. Any differences are resolved through discussion or consultation with a third independent examiner (SYY).

Statistical analysis

Our network meta-analysis was designed and conducted by NMA's Systematic evaluation and The Preferred Report Project (PRISMA) Reporting Guidelines for Meta-Analysis (Hutton et al., 2015) (Supplementary Table S1). Our team registered the master agreement on PROSPERO, with the registration number CRD42022301423. The method described in this study was accomplished using Stata 16.0 Software, and the data and code for the analysis can be accessed from our Giuhub Repositories (https://github.com/DongYangGao/Dongyang.github.io.git).

Odds ratio (OR) was used as effect size and 95% confidence interval (CI) was calculated. Stata 16.0 software network group command data preprocessing. The inconsistency test is mainly used to evaluate the difference between direct and indirect comparison results. When there is a closed ring, the consistency test is carried out by the node analysis method, if $p > 0.05$, indicating good consistency, the consistency model was used for analysis; otherwise, the inconsistency model was used for analysis. A network diagram of different outcome indicators was drawn for comparison between different cyclosporine A products. Dot area represented the number of clinical trial participants using the product, and the thickness of the line between dots represented the number of included studies (Salanti et al., 2011). The surface under cumulative ranking (SUCRA) represents the overall probability that an intervention is one of the best treatments based on the ranking of all interventions. SUCRA is expressed as a percentage. When SUCRA is 100%, intervention is effective; when SUCRA is 0, intervention is ineffective (Cope and JANSEN, 2013; Shim et al., 2017). Finally, a funnel plot is used to identify the existence of a small sample effect.

Results

Literature retrieval and inclusion features

A total of 1,528 articles were retrieved from the electronic database, 512 duplicate studies were deleted, and 971 articles were excluded after reading titles and abstracts. After reading the full text of the 45 articles, 24 of the studies were excluded according to exclusion criteria, such as seven studies that did not meet the criteria that "controls should be treated with artificial tears, excipients, or placebo." Finally, 21 eligible studies were included. The literature retrieval process (Page et al., 2021) is shown in Figure 2.

The 21 studies that were eventually included were published between 2000 and 2021 and were shown to have been conducted globally, with seven in Europe (including two in collaboration between the United States and Germany), eight in Asia, and six in the United States alone. A total of 4,107 participants were

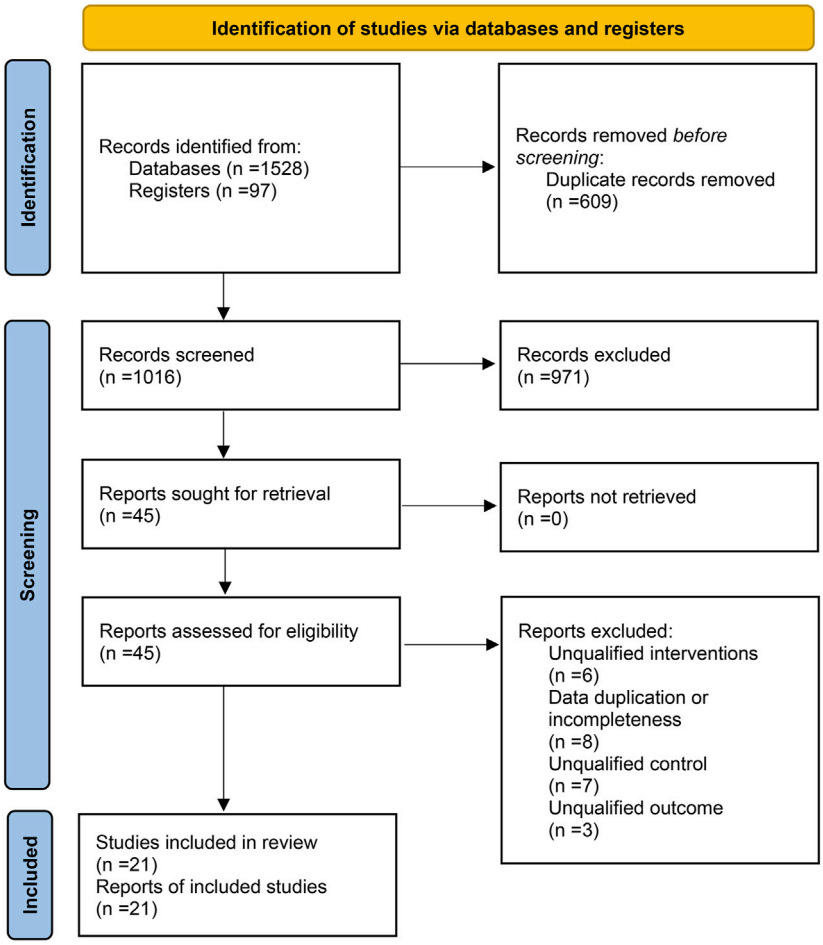

FIGURE 2
Literature retrieval process.

recruited and followed for 8 weeks to 6 months. All studies included adults older than 18 years of age. All studies included at least one outcome measure for comparison. Table 2 described characteristics of these included studies.

Risk assessment of bias

The risk of bias was assessed for 21 included studies (Figure 3). For selection bias, all included studies were randomized, but seven studies (Willen et al., 2008; Altiparmak et al., 2010; Demiryay et al., 2011; Leonardi et al., 2016; Baudouin et al., 2017; Tauber et al., 2018; Wirta et al., 2019) did not describe the specific generation method of random sequences. Eight studies (Perry et al., 2006; Guzey et al., 2009; Kim et al., 2009; Altiparmak et al., 2010; Demiryay et al., 2011; Leonardi et al., 2016; Baudouin et al., 2017; Kang et al., 2020) did not provide detailed information about allocation hiding methods, and were all unable to determine the choice bias and rated as "unclear risk." In terms of implementation bias and detection bias, three studies (Kim et al., 2009; Altiparmak et al., 2010; Demiryay et al., 2011) did not report the use of the blind method and were rated as "unclear risk," and two studies (Rao, 2010; Park et al., 2019) were rated as "high risk" because researchers were single-blind. All 21 studies were considered to have a low risk of loss of follow-up bias because the number of participants who dropped out of the study was reported, and all studies reported all outcome measures described in their respective methods, with no bias reported. None of the 21 studies described other bias in detail and was rated as "unclear risk."

TABLE 2 Basic features of the included studies.

Study	Year	Country	Interventions and control		Number of patients (baseline)	Mean age (SD)	Duration	Outcomes reported
Sall et al. (2020)	2000	United States	Restasis®	BID	293	58.7 (13.9)	6 months	③④
			Artificial tears	BID	292	59.9 (14.3)		
Stevenson et al. (2000)	2000	United States	Restasis®	BID	33	N/A	12 weeks	④
			Artificial tears	BID	31	N/A		
Perry et al. (2006)	2006	United States	Restasis®	BID	16	N/A	3 months	②③④
			Artificial tears	BID	17	N/A		
Willen et al. (2008)	2008	United States	Restasis®	BID	22	44.0 (12.6)	3 months	①②③
			Artificial tears	BID	22	42.2 (14.8)		
Kim et al. (2009)	2009	Korea	Restasis®	BID	50	41.3 (9.7)	3 months	②③④
			Artificial tears	QID	50	35.9 (8.5)		
Guzey et al. (2009)	2009	Turkey	Restasis®	BID	32	61.5 (6.9)	6 months	①②③
			Vehicle	BID	32	60.5 (8.2)		
Altiparmak et al. (2010)	2010	Turkey	Restasis®	BID	25	41.0 (1.1)	6 months	②③④
			Artificial tears	BID	48	40.9 (8.8)		
Chen et al. (2010)	2010	China	Restasis®	BID	116	46.6 (11.1)	8 weeks	②③④
			Vehicle	BID	117	46.0 (12.1)		
Rao (2010)	2010	China	Restasis®	BID	41	47.5 (5.9)	12 months	①②③④
			Artificial tears	BID	33	48.2 (6.3)		
Demiryay et al. (2011)	2011	Turkey	Restasis® +Artificial Tears	BID	22	46.6 (12.3)	4 months	②③④
			Artificial Tears	QID	20	44.3 (14.4)		
Prabhasawat et al. (2012)	2012	Thailand	Restasis®	BID	36	48.1 (13.9)	12 weeks	①②④
			Artificial tears	BID	34	55.0 (13.0)		
Kang et al. (2020)	2019	Korea	TJ Cyporin®	BID	18	55.1 (13.5)	12 weeks	①②③④
			Restasis®	BID	18	53.5 (9.7)		
Park et al. (2019)	2019	Korea	TJ Cyporin®	BID	58	N/A	12 weeks	①②③④
			Restasis®	BID	58	N/A		
Leonardi et al. (2016)	2016	9 European countries	Ikervis®	QD	154	60.8 (13.5)	6 months	①②③④
			Vehicle	QD	91	62.1 (11.8)		
Baudouin et al. (2017)	2017	6 European countries	Ikervis®	QD	241	57.6 (12.9)	6 months	②④
			Vehicle	QD	248	58.8 (12.7)		
Kim et al. (2017)	2017	Korea	Clacier®	BID	34	N/A	12 weeks	①②③④
			Restasis®	BID	39	N/A		
Tauber et al. (2018)	2018	United States	Cequa®	BID	152	59.2 (14.6)	12 weeks	④
			Vehicle	BID	152	59.3 (13.8)		
Goldberg et al. (2019)	2019	United States	Cequa®	BID	371	58.4 (14.1)	12 weeks	④
			Vehicle	BID	373	59.5 (14.7)		
Chen et al. (2019)	2019	China	Zirun®	BID	119	46.3 (12.5)	12 weeks	①②③④
			Vehicle	BID	115	45.0 (12.4)		
Wirta et al. (2019)	2019	The United States and Germany	CyclASol®	BID	51	64.3 (10.7)	16 weeks	①④
			Restasis®	BID	53	62.8 (11.9)		

(Continued on following page)

TABLE 2 (*Continued*) Basic features of the included studies.

Study	Year	Country	Interventions and control		Number of patients (baseline)	Mean age (SD)	Duration	Outcomes reported
			Vehicle	BID	52	61.3 (10.5)		
Sheppard et al. (2021)	2021	The United States and Germany	CyclASol®	BID	162	61.5 (13.6)	12 weeks	③④
			Vehicle	BID	166	61.3 (12.7)		

Vehicle (the same ophthalmic emulsion formulation without cyclosporine); N/A, data not available; ① Ocular surface disease index (OSDI) score; ② Schirmer's test (ST) with or without anesthesia; ③ Tear film break-up time (BUT); ④ Treatment-Emergent AEs (TEAEs).

Ocular surface disease index score change from baseline

Ten studies with a total of 1,090 participants reported changes in OSDI scores from baseline across eight treatments, as shown in Figure 4. The changes of Restasis® (OR-4.82, 95% CI-6.18 to −3.45) and CyclASol® (OR-3.40, 95% CI-4.94 to −1.86) from baseline were significantly lower than those of Placebo. Other comparisons found no significant difference. A league chart showing the relative impact of different formulations is shown in Table 3. The SUCRA probability ranking of all treatments with reduced OSDI score showed that Restasis® may be the most effective commercially available formulation. The ranking result of SUCRA probability from high to low is Restasis® > Zirun® > TJ Cyporin® > CyclASol® > Clacier® > Ikervis® > Placebo. The details are shown in Figure 5. The comparison adjustment funnel of OSDI score changes is shown in Figure 6, and no significant visual asymmetry is found.

Schirmer's test score changes

Fourteen studies with a total of 1,913 participants reported changes in ST scores involving seven treatments, as shown in Figure 4. There was no significant difference in baseline changes in ST scores between treatments. A league chart showing the relative effects of different treatments is shown in Table 3. The SUCRA probability ranking results for all treatments that improved ST scores showed that Zirun® was probably the most effective commercially available formulation, with the SUCRA probability ranking from high to low as Zirun® > Clacier® > Restasis® > Ikervis® > TJ Cyporin® > Placebo; The details are shown in Figure 5. The comparison adjustment funnel plot of ST score changes is shown in Figure 6. The funnel plot results show poor symmetry, suggesting that there may be a certain publication bias.

Tear film break-up time changes from baseline

Fifteen studies with a total of 1,881 participants reported the results of changes in BUT involving seven treatments, as shown in Figure 4. There was no significant difference in BUT among all comparisons. A league chart showing the relative impact of different formulations is shown in Table 4. The SUCRA probability ranking of all formulations that improved BUT scores showed that TJ Cyporin® was probably the most efficient commercial formulation, and the SUCRA probability ranking from high to low was TJ Cyporin® > Clacier® > Zirun® > Restasis® > Ikervis® > Placebo. The details are shown in Figure 5. The comparison adjustment funnel diagram of OSDI score changes is shown in Figure 6. The funnel diagram results show poor symmetry, suggesting that there may be a certain publication bias.

Treatment-emergent AEs

Nineteen studies with a total of 4,032 participants reported the results of TEAEs, involving eight treatments, as shown in Figure 4. The league chart of the relative effects of the treatments is shown in Table 4. Placebo (SUCRA, 82.7%) showed the lowest incidence of TEAEs compared to the other formulations except for Clacier®, and the difference was significant. There was no significant difference between Placebo (OR-0.02, 95% CI-1.04 to

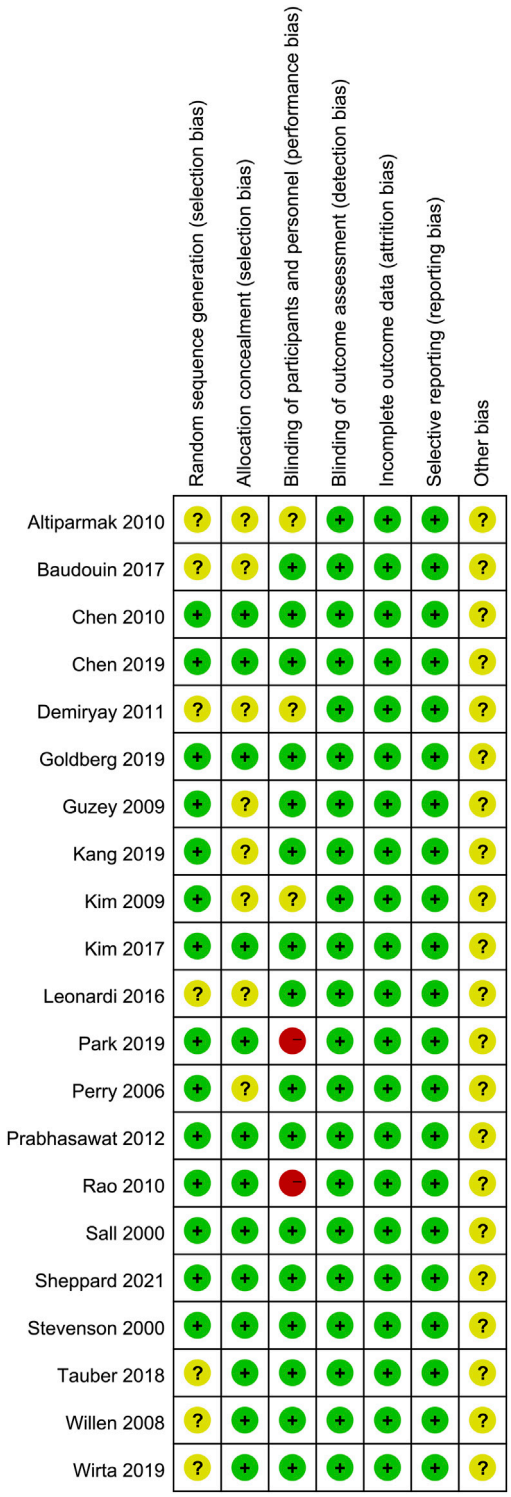

FIGURE 3
Literature bias risk assessment results.

1.01) and Clacier®. The SUCRA probability of TEAEs incidence in each treatment ranked from high to low as Placebo > Clacier® > CyclASol® > Zirun® > Cequa® > Restasis® > TJ Cyporin® > Ikervis® (Figure 5). The comparison adjustment funnel diagram of TEAEs is shown in Figure 6, and no significant visual asymmetry is found.

Discussion

To our knowledge, this is the first study to comprehensively compare the efficacy and safety of different commercial cyclosporine A formulations in the treatment of the dry eye. Previous systematic evaluations have shown that although local CsA can improve some objective and subjective outcomes of patients with dry eye, there will be an inconsistent improvement of outcome indicators and an increase in treatment-emergent AEs (Zhou and WEI, 2014; Wan et al., 2015; De Paiva et al., 2019; Tuan et al., 2020). To weigh the pros and cons of different types of commercial CsA and help clinicians make decisions, we compared different application strategies of direct or indirect evidence, using frequency theory framework network meta-analysis, screening of RCT, participants included 21 eligible studies, evaluated the four outcome indicators: OSDI score changes, ST score changes, (BUT) changes, treatment-emergent AEs (TEAEs) incidence. The ranking of all formulations and the accuracy of estimation was obtained (Dias et al., 2013).

Topical use of cyclosporine A is a highly effective treatment strategy for direct exposure to the surface of the eye. However, due to the low bioavailability of the eye for the sake of its good protective mechanisms (eye barrier, tear dilution, blinking and tear removal) (Davies, 2000; Gaudana et al., 2010), and the high lipophilic nature of CsA, the toxicity shown by the use of osmotic enhancers and surfactants in formulations and the discomfort caused by oil-based formulations (Cholkar et al., 2012; Rodriguez-Aller et al., 2013), formula reform is imperative. Currently, these products are only approved for marketing in some regions (Lallemand et al., 2017), and it is not clear whether the differences in formulations translate into differences in clinical efficacy and safety (Tong et al., 2020).

Our network meta-analysis of 4,107 participants showed that Restasis®, Zirun®, TJ Cyporin®, CyclASol®, Clacier®, and Ikervis® were more effective than placebo on three subjective and objective measures of effectiveness: OSDI score, ST, and BUT. Although Cequa® has completed phase 2/3 and Phase 3 trials, it could not be included because the outcome measure was the number of people who improved. Restasis® (OR-4.82, 95% CI-6.18 to 3.45, SUCRA 77.2%) was the most effective formulation for reducing OSDI, superior to other commercially available formulations, and the difference was significant. OSDI questionnaire evaluates subjective symptoms in patients with dry eye (Grubbs et al., 2014; Pult and WOLFFSOHN, 2019). Dryness and discomfort were the symptoms that scored highest on the questionnaire (Begley et al., 2002). Restasis® (Allergan Inc., Irvine, CA), the first commercial CsA emulsion, was used

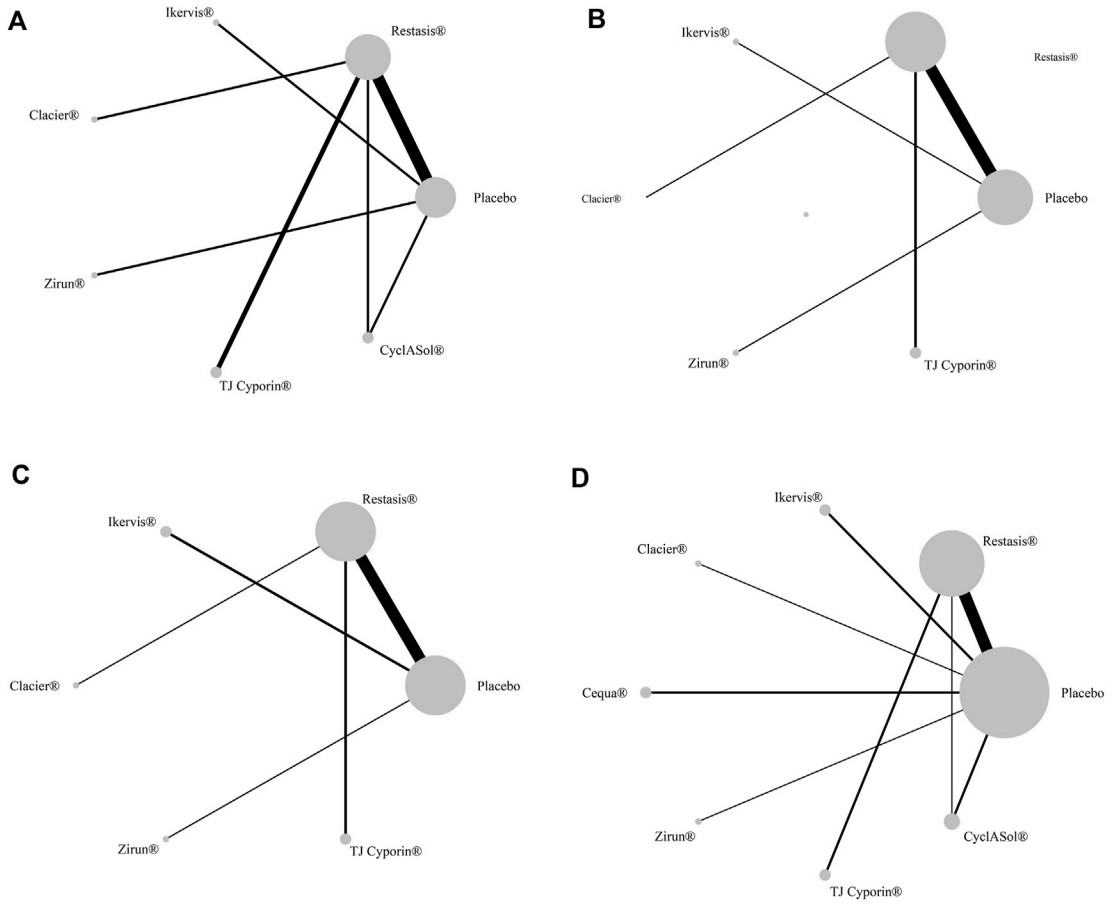

FIGURE 4
Network comparison of the four indicators. **(A)**: ocular surface disease index (OSDI) change from baseline; **(B)**: Schirmer's test (ST) change from baseline; **(C)**: tear film break-up time (BUT) change from baseline; **(D)**: Treatment-emergent AEs (TEAEs). The node size represents the sample size of intervention measures, and the line represents the number of RCTs between the two intervention measures.

TABLE 3 League table of results for OSDI and ST score change from baseline.

ST score change from baseline

OSDI score change from baseline	**Restasis®**	1.73 (−3.81, 7.27)	0.45 (−3.53, 4.42)	N/A	0.36 (−4.96, 5.68)	0.48 (−5.07, 6.03)	1.18 (−0.68, 3.04)
	0.51 (−7.07, 8.09)	**Zirun®**	2.18 (−4.64, 9.00)	N/A	1.37 (−6.31, 9.05)	2.21 (−5.18, 9.60)	2.91 (−2.31, 8.13)
	−0.32 (−6.45, 5.81)	−0.83 (−10.57, 8.91)	**TJ Cyporin®**	N/A	0.81 (−5.83, 7.45)	−0.03 (−6.86, 6.79)	0.73 (−3.65, 5.12)
	−1.42 (−2.96, 0.12)	−1.93 (−9.55, 5.68)	−1.10 (−7.41, 5.21)	**CyclASol®**	N/A	N/A	N/A
	−3.14 (−11.68, 5.40)	−3.65 (−15.07, 7.76)	−2.82 (−13.33, 7.69)	−1.72 (−10.39, 6.96)	**Clacier®**	0.84 (−6.85, 8.53)	1.54 (−4.10, 7.18)
	−3.72 (−9.18, 1.75)	−4.23 (−13.37, 4.91)	−3.40 (−11.60, 4.80)	−2.30 (−7.81, 3.21)	−0.58 (−10.71, 9.56)	**Ikervis®**	0.70 (−4.53, 5.93)
	−4.82 (−6.18,−3.45)	−5.33 (−12.79, 2.13)	−4.50 (−10.76, 1.77)	**−3.40 (−4.94, −1.86)**	−1.68 (−10.32, 6.97)	−1.10 (−6.39, 4.19)	**Placebo**

Each cell contains the odds ratio (OR) and 95% confidence interval for OSDI changes and ST changes; comparisons should be read from left to right. Bold numbers indicate statistically significant differences. OSDI score change from baseline, ST score change from baseline; N/A, data not available.

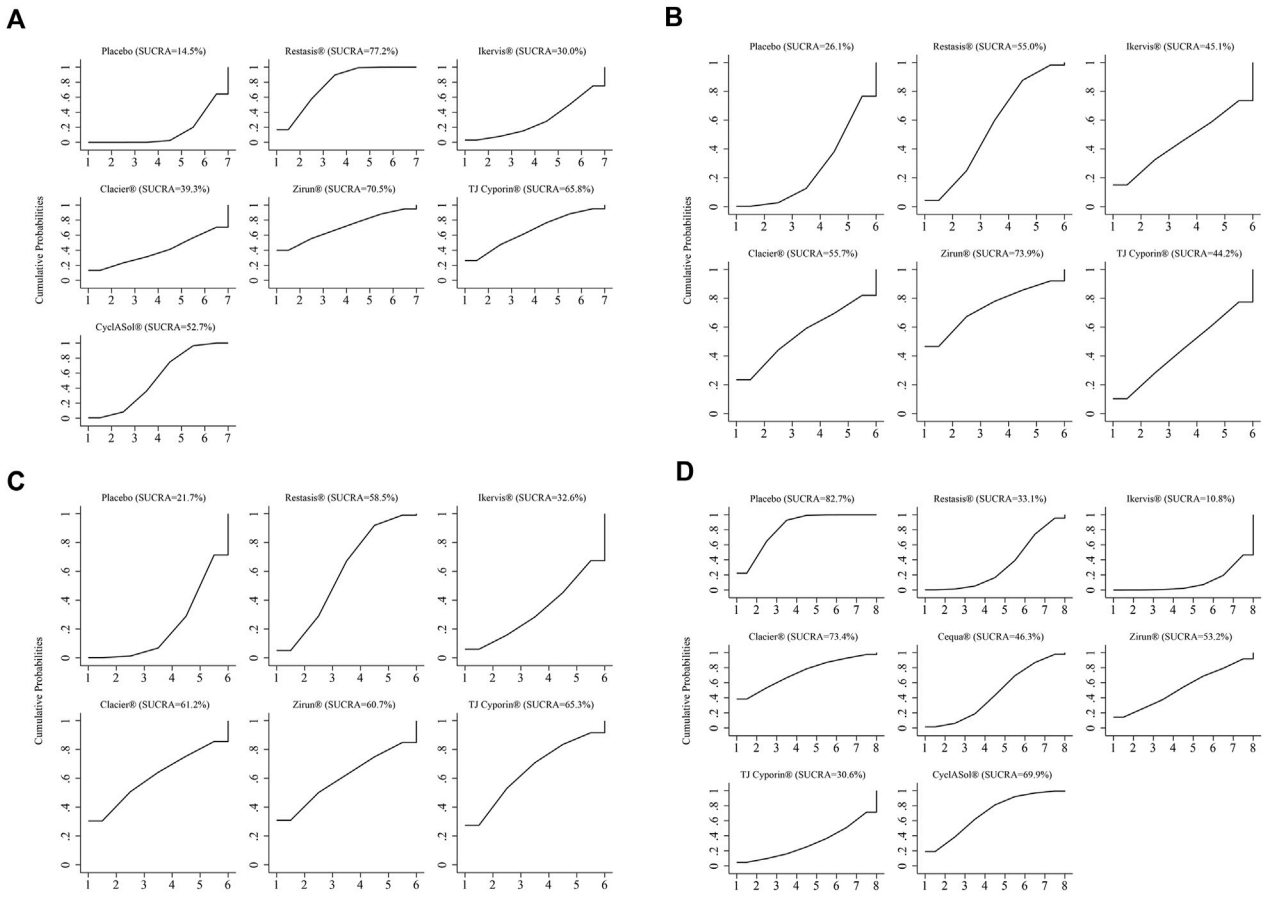

FIGURE 5
Surface plot under the cumulative ranking curve (SUCRA) of all outcome measures. **(A)**: ocular surface disease index (OSDI) change from baseline; **(B)**: Schirmer's teat (ST) change from baseline; **(C)**: tear film break-up time (BUT) change from baseline; **(D)**: Treatment-emergent AEs (TEAEs). A larger SUCRA score indicates that the intervention is more effective.

for the treatment of DED (Tatlipinar and Akpek, 2005). It is a preservative-free anionic oil-in-water nanoemulsion with castor oil as solvent, polysorbate 80 as an emulsifier, and carbomer copolymer as a stabilizer (Lallemand et al., 2017). The advantage of Restasis® in improving subjective symptoms is mainly due to the maturity of its preparation process, which is consistent with previous literature reports (Tong et al., 2020).

The application of new excipients (such as semi-fluorinated Alkanes) and the change of dosage form (like cationic emulsion and nano-micellar aqueous solution) are the main directions. Zirun®(Sinqi Pharmaceutical, Shenyang, China) uses new micelles as nanocarriers for drug delivery (Yu et al., 2018) and is an ophthalmic emulsion approved by NMPA in China in 2020 (Chen et al., 2019). Zirun® (SUCRA 73.9%) was the best choice for improving Schirmer's Test (ST). ST primarily assesses the secretion of basic tear and the function of the main lacrimal gland developed in 1903 (Erickson et al., 1958; Li et al., 2012). According to current information disclosed by Zirun®, the retention effect of the new micellar preparation in the eye is 4.5 times higher than that of the traditional cyclosporine A preparation (Yu et al., 2018), which may play A major role in repairing lacrimal gland function. Our study also shows that TJ Cyporin® (SUCRA 65.3%) ranked first in terms of improved BUT values. The dropper size is 20 nm–200 nm, with acceptable stability and bioavailability (Lallemand et al., 2017). Tear film instability may be the relative abnormality of the mucin/water layer attached to calyx glycose (Tsubota, 2018). Similar to previous reports, TJ Cyporin® has an obvious repair function on calyx glycose in previous reports (Kang et al., 2020), so it makes sense. For security indicator TEAEs, placebo was unquestionably the lowest. Our results also showed no difference in safety between Clacier® (SUCRA 73.4%) and placebo. All dosage forms have been reported to cause certain adverse reactions (Leonardi et al., 2015), but study with similar results have been analyzed that hydrophilic agent (ethylene oxide) used in Clacier® forms nano-emulsion with small and

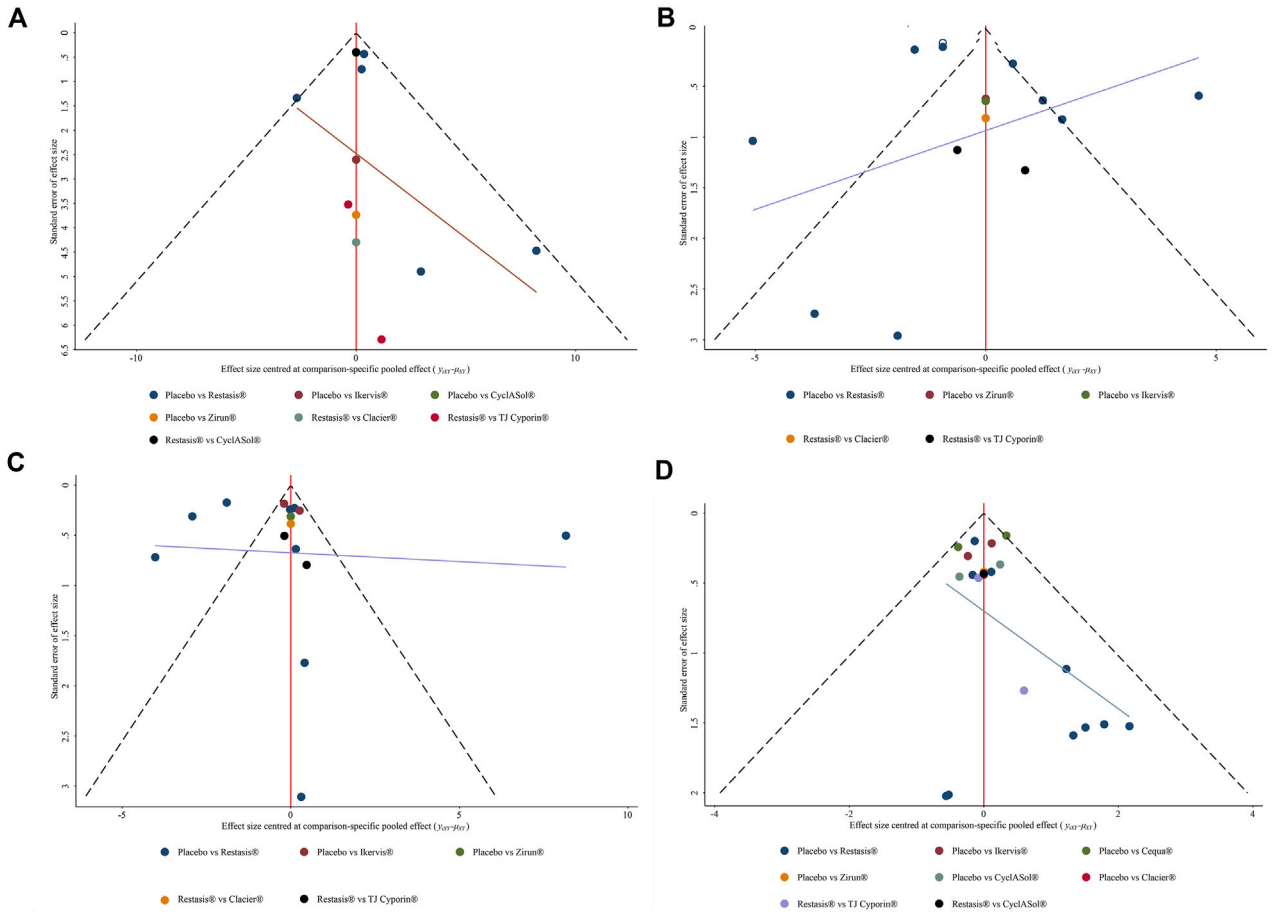

FIGURE 6
Comparison-adjusted funnel plot for all outcome measures. The red line represents the invalid hypothesis. Different colors correspond to different comparisons. **(A)**: ocular surface disease index (OSDI) change from baseline; **(B)**: Schirmer's test (ST) change from baseline; **(C)**: tear film break-up time (BUT) change from baseline; **(D)**: Treatment-emergent AEs (TEAEs).

TABLE 4 League table of results for BUT score change from baseline and TEAEs.

BUT change from baseline

Treatment-emergent AEs							
Placebo	−0.02 (−1.04, 1.01)	−0.13 (−0.78, 0.52)	−0.35 (−1.34, 0.64)	−0.44 (−0.92, 0.05)	−0.62 (−1.10,−0.14)	−0.74 (−1.84, 0.36)	−0.99 (−1.52,−0.46)
2.41 (−3.78, 8.60)	**Clacier®**	−0.12 (−1.33, 1.10)	−0.33 (−1.76, 1.09)	**−0.42 (−1.55, 0.71)**	**−0.60 (−1.74, 0.53)**	**−0.72 (−2.22, 0.78)**	**−0.98 (−2.13, 0.18)**
N/A	N/A	**CyclASol®**	**−0.22 (−1.40, 0.97)**	**−0.30 (−1.12, 0.51)**	**−0.49 (−1.22, 0.25)**	**−0.60 (−1.84, 0.63)**	**−0.86 (−1.70, −0.02)**
2.55 (−4.02, 9.12)	0.14 (−8.89, 9.16)	N/A	**Zirun®**	−0.09 (−1.19, 1.01)	**−0.27 (−1.37, 0.83)**	−0.39 (−1.87, 1.09)	**−0.64 (−1.77, 0.48)**
N/A	N/A	N/A	N/A	**Cequa®**	**−0.18 (−0.88, 0.52)**	**−0.30 (−1.51, 0.92)**	**−0.55 (−1.27, 0.16)**
1.90 (−0.26, 4.06)	0.51 (−6.04, 7.07)	N/A	0.65 (−5.55, 6.85)	N/A	**Restasis®**	**−0.12 (−1.10, 0.86)**	**−0.37 (−1.10, 0.35)**
2.62 (−2.33, 7.57)	0.21 (−7.71, 8.13)	N/A	0.07 (−7.56, 7.71)	N/A	0.72 (−3.73, 5.17)	**TJ Cyporin®**	**−0.26 (−1.48, 0.97)**
0.27 (−4.10, 4.63)	2.14 (−5.43, 9.71)	N/A	2.28 (−5.61, 10.16)	N/A	1.63 (−3.24, 6.50)	2.35 (−4.25, 8.95)	**Ikervis®**

Each cell contains the odds ratio (OR) and 95% confidence interval for BUT changes and TEAEs; the comparison should be read from left to right. Bold numbers indicate statistically significant differences. BUT score change from baseline, ST score change from baseline; N/A, data not available.

uniform particle sizes, may reduce irritation and blur (Kang et al., 2020).

There are some limitations to this study. First, two of the included studies were single-blind and rated as high risk, which may have a certain bias. Second, although authoritative databases and registered websites were selected, RCTs for which we did not find commercial CsA formulations for the dry eye could not be included due to language or literature publication restrictions in some countries. Third, there are some confounding factors in the outcomes that may affect the stability of the results. For example, OSDI evaluation is subjective to a certain extent, and ST and BUT test personnel may have certain experience and technical deviations. Fourth, some dosage forms are once a day, while others are twice a day. This difference in the frequency of dosage use may cause some uncertainty, and future studies with larger sample sizes will be required to conduct further analysis of the difference in the frequency of dosage. Due to some differences in the baseline characteristics of the included trials, the selection of formulations determined by disease characteristics cannot be fully confirmed. In future studies, subgroup analyses based on different baseline characteristics should be feasible after the inclusion of high-quality randomized controlled studies. In addition, we have not found any cost-benefit comparison between different formulations at present, and the advantages and disadvantages of different CsA formulations should be further explored and compared from the perspective of health economics in the future. Finally, if other immunosuppressants can be included in a larger range of statistical comparison, more statistical results may be obtained.

In summary, the network meta-analysis of this study was designed to resolves discrepancies between published studies, the results of this network meta-analysis suggest that various commercial formulations of CsA have good efficacy in the treatment of patients with dry eye. Restasis® is the best choice for reducing the Ocular Surface Disease Index (OSDI) score. Zirun® and TJ Cyporin® were the most effective in improving Schirmer's Test (ST) and tear film break-up time (BUT) values, respectively. In terms of safety, Clacier® is similar to placebo, although other dosage forms may be associated with some adverse effects. The optimal order of various commercial CsA formulations was different among individual outcomes, so it was difficult to select the optimal formula. More double-blind, multi-center, large-sample, and high-quality clinical trials are still needed for supplementary validation to provide stronger evidence support.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

Study concept design, DG and YS; Data collection and analysis, DG and ZD; Drafting of the manuscript: DG and ZD; Critical revision of the manuscript, YS and KY; Approval of the final manuscript, DG, ZD, KY, and YS.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.882803/full#supplementary-material

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Efficacy and safety of the dexamethasone implant in vitrectomized and nonvitrectomized eyes with diabetic macular edema: A systematic review and meta-analysis

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Purpose: To compare the efficacy and safety of the intravitreal dexamethasone (DEX) implant for the treatment of diabetic macular edema (DME) in vitrectomized and nonvitrectomized eyes.

Methods: We performed a literature search in four electronic databases (PubMed, EMBASE, MEDLINE, and Cochrane Library) from inception to 22 May 2022. Studies comparing the efficacy of the DEX implant in vitrectomized and nonvitrectomized eyes with DME with at least 3 months of follow-up were included. The main outcomes included comparison of the mean change in the best-corrected visual acuity (BCVA) and central macular thickness (CMT) from baseline to different follow-up endpoints between the vitrectomized and nonvitrectomized groups. The secondary outcomes were the mean duration of action for the first DEX implantation and the number of required injections throughout the follow-up period. Safety data were collected and compared.

Results: The final analysis included 7 studies involving 582 eyes, 208 vitrectomized eyes and 374 nonvitrectomized eyes. The mean between-group differences in BCVA improvement were not significant at any endpoint, with averages difference of −0.07 logarithm of the minimum angle of resolution (logMAR) (p = 0.088) at 1 month, −0.03 logMAR (p = 0.472) 3 months, −0.07 logMAR (p = 0.066) 6 months, and −0.04 logMAR (p = 0.486) 12 months. The mean between-group differences in CMT reduction were not statistically significant, with mean differences of 7.17 μm (p = 0.685) at 1 month,

Abbreviations: AEs, adverse events; CMT, central macular thickness; DEX, dexamethasone; DME, diabetic macular edema; DR, diabetic retinopathy; ME, macular edema.

20.03 μm (p = 0.632) 3 months, −1.80 μm (p = 0.935) 6 months, and −25.65 μm (p = 0.542) 12 months. However, the vitrectomized group had a significantly shorter duration of action during the first DEX implantation than the nonvitrectomized group, with a mean difference of 0.8 months (p = 0.005). No significant between-group differences were detected for the number of required injections or safety profile.

Conclusion: This meta-analysis showed similar efficacy and safety of the sustained-release DEX intravitreal implant for vitrectomized and nonvitrectomized eyes with DME. The intravitreal DEX implant could be considered an effective choice for DME treatment in eyes with prior vitrectomy.

KEYWORDS

dexamethasone implant, vitrectomized, nonvitrectomized, diabetic macular edema, meta-analysis

1 Introduction

Diabetic retinopathy (DR) is the most common vascular retinopathy affecting working-age individuals worldwide. Among patients with DR, diabetic macular edema (DME) is the major cause of vision deterioration (Le et al., 2021). DME, the accumulation of fluid exudation within the retinal layers around the macular area, can occur at any stage of DR (Wong et al., 2016; Tan et al., 2017). Vascular endothelial growth factor (VEGF) and inflammatory factors play important roles in DME formation.

Pars plana vitrectomy is a mainstay and beneficial surgery for the treatment of patients with DR with complicated conditions, such as vitreous hemorrhage, epiretinal membrane, vitreomacular traction, and retinal detachment. After vitrectomy, the vitreous gel is replaced with less viscous liquids, which enhance the transport of oxygen to the ischemic retina and clearance of cytokines, such as VEGF, thereby relieving macular edema (ME) and neovascularization (Stefánsson, 2009; Yoshida et al., 2010). However, due to the chronic nature of DR, many patients may develop recurrent or persistent DME after the surgical procedure, requiring subsequent intravitreal drug therapy.

Pharmacokinetic changes in vitrectomized eyes may have unfavorable effects on the efficacy and duration of intravitreal medications. Anti-VEGF agents and other intravitreal drugs (5 fluorouracil, triamcinolone, and amphotericin B) have been washed out more rapidly in vitrectomized eyes than in nonvitrectomized eyes (Doft et al., 1985; Jarus et al., 1985; Chin et al., 2005; Christoforidis et al., 2013). Although intravitreal anti-VEGF drugs have been recommended as the first-line therapy for DME, their efficacy in vitrectomized eyes is not ideal. Chen and co-workers reported greater anatomical and functional improvements, along with fewer injections in nonvitrectomized eyes than in vitrectomized eyes after injection of ranibizumab (Chen et al., 2018). Several studies have demonstrated no significant anatomical and functional improvements with bevacizumab and aflibercept in vitrectomized eyes (Yanyali et al., 2007; Okamoto et al., 2014; Chen et al., 2017).

The dexamethasone (DEX) intravitreal implant (Ozurdex; Allergan, Irvine Inc., CA, United States), a biodegradable device designed to slowly release DEX for up to 6 months after injection, has been approved for the management of DME and ME following retinal vein occlusion and noninfectious posterior uveitis (Lowder et al., 2011; Boyer et al., 2014; Wecker et al., 2021). Its efficacy and safety have been well demonstrated not only for treatment-naive DME (Boyer et al., 2014; Mathis et al., 2020) but also for persistent DME refractory to intravitreal anti-VEGF medications (Zhioua et al., 2015; Shah et al., 2016; Yuan et al., 2022). Due to the slow-release properties of the DEX implant, its duration of action and efficacy remain satisfactory in post-vitrectomy eyes. A prospective clinical trial investigated the efficacy and tolerability profiles of intravitreal DEX implantation in 55 vitrectomized eyes with DME over a 26-week period (Boyer et al., 2011). They reported significant BCVA and CMT improvement at 8 and 26 weeks after receiving a single intravitreal injection. Additionally, a retrospective study demonstrated that the DEX implant achieved significantly better anatomical/visual improvement and fewer injections than intravitreal ranibizumab in the treatment of vitrectomized eyes with DME (Wang et al., 2021).

Although several studies have compared the safety and effectiveness of the DEX intravitreal implant for treating DME in nonvitrectomized and vitrectomized eyes, no comprehensive synthesis of available data has been published. Therefore, we conducted this systematic review and meta-analysis to compare the visual and anatomical improvements of the sustained-release DEX implant in nonvitrectomized and vitrectomized eyes for DME therapy.

2 Materials and methods

2.1 Literature search

We performed a systematic search of relevant topics in 4 electronic databases (PubMed, EMBASE, MEDLINE, and

Cochrane Library) from inception to 22 May 2022. The literature search strategy included a combination of the following terms: "dexamethasone," "Ozurdex," "vitrectomized," "diabetic macular edema" and "DME." Studies published in English that compared the efficacy of the DEX intravitreal implant in nonvitrectomized and vitrectomized eyes with DME were reviewed. We further investigated the references of eligible articles to find any relevant studies. We performed this meta-analysis in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (Liberati et al., 2009).

2.2 Eligibility criteria

Inclusion criteria were as follows: (1) patients with DME older than 18 years; (2) studies with a comparison of the efficacy of the DEX intravitreal implant between nonvitrectomized and vitrectomized eyes; (3) studies with follow-up for at least 3 months; and (4) the primary measures, central macular thickness (CMT) and best-corrected visual acuity (BCVA), were presented as mean ± standard deviation. Reviews, letters without data, case reports, and conference abstracts were excluded. The most recently published studies were included when the same study patients were presented in various publications.

2.3 Outcome measures

The primary outcomes included a comparison of the mean changes in BCVA and CMT at different follow-up endpoints (1, 3, 6, and 12 months) between the nonvitrectomized and vitrectomized groups after intravitreal DEX implant therapy. We compared the mean duration of action during the first DEX implantation and the mean number of injections required over the follow-up period between the 2 groups as secondary outcomes. We presented the BCVA data as the logarithm of the minimum angle of resolution (logMAR). Safety data, including ocular and systemic adverse events (AEs) during the follow-up period, were also collected.

2.4 Data extraction and quality assessment

Two independent investigators (QY and YG) conducted a full-text assessment of the included studies and extracted relevant information from each study. The following information were collected: first author, publication year, research location, number of samples (patients/eyes), mean age, number of DEX injections, follow-up duration, mean BCVA and change in CMT, duration of action, rate of elevated intraocular pressure (IOP), and other recorded AEs.

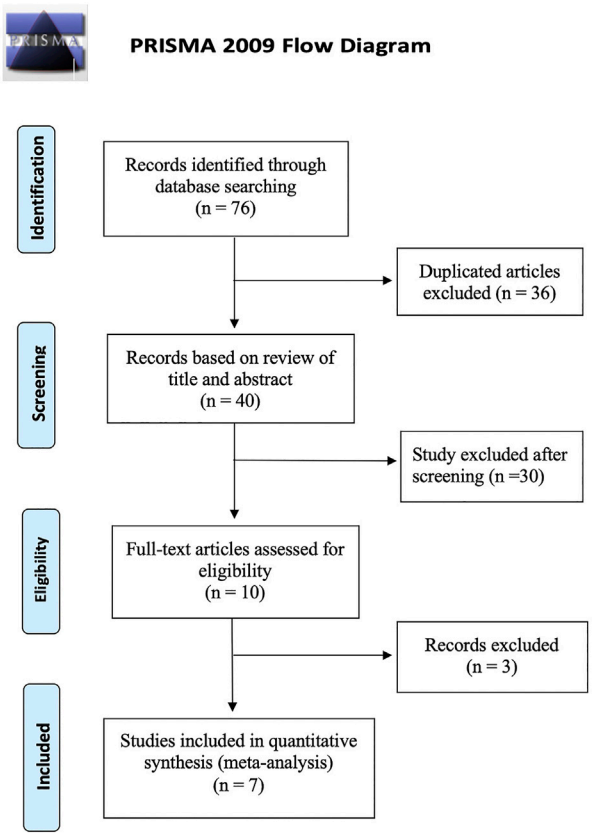

FIGURE 1
Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow chart of study identification and selection process.

The same 2 reviewers independently assessed the quality of all included studies based on the modified Downs and Black checklist (Downs and Black, 1998). This evaluation tool is suitable for both randomized controlled trials and nonrandomized trials. The score range provides the corresponding levels of quality: excellent quality (26–28 of a maximum of 28 points), fair quality (15–19), and poor quality (0–14). The higher the score, the lower is the risk of bias. All included trials were classified as having fair quality. We consulted a third reviewer (HX) to reach a consensus in case of any discrepancies.

2.5 Statistical analyses

Statistical analyses were performed using STATA software (version 15.0; Stata Corporation, College Station, TX, United States). In terms of continuous data, the weighted mean difference was calculated, and the pooled results are presented as the mean difference with a 95% confidence interval (CI). Statistical heterogeneity was assessed using

the Cochran Q test, along with the statistical value I^2. Heterogeneity across studies was considered acceptable if $p > 0.1$ and $I^2 < 50\%$. The random-effects model was adopted even in the absence of statistically significant inter-study heterogeneity because it can provide more conservative effect estimates in the case of residual heterogeneity. Funnel plots and the Egger test were used to assess potential publication bias in all included studies. We performed sensitivity analysis based on the leave-one-out approach. A 2-sided alpha level of $p < 0.05$ was regarded to be statistically significant.

3 Results

3.1 Study selection and description of the studies

The study identification and selection process based on the PRISMA flow chart is presented in Figure 1. We identified 76 studies by database searching and eliminated 36 duplications. After screening titles and abstracts, 30 studies were removed because they were on improper topics or were case reports, reviews, and letters. Three publications were excluded after full-text review. Finally, 7 articles that met the criteria were included (Medeiros et al., 2014; Bonnin et al., 2015; Çevik et al., 2018; Bastakis et al., 2019; Wang et al., 2020; Iglicki et al., 2022; Kwon and Park, 2022). All eligible studies were retrospective in design, with follow-up durations ranging from 4 to 36 months.

3.2 Baseline characteristics

The basic characteristics of the 7 included studies are shown in Supplementary Table S1. In total, 582 eyes were included in our analyses, 208 vitrectomized and 374 non-vitrectomized eyes. The sample sizes ranged from 18 to 236 eyes, with a mean patient age ranging from 57.82 to 76 years. Average baseline BCVA values ranged from 0.57 to 0.98 logMAR in the vitrectomized group and from 0.57 to 0.88 logMAR in the nonvitrectomized group, without significant between-group difference ($p = 0.647$). Average baseline CMT values ranged from 462.19 to 635.55 μm in the vitrectomized group and from 475.11 to 640 μm in the nonvitrectomized group, without significant between-group difference ($p = 0.905$). The average number of DEX implantations ranged from 1 to 3.41 times in the vitrectomized group and from 1 to 3.54 times in the nonvitrectomized group.

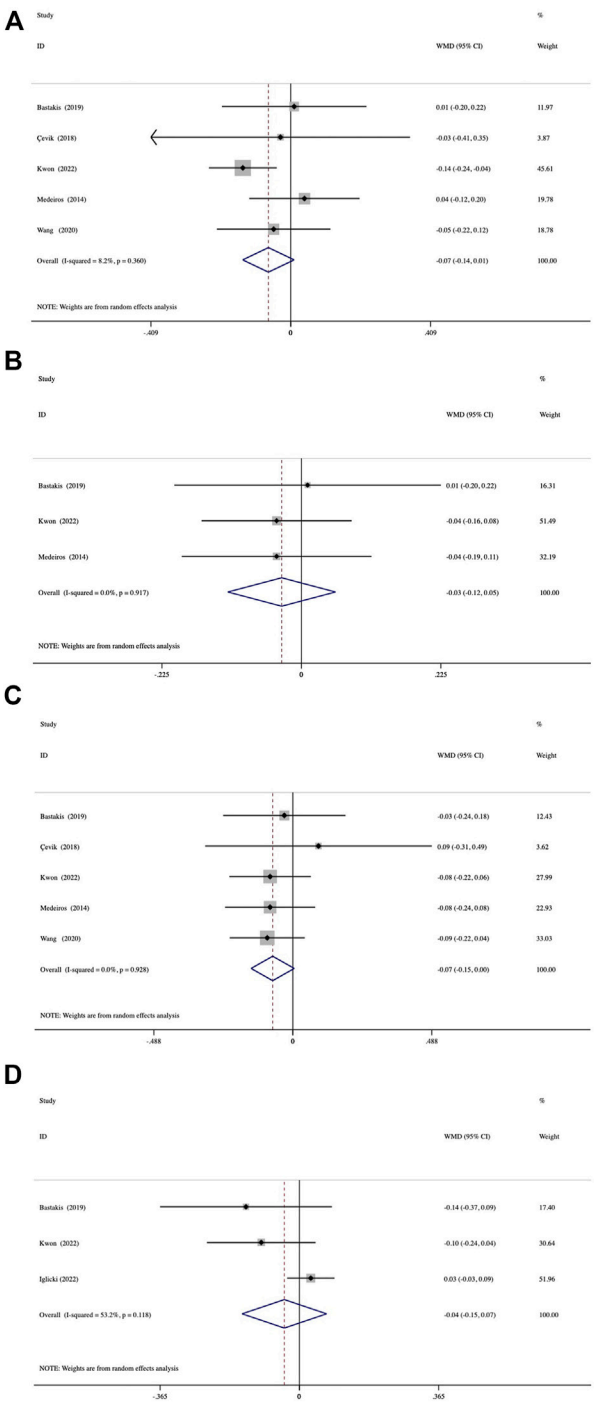

FIGURE 2
Forest plots of the comparison of mean BCVA (best-corrected visual acuity) improvement between the vitrectomized and nonvitrectomized groups **(A)** 1 month, **(B)** 3 months, **(C)** 6 months, and **(D)** 12 months.

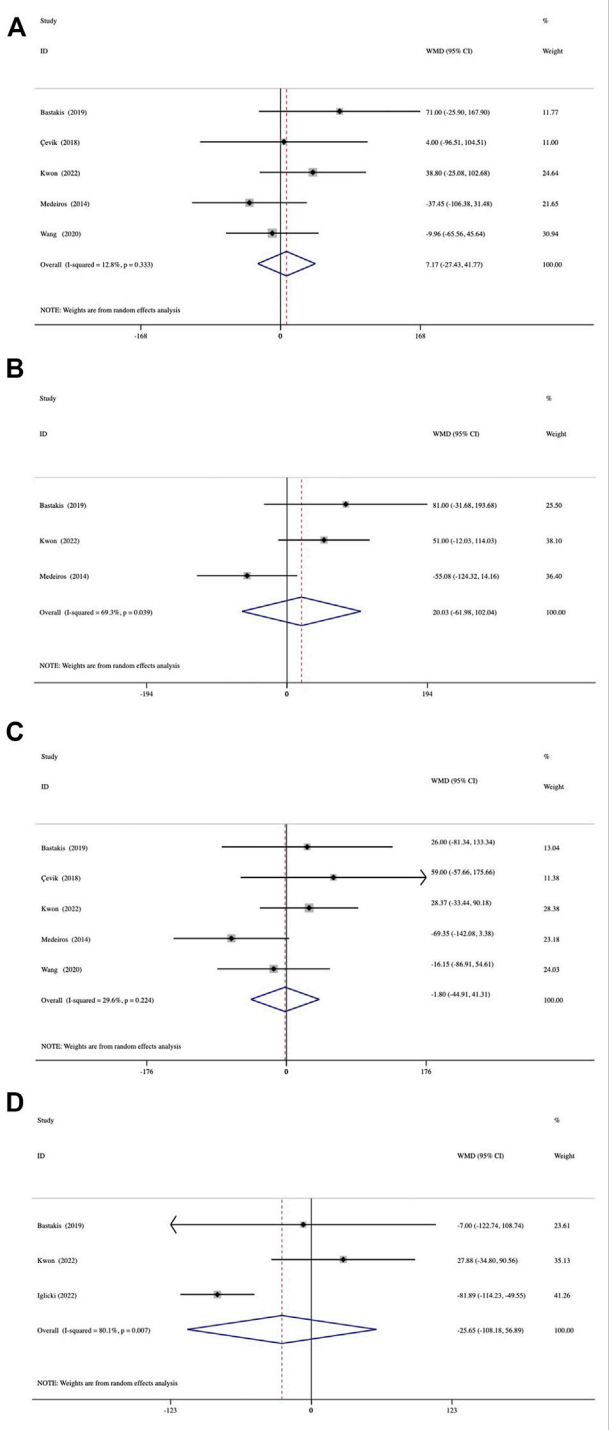

FIGURE 3
Forest plots of the comparison of mean CMT (central macular thickness) improvement between the vitrectomized and nonvitrectomized groups **(A)** 1 month, **(B)** 3 months, **(C)** 6 months, and **(D)** 12 months.

3.3 Best-corrected visual acuity

Both groups achieved significant BCVA improvement at 1, 3, and 6 months (all, $p < 0.05$; Supplementary Table S2). At 12 months, the average BCVA gain was also significant in the vitrectomized group but not in the nonvitrectomized group ($p = 0.059$). The mean BCVA gain from baseline to the 4 follow-up visits was compared between the groups (Figure 2). The assessment of mean BCVA improvement from baseline to the first month was performed across 5 studies and showed no significant between-group differences of −0.07 logMAR (95% CI, −0.14 to 0.01; $p = 0.088$) (Figure 2A). We conducted the comparison of mean BCVA change at 3 months in 3 studies, which presented a nonsignificant difference of -0.03 logMAR (95% CI, −0.12 to 0.05; $p = 0.472$) (Figure 2B). In 5 studies followed up for 6 months, we detected no significant between-group differences in mean BCVA gain, with an average difference of −0.07 logMAR (95% CI, −0.15 to 0.00; $p = 0.09$) (Figure 2C). At 12 months, the comparison of mean BCVA change included 3 studies and demonstrated no significant between-group differences (−0.04 logMAR; 95% CI, -0.15 to 0.07; $p = 0.486$) (Figure 2D). We detected no significant inter-study heterogeneity between the studies at 1 month ($I^2 = 8.2\%$, $p = 0.360$), 3 months ($I^2 = 0\%$, $p = 0.917$), or 6 months ($I^2 = 0\%$, $p = 0.928$).

3.4 Central macular thickness

Both groups showed significant reductions in CMT at 1, 3, 6, and 12 months (all, $p < 0.001$) (Supplementary Table S2). A comparison of the mean reduction in CMT at different follow-up endpoints from baseline between the groups is shown in Figure 3. The assessment of 1-month reduction in CMT between the groups was based on data from 5 studies. The pooled results demonstrated no significant difference by 7.17 μm (95% CI, −27.43 to 41.77; $p = 0.685$) (Figure 3A). At 3 months, analysis of data from 3 studies showed a mean difference of 20.03 μm (95% CI, −61.98 to 102.04; $p = 0.632$) (Figure 3B). A comparison of 5 studies at 6 months was conducted, which presented a mean difference of −1.80−μm (95% CI, −44.91 to 41.31; $p = 0.935$) (Figure 3C). In 3 studies with a 12-month follow-up duration, the mean between-group differences in reduction in CMT was -25.65 μm (95% CI, −108.18 to 56.89; $p = 0.542$) (Figure 3D). No significant inter-study heterogeneity was found between the studies at 1 month ($I^2 = 12.8\%$, $p = 0.333$) or 6 months ($I^2 = 29.6\%$, $p = 0.224$).

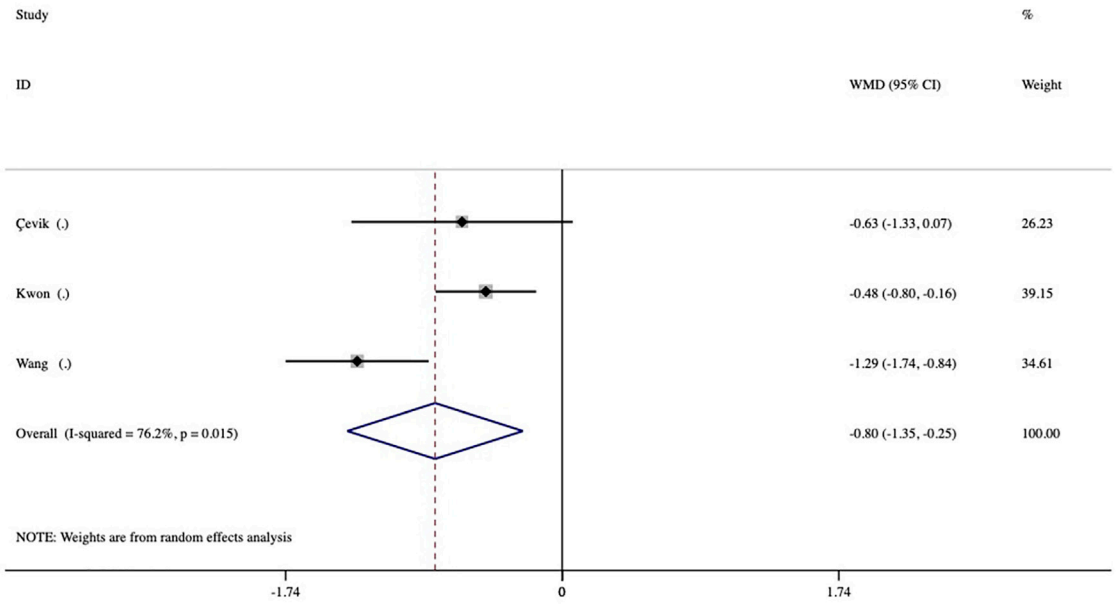

FIGURE 4
Forest plots of the comparison of the mean duration of action (intervals of macular edema recurrence) between the vitrectomized and nonvitrectomized groups.

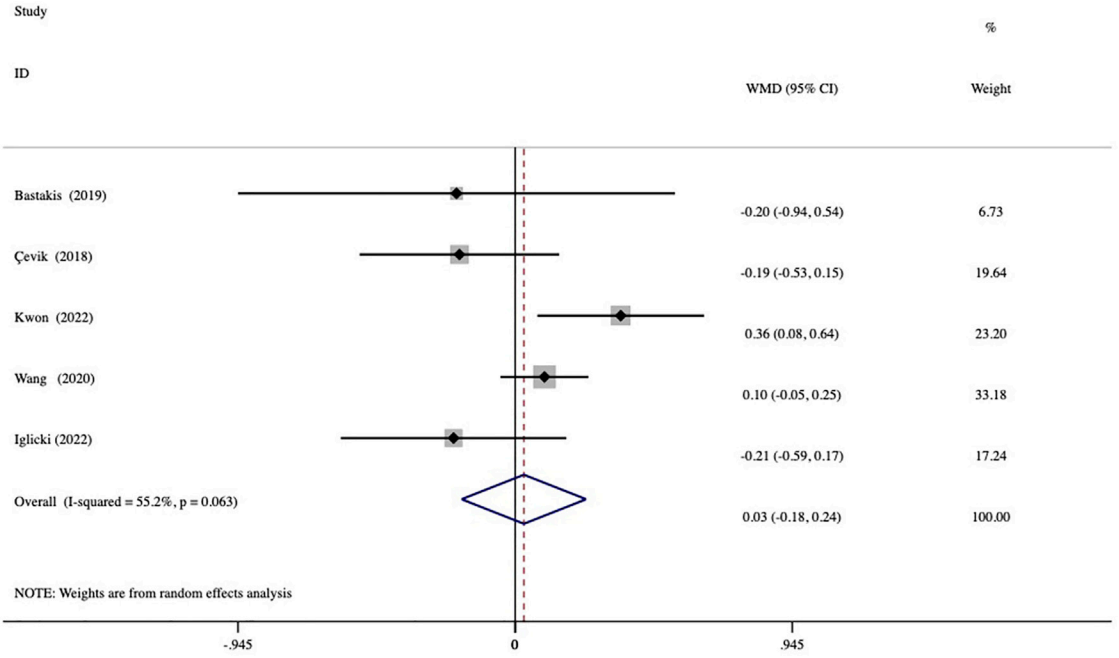

FIGURE 5
Forest plots of the comparison of the mean number of injections between the vitrectomized and nonvitrectomized groups during the follow-up period.

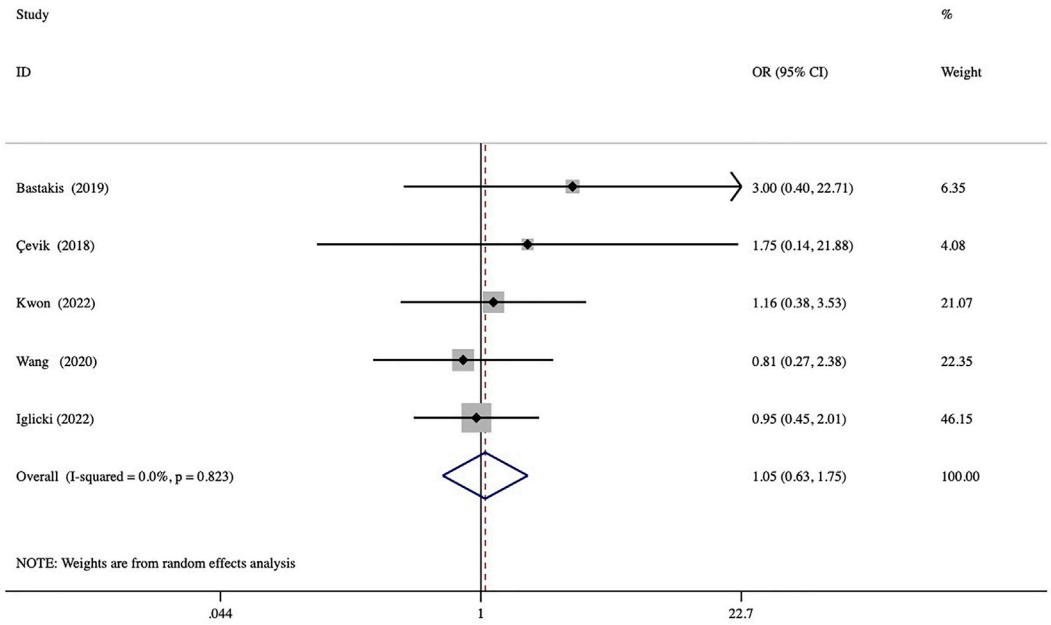

FIGURE 6
Forest plots of the comparison of the rate of elevated IOP (intraocular pressure) between the vitrectomized and nonvitrectomized groups.

3.5 Duration of dexamethasone action and the number of injections

The duration of action during the first DEX implantation (interval of macular edema recurrence) was reported in 3 studies. The duration of action was significantly shorter in the vitrectomized group than in the nonvitrectomized group with an average difference of -0.80 months (95% CI, −1.35 to −0.25; p = 0.005) (Figure 4). Analysis of the mean number of DEX intravitreal injections over the follow-up period included 5 studies and no significant between-group differences in the number of injections was detected, with a mean of 0.03 times (p = 0.784) (Figure 5).

3.6 Quality assessment

All included studies were estimated as being of fair quality with a moderate risk of bias (score range, 15–18 points). In the assessment of BCVA, possible publication bias was detected by inspection of the funnel plot (Supplementary Figure S1A) and Egger test (p = 0.001). However, studies included in the CMT assessment presented no possible publication bias, as indicated by the funnel plot (Supplementary Figure S1B) and Egger test (p = 0.597). A considerable level of inter-study heterogeneity was detected in the comparison of BCVA gains at 12 months and reductions in CMT at 3 and 12 months. The sensitivity analysis indicated that the pooled results were stable and did not significantly change by eliminating any single study.

3.7 Safety

No serious ocular and systematic AEs associated with DEX implantation were observed in any of the included studies. Among the several AEs reported, elevated IOP was the most frequent, and most patients were satisfactorily controlled with IOP-lowering drugs or observation. A comparison of the rates of elevated IOP showed no significant between-group differences (odd ratio = 1.05, p = 0.844) (Figure 6). Three studies reported cases of cataract formation or progression (Çevik et al., 2018; Iglicki et al., 2022; Kwon and Park, 2022). In addition, other minor AEs associated with injections were conjunctival hemorrhage, mild ocular pain, local hyperemia, and foreign body sensation.

4 Discussion

This meta-analysis was the first to comprehensively compare the efficacy and safety of intravitreal DEX

implantation between vitrectomized and nonvitrectomized eyes with DME. The pooled results demonstrated no significant difference in BCVA gains and reductions in CMT at 1, 3, 6, and 12 months, indicating similar efficacy between the groups. However, the duration of action of the intravitreal DEX implant in the vitrectomized eyes was significantly shorter than that in the nonvitrectomized eyes. In addition, we found no significant between-group differences in terms of the mean number of required injections during the same follow-up period. Regarding safety data, the rate of a high IOP also was not significantly different between the groups.

The similar efficacy of the DEX intravitreal implant in the 2 groups could mainly be attributed to its sustained-release property. This biodegradable device was developed to slowly release DEX within 6 months. Theoretically, its efficacy should not be significantly affected by the microenvironment in the vitreous cavity. A study in rabbits showed a similar pharmacokinetic of the DEX intravitreal implant in nonvitrectomized and vitrectomized rabbit eyes, with DEX remaining for at least 31 days in both groups (Chang-Lin et al., 2011). The effectiveness and safety of the DEX implant in vitrectomized eyes with DME and ME associated with other retinal diseases, such as retinal vein occlusion and uveitis have been well demonstrated (Adán et al., 2013; Novais et al., 2016; Rezkallah et al., 2018).

Moreover, vitrectomy may alter the levels of some cytokines. Several studies have reported that angiogenesis-related factors, such as VEGF, hepatocyte growth factor, angiopoietin-2, and erythropoietin, were reduced *via* vitrectomy in eyes with proliferative diabetic retinopathy (Yoshida et al., 2010; Yoshida et al., 2012). However, some proinflammatory cytokines, such as monocyte chemoattractant protein-1 and interleukin-6, increase after vitrectomy in patients with proliferative diabetic retinopathy (Yoshida et al., 2015). Monocyte chemoattractant protein-1 has been reported to be a contributing factor to postoperative DME in vitrectomized eyes. Therefore, the anti-inflammatory effect of DEX implants may offset some of the effects of the increased rate of drug clearance and may be a beneficial therapy for vitrectomized eyes with DME.

This meta-analysis showed that the vitrectomized group presented a significantly shorter duration of action than the nonvitrectomized group. An *in vitro* experiment showed that DEX diffused 4 times faster through a saline solution than through a vitreous solution (Gisladottir et al., 2009). A retrospective, multicenter study also demonstrated significant shorter mean rejection intervals in baseline vitrectomized groups than in nonvitrectomized groups (5.2 months *versus* 6.9 months) (Rezkallah et al., 2018). However, the authors considered that a nearly 1-month difference between groups may not be clinically relevant. Given the small average duration difference of 0.8 months (<1 month) and nearly equal number of required injections during the same follow-up period in this meta-analysis, we still believe that DEX implantation was similarly effective in nonvitrectomized and vitrectomized eyes with DME. Nevertheless, well-designed prospective trials, especially randomized controlled trials, are required to confirm these findings.

No severe AEs were observed in any of the included studies. Increased IOP and cataract development were the most frequently reported AEs among the eligible trials. Most IOP increases could be controlled with topical IOP-lowering medications. We compared the rates of increased IOP between the vitrectomized and nonvitrectomized eyes. The pooled results suggested that the risk of increased IOP did not increase after vitrectomy, which is consistent with most findings of previous studies. Additionally, Kwon and Park reported that the maximal average IOP presented 1 month earlier in the vitrectomized eyes than in the non-vitrectomized eyes, although they detected no significant differences between the groups in the prevalence of increased IOP (Kwon and Park, 2022). DEX implants should be used cautiously in eyes with clear lenses and elevated IOP.

This meta-analysis has several limitations. First, our final analysis included a limited number of studies that were all retrospective in design. Second, all included trials were estimated as being of fair quality for having moderate risk of bias. Lastly, possible publication bias was detected in the BCVA data analysis.

5 Conclusion

In conclusion, our analysis showed no significant differences in anatomical and functional improvement between vitrectomized and nonvitrectomized eyes with DME treated with a DEX implant. The safety profile of the DEX intravitreal implant was well-balanced in both groups. Thus, the intravitreal DEX implant could be considered an effective and safe alternative in vitrectomized eyes for treatment of patients with DME.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

QY and YL conceived and designed the study. QY wrote the initial version of the manuscript. QY, YG, and HX performed the literature searches and selected all eligible articles. QY and YG conducted the data extraction and quality assessment. All authors participated in data analysis and interpretation. MZ and YL supervised the study and revised the manuscript. All authors approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.1029584/full#supplementary-material

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Corneal wound healing and nerve regeneration by novel ophthalmic formulations based on cross-linked sodium hyaluronate, taurine, vitamin B6, and vitamin B12

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Introduction: To evaluate the pharmacological profile of ocular formulations based on cross-linked sodium hyaluronate (CL-SH), taurine (Tau), vitamin B6 (Vit B6) and vitamin B12 (Vit B12) using *in vitro* and *in vivo* paradigms.

Methods: Rabbit corneal epithelial cells were used to assess wound healing and reactive oxygen species (ROS) formation by scratch assay and oxidative stress (0.3 mM H_2O_2; 30 min), respectively with or without ocular formulations exposure. *In vivo* studies were carried out on albino rabbits to evaluate corneal nerve regeneration and corneal wound healing with or without treatment with six different formulations. Animals were anesthetized, the corneal epithelium was removed, and formulations were topically administered (30 µL/eye; 3 times/day for 6 days). Slit-lamp observation was carried out at different time points. After 6 days the animals were killed, and corneas were collected to evaluate corneal re-innervation by immunohistochemistry of selective neuronal marker β-III tubulin.

Results: Formulations containing the concentrations 0.16% or 0.32% of cross-linked sodium hyaluronate, taurine, vitamin B6 and vitamin B12 accelerated corneal wound healing. Cells exposed to H_2O_2 led to significant ($p < 0.05$) increase of reactive oxygen species concentration that was significantly ($p < 0.05$) counteract by formulations containing cross-linked sodium hyaluronate (0.32%) and taurine with or without vitamins. The extent of re-innervation, in terms of β-III tubulin staining, was 5-fold greater ($p < 0.01$) in the eye of rabbits treated with formulation containing 0.32% cross-linked sodium hyaluronate, taurine, vitamins (RenerviX®) compared with the control group (no treatment). Furthermore, re-innervation elicited by RenerviX® was significantly greater ($p < 0.01$) compared with the group treated with the formulation containing 0.32% cross-linked sodium hyaluronate and taurine without vitamins, and with the group treated with the formulation containing 0.5% linear sodium hyaluronate (SH), taurine, and vitamin B12, respectively.

Discussion: In conclusion, among the formulations tested, the new ophthalmic gel RenerviX® was able to contrast oxidative stress, to accelerate corneal re-epithelization and to promote nerve regeneration.

KEYWORDS

corneal wound healing, nerve regeneration, vitamin B6, vitamin B12, taurine, sodium hyaluronate

Introduction

Corneal damage represents a frequent clinical problem consequent to various chemical, physical, and pathological insults, including, but not limited to, dry eye disease and refractive surgery (Ljubimov and Saghizadeh, 2015; Bandeira et al., 2019), that generate a potent inflammatory response (Mohan et al., 2022). Oxidative stress has been demonstrated to play a central role in ocular inflammation eliciting reactive oxygen species that contribute to ocular surface damage (Cejkova and Cejka, 2015). Based on these premises, antioxidants may represent a potential option to handle corneal damage (Dogru et al., 2018) elicited by inflammatory process (Buddi et al., 2002; Jurkunas et al., 2010; Shetty et al., 2017; Fresta et al., 2020). Corneal wound healing is a complex and dynamic process which helps to preserve the integrity of the corneal epithelial to ensure corneal transparency and clear vision. This process includes, above all, the migration, proliferation, adhesion, and differentiation of the stem cell of the corneoscleral junction, and the remodeling of extracellular matrix (Mei et al., 2012; Di Girolamo et al., 2015; Ljubimov and Saghizadeh, 2015; West et al., 2015; Chou et al., 2018), regulated by many cytokines, growth factors, and signaling pathways (Mohan et al., 2022). Furthermore, the preservation of corneal nerves is crucial for normal corneal function but also in promoting epithelial wound healing thanks to the release of essential neurotrophins for corneal homeostasis (Bucolo et al., 2009; Cortina et al., 2010; Bucolo et al., 2019; Puglia et al., 2021). Therefore, after a corneal damage, it is essential to restore the epithelium, the stroma, but also the nervous components (Bukowiecki et al., 2017; Wilson et al., 2017). As a result, corneal repair and regenerative strategies should target multiple pathways and mechanisms, and several approaches have been investigated to maintain corneal homeostasis and healing process. For example, the extracellular matrix components (such as proteoglycans) regulate collagen deposition and matrix assembly, and while sodium hyaluronate demonstrated to accelerate the healing of corneal epithelial after injury (Mohan et al., 2003; Borderie et al., 2006; Yang et al., 2010; Wu et al., 2013; Gupta et al., 2022). Moreover, vitamins have a role in promoting the healing after damage and in maintaining the normal cell growth, replication processes and reinnervation (Kim et al., 2012; Romano et al., 2014; Reins et al., 2016; Fernandez-Villa et al., 2018; Fogagnolo et al., 2020; Gujral et al., 2020). The research of topical products able to modulate the wound healing is growing fast to find new approaches to handle corneal damage. This study aims to evaluate the pharmacological profile of different ocular formulations based on sodium hyaluronate (linear and cross-linked) at different concentrations, taurine, vitamin B6 and vitamin B12 using *in vitro* and *in vivo* paradigms.

Material and methods

Cell culture

Statens Seruminstitut rabbit corneal (SIRC) epithelial cells (ATCC CCL-60) were cultured in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich, Milan, Italy) supplemented with 10% of fetal bovine serum (FBS, Sigma-Aldrich), 1X Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA, Thermo Fisher Scientific, Waltham, MA, United States) and 1X Penicillin/Streptomycin (P/S, Sigma-Aldrich) at 37 °C in 5% CO_2 in humid air. Cell culture plates were coated with 5–10 µL gelatin solution/cm^2 (i.e., 0.1–0.2 mg/cm^2 gelatin, G1393, Sigma-Aldrich) to promote cell adhesion. SIRCs (P6) were cultured with or without test formulations. All media were filtered with syringe filters, 0.45 µm (Corning® 28 mm Diameter Pore SFCA Membrane, Cat. No. 431220, Arizona, United States) to ensure sterile conditions.

Ophthalmic formulations

Six different ophthalmic formulations were used: formulation #1 (F1), containing 0.5% SH-L, Tau and 0.05% Vit B12; formulation #2 (F2), containing 0.48% cross-linked SH-CL, 0.5% Tau, 0.05% Vit B6 and 0.05% Vit B12; formulation #3 (F3), containing 0.32% SH-CL, 0.5% Tau, Vit B6 and Vit B12 (Renervix® Alfa Intes I.T.S. s.r.l); formulation #4 (F4), containing 0.16% SH-CL, 0.5% Tau, Vit B6 and Vit B12; formulation #5 (F5), containing 0.02% SH-CL, 0.5% Tau, Vit B6 and Vit B12; formulation #6 (F6), containing 0.32% SH-CL, 0.5% Tau (no vitamins).

Scratch wound healing assay

The SIRC cells were grown to confluence in six-well dishes (5×10^4 cells/well). Reached the confluence, cells were washed twice with warm phosphate saline buffer (PBS, 1X) and then incubated with a serum-free medium for 5 h. Then, the confluent monolayer of cells was scratched with a 200 µL pipette tip. All the wells were washed with fresh medium to remove detached cells before incubation in a serum-free medium containing formulation #1, formulation #2, formulation #3, formulation #4, formulation #5 or formulation #6. To be sure the wounds with the same wound area were compared, a couple of lines were made at two points of the well to link the opposite points in the well with a marking pen, using the lines as a reference for the photographic report at the time of the beginning of the experiment (T0) and for 12 h (T12), 24 h (T24), 36 h (T36), 48 h (T48) and 72 h (T72). Wound area was analyzed from six different wells for each treatment, and all images were acquired with a Leica microscope using a ×20 magnification. The average wound area, expressed in the percentage of control (CTR), was determined using ImageJ Software (Broken Symmetry Software, Bethesda, MD, United States).

Detection of ROS

ROS generation was evaluated in SIRC cells, after oxidative stress induction by treatment with H_2O_2, by using the 2′,7′–dichlorofluorescin diacetate (DCFDA)– Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol, as previously described by Maugeri et al. (Antioxidants 2022. PMID: 35052632). Briefly, SIRC cells were plated into 96-well plates (1×10^4 cells/well). After overnight growth, cells were cultured for 60 min in the control medium (CTR); or in the presence of the formulation #3, containing 0.32% sodium hyaluronate (SH-CL), 0.5% taurine, 0.05% vitamin B6 and 0.05% vitamin B12 (RenerviX®); or in the presence of formulation #6, containing 0.32% sodium hyaluronate (SH-CL) and 0.5% taurine. Then, oxidative stress was induced with 0.3 mM H_2O_2 treatment for 30 min. Subsequently, cells were washed gently in PBS twice and incubated with 25 µM DCFDA previously dissolved in a buffer solution for 45 min in the dark. ROS concentration was detected by fluorescence spectroscopy with excitation and emission wavelength of 495 nm and 529 nm, respectively, using Varioskan Flash Multimode Reader (Thermo Fisher Scientific). Twelve replicate wells were used for each group.

Corneal epithelial wound healing

Male New Zealand albino rabbits (1.8–2.0 kg) were purchased from Envigo (Udine, Italy). Animals were housed under standard conditions with food and water provided *ad libitum* in a light-controlled room and set temperature and humidity. Animal care and experimental procedures were carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Protocols were

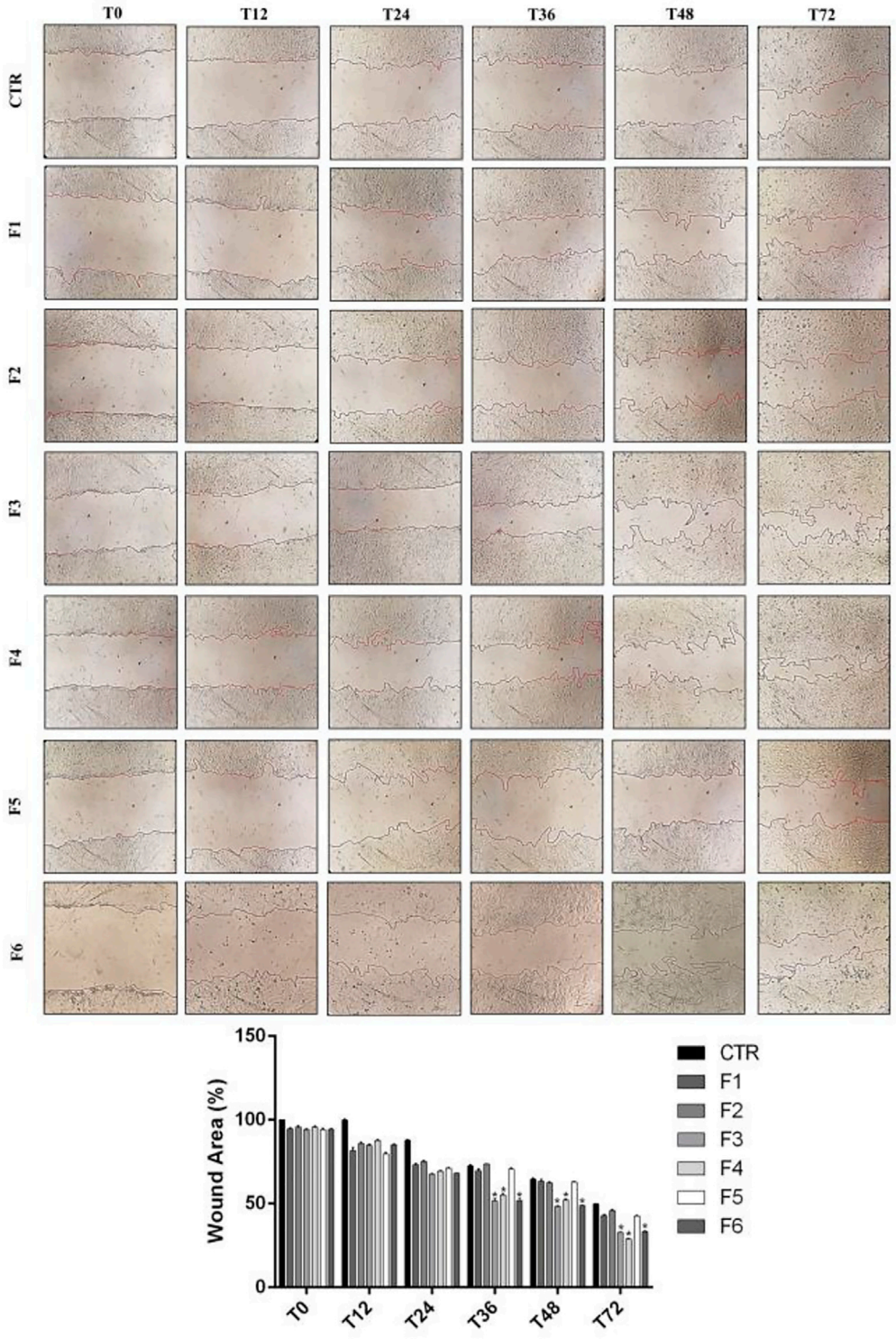

FIGURE 1
Wound healing in SIRCs monolayer. (Top Panel) Representative images of wound healing assays performed in SIRCs exposed to the six different formulations at 0, 12, 24, 36, 48 and 72 h. (Bottom Panel) The bar graph shows the average wound area expressed in the percentage of CTR. *$p < 0.01$ vs. F1, F2 and F5 as determined by one-way ANOVA followed by the Tukey's *post hoc* test. Data are shown as mean ± SD of five independent experiments ($n = 5$).

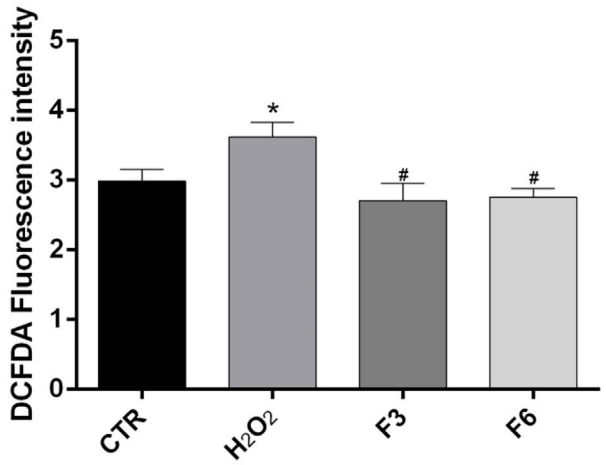

FIGURE 2
Detection of intracellular ROS production in corneal cells.

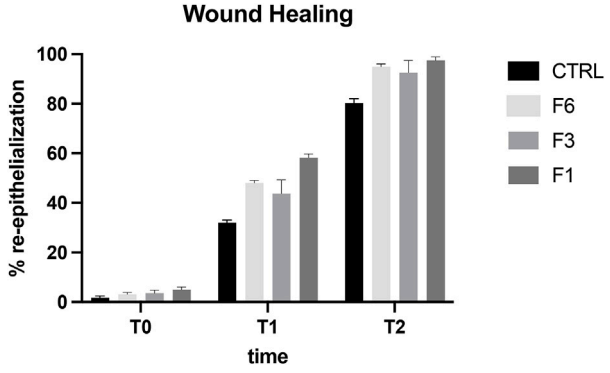

FIGURE 3
Wound healing in rabbit eye. T0 (day 1), T1 (day 2), T2 (day 4). Formulation 6 (F6); RenerviX® (F3); Formulation 1 (F1); Control group. Data are showed as % of corneal re-epithelialization. No statistical differences were observed between treated groups. Data are shown as mean ± SD (n = 4).

approved by the Institutional Animal Care and Use Committee of the University of Catania (project #303). Animals were anesthetized and the corneal epithelium was removed with 0.5 mm corneal rust ring remover (Algerbrush, EyeBM Vet, Milan, Italy), under a dissecting microscope. The eyes were treated as follow: group 1) Formulation #6 containing 0.32% SH-CL and 0.5% Tau; group 2) Formulation #3 containing 0.32% SH-CL, 0.5% Tau, 0.05% Vit B6 and 0.05% Vit B12, RenerviX®); group 3) Formulation #1 containing 0.5% SH-L, 0.5% Tau and 0.5% Vit B12. All formulations were topically administered (one drop, three times per day for 6 days) starting the same day of corneal epithelial debridement. Slit-lamp observation was carried out at different time points. After 6 days, the animals were killed, and corneas were collected for the immunohistochemical analysis to evaluate corneal re-innervation by immunohistochemical analysis of the selective neuronal marker beta-III tubulin.

Immunohistochemistry analysis

The expression and distribution of ß-III tubulin in rabbit cornea were evaluated through immunohistochemical analysis. Briefly, after dewaxing in xylene, the corneal slides were hydrated through graded ethanol and incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline. The sections were then heated in a thermoregulated bath (80° for 30 min) with rodent decloaker (Biocare Medical, Pacheco, CA, United States), to perform antigen retrieval. The blocking step to prevent non-specific binding of the antibody was performed before application of the primary antibody with 1% bovine serum albumin (BSA, Sigma, Milan, Italy) in PBS for 1 h in a moist chamber. After blocking, the sections were incubated overnight at 4 °C with ß-III Tubulin antibody (ab78078, Abcam, Cambridge United Kingdom), work dilution in PBS and 1%BSA 1:100. Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-rabbit was used as secondary antibodies) and then detected with peroxidase labeled streptavin, both incubated for 10 min at room temperature (LSAB + System-HRP, K0690, Dako, Denmark). The immunoreaction was visualized by incubating the sections for 3 min in 3,3′-diaminobenzidine solution (DAB substrate Kit; SK-4100, Vector Laboratories, Burlingame, CA, United States). The samples were lightly counterstained with hematoxylin, mounted in vecta mount (Vector Laboratories) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss). Densitometric analysis was carried with ImageJ. ß-III Tubulin staining in the corneal epithelium was quantified as previously described by Romano et al. (2014).

Statistical analysis

Statistical analysis was performed by GraphPad prism 7 (GraphPad software La Jolla, California). The data generated by all experiments are reported as mean ± SD. One-way analysis of variance (ANOVA) was carried out, and Tukey's *post hoc* test was used for multiple comparisons. Differences between groups were considered statistically significant for p-values <0.05.

Results

Wound healing in SIRCs

We performed wound healing assay to evaluate the impact of the formulations in the wound repair capability of SIRC cells. As shown in Figure 1, at 12 and 24 h after confluent SIRCs were scratched, all formulations produced a significant ($p < 0.01$) reduction of the average wound area as compared to control. However, starting from 36 h until to 72 h, the corneal cells exposed to F3, F4, and F6 showed the best performance in terms of wound closure compared to control group and the other formulations ($p < 0.01$ vs. F1, F2, F5). No significant differences were observed between F3, F4, and F6. These findings suggest that these formulations exert comparable positive effects on the wound healing rate in SIRC cells. We then analyzed the effect of F3 against oxidative stress induced by treatment with H_2O_2 (0.3 mM) for 30 min using a DCFDA assay. To assess the role of vitamins contained in F3, we also tested F6, containing similarly to F3, 0.32% SH-CL and 0.5% Tau, but no vitamins. As

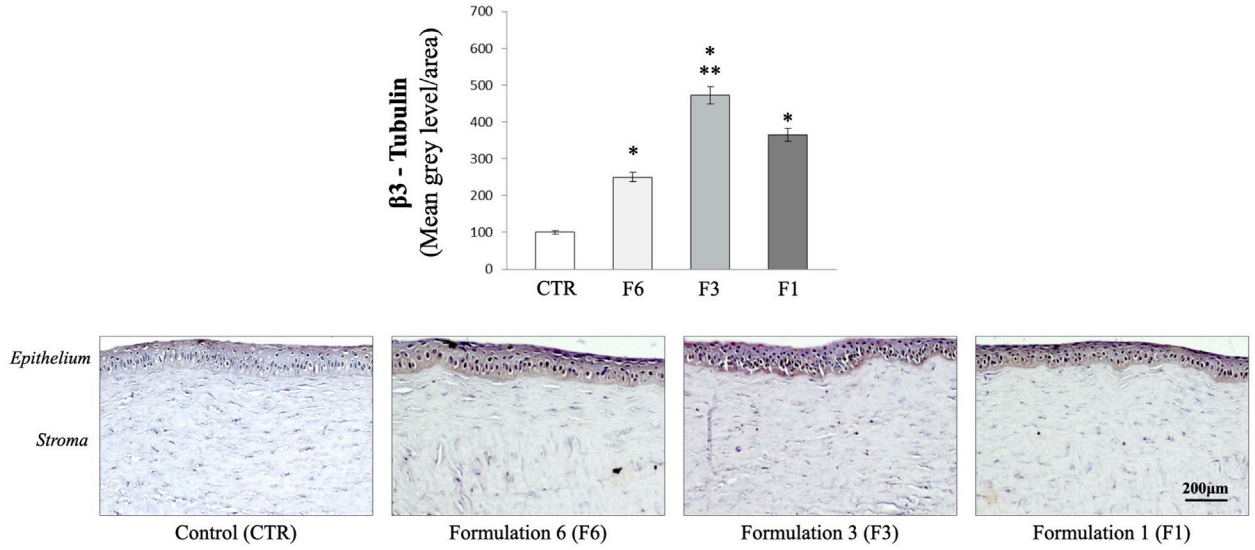

FIGURE 4
Immunohistochemical analysis. Measurement of corneal ß-III tubulin expression. Formulation 6 (F6); Formulation 3 (F3, RenerviX®); Formulation 1 (F1); Control group. *$p < 0.01$ vs. CTR; **$p < 0.01$ vs. F6 and F1. Data are shown as mean ± SD ($n = 4$).

shown in Figure 2, cellular ROS levels significantly ($p < 0.05$) increased in SIRC cells after H_2O_2 treatment compared to control. The treatment with F3 and F6 significantly ($p < 0.05$) reduced ROS formation after H_2O_2 stress.

ROS levels were measured in SIRCs after 0.3 mM H_2O_2 treatment for 30 min alone or in cells previously treated for 60 min with F3 or F6, using the cytoplasmic probe, DCFDA. *$p < 0.05$ vs. CTR; #$p < 0.05$ vs. H_2O_2 as determined by one-way ANOVA followed by Türkiye's multiple comparison test. Data are shown as mean ± SD of five independent experiments ($n = 5$).

Corneal epithelial wound healing *in vivo* study

The aim of the *in vivo* study was to evaluate the effects of RenerviX® on corneal wound healing and to evaluate the expression and the localization of regenerated nerve fibers after corneal abrasion in rabbit eye. As showed in Figure 3 all the ophthalmic formulations tested [Formulation 6 (F6) containing 0.32% SH-CL and 0.5% Tau; Formulation 3 (F3) (RenerviX®) containing 0.32% SH-CL, 0.5% Tau, 0.05% Vit B6 and 0.05% Vit B12; Formulation 1 (F1) containing 0.5% SH-L, 0.5% Tau and 0.5% Vit B12] contribute to the corneal wound healing even though no statistical differences were observed between treated groups (Figure 3).

Effects of ocular formulations on corneal nerve regeneration

Corneal re-innervation was examined by immunohistochemical analysis of the selective neuronal marker, beta-III tubulin after mechanical injury. As shown in Figure 4, immunohistochemical analysis demonstrated the presence of regenerating nerve fibers expressing β-III tubulin in the apical areas of the cornea of eyes treated with all three formulations (F1; F3 and F6) ($p < 0.05$ and $p < 0.01$ vs. CTR). However, the extent of re-innervation was significantly greater in the eye of rabbits treated with RenerviX® compared with the group control and the groups treated with formulations containing 0.32% SH-CL and Tau (F6) and formulation containing 0.5% SH-L, 0.5% taurine and 0.5% vitamin B12 (F1) ($p < 0.05$ vs. F6 and F1).

Discussion

In the present study we demonstrated that RenerviX®, was able to improve corneal wound healing, to restore functional corneal nerves, and to protect corneal cells from oxidative stress. Treatment with RenerviX® stimulates re-innervation of the injured cornea in rabbit eye with a significant difference when compared to formulation 6 and formulation 1. Finally, no levels of taurine, pyridoxine (vit B6) and cyanocobalamin (vit B12) were detected after 18 h in the cornea samples of rabbit eyes treated with a single instillation of RenerviX®, suggesting that no deposit of these molecules occurred after topical administration. Altogether, these findings suggest that pyridoxine (vitamin B6) present in RenerviX®, significantly contributes to the corneal preservation and recovery after an insult.

Previous studies demonstrated the importance of vitamins in maintaining ocular surface homeostasis, suggesting the possible protective effects against damages (Lasagni Vitar et al., 2022). Vitamins are essential for many corneal functions and help ensuring corneal integrity supporting the epithelial barrier and cells survival (Yin et al., 2011; Bucolo et al., 2015; Reins et al., 2015; Gozzo et al., 2021; Kaminska et al., 2021; Lasagni Vitar et al., 2022). Moreover, their anti-inflammatory, antimicrobial and antioxidant properties have been demonstrated (Wimalawansa, 2019). Vit B6 role is important for several biosynthetic pathways such as purine, pyrimidine, and amino acids syntheses and in maintaining the normal cell growth and replication processes (Fernandez-Villa et al., 2018; Fiorillo and Romano, 2020; Fogagnolo et al., 2020). For example, the local treatment with Vit B12 led to faster repair of corneal

damage and facilitated reinnervation (Romano et al., 2014). This is in line with the evidence that Vit B12 promotes the synthesis of neurotrophic factors, supporting neurite growth and survival (Scalabrino and Peracchi, 2006; Okada et al., 2010). Indeed, Vit B12 deficiency is associated with sensory innervation impairment, optic neuropathy, eye movement disorders and corneal damage (Chavala et al., 2005; Akdal et al., 2007; Jurkunas et al., 2011; Conti et al., 2021).

Moreover, Vit B6 has been recognized as a potent antioxidant as well as an established cofactor for several metabolic enzymes, including, among others, those involved in protein metabolism, conversion of tryptophan to niacin, and neurotransmitter function (Kannan and Jain, 2004; Tunali, 2014; Hsu et al., 2015).

The role of Vit B6 as a therapeutic agent has been demonstrated in several disorders such as diabetes (Jain, 2007; Amato et al., 2021) and cardiovascular diseases (Wierzbicki, 2007). For example, the antioxidant and scavenging properties have been considered in reducing oxidative stress markers associated with homocysteinemia or in preventing free radicals formation and lipid peroxidation in cellular models (Mahfouz and Kummerow, 2004).

In addition, Vit B6 is involved in the immune system regulation and the regulation of neurotransmitters (Baltrusch, 2021). Being essential for the amino-acid metabolism, Vit B6 regulates the synthesis of neurotransmitters, responsible for signal transmission (Yang and Wang, 2009; Baltrusch, 2021). In preclinical models, vitamin B6 showed neuroprotective effects against glutamate damage stimulating nerve regeneration, and prevention of neuronal death in the retina after ischemic damage (Wang et al., 2002; Yang and Wang, 2009). Furthermore, some clinical evidence supported the regenerative effect of Vit B6 (Talebi et al., 2013). These evidences are important with an impact on corneal nerves protection necessary for the maintenance of a healthy ocular surface (Muller et al., 2003) and support corneal healing (Yu and Rosenblatt, 2007; He et al., 2010; Marfurt et al., 2010; Toro et al., 2021).

In conclusion, among the formulations tested, the new ophthalmic gel based on 0.32% SH-CL, 0.5% taurine, 0.05% vitamin B6 and 0.05% Vitamin B12 (RenerviX®), demonstrated to better contrast oxidative stress, to accelerate corneal re-epithelization and to promote nerve regeneration, suggesting an important advantage in clinical practice, ranging from corneal abrasion and/or neuropathy (diabetes or severe dry-eye) to help patients' recovery after eye surgery.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Catania (project #303).

Author contributions

CB and GR made substantial contributions to conception, design, and interpretation of data. GM, SG, VD, GR carried out formal analysis of data. CB and GR wrote initial draft of the manuscript. GM, SG, VA, GR, FD, and CB reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Anti-vascular endothelial growth factor monotherapy or combined with verteporfin photodynamic therapy for retinal angiomatous proliferation: a systematic review with meta-analysis

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Purpose: To assess functional and anatomical outcomes of intravitreal anti-Vascular Endothelial Growth Factor (anti-VEGF) monotherapy *versus* combined with verteporfin Photodynamic Therapy (PDT) for Retinal Angiomatous Proliferation (RAP).

Methods: Studies reporting outcomes of intravitreal anti-VEGF monotherapy and/or in combination with verteporfin PDT in RAP eyes with a follow-up ≥ 12 months were searched. The primary outcome was the mean change in best corrected visual acuity (BCVA) at 12 months. Mean change in central macular thickness (CMT) and mean number of injections were considered as secondary outcomes. The mean difference (MD) between pre- and post-treatment values was calculated along with 95% Confidence Interval (95% CI). Meta-regressions were performed to assess the influence of anti-VEGF number of injections on BCVA and CMT outcomes.

Results: Thirty-four studies were included. A mean gain of 5.16 letters (95% CI = 3.30–7.01) and 10.38 letters (95% CI = 8.02–12.75) was shown in the anti-VEGF group and combined group, respectively (anti-VEGF group vs. combined group, $p < 0.01$). A mean CMT reduction of 132.45 μm (95% CI = from –154.99 to –109.90) and 213.93 μm (95% CI = from –280.04 to –147.83) was shown in the anti-VEGF group and combined group, respectively (anti-VEGF group vs. combined group, $p < 0.02$). A mean of 4.9 injections (95% CI = 4.2–5.6) and 2.8 injections (95% CI = 1.3–4.4) were administered over a 12-month period in the anti-VEGF group and combined group, respectively. Meta-regression analyses showed no influence of

injection number on visual and CMT outcomes. High heterogeneity was found across studies for both functional and anatomical outcomes.

Conclusion: A combined approach with anti-VEGF and PDT could provide better functional and anatomical outcomes in RAP eyes compared with anti-VEGF monotherapy.

KEYWORDS

retinal angiomatous proliferation (RAP), anti vascular endothelial growth factor, verteporfin photodynamic therapy (V-PDT), monotherapy, combined therapy

1 Introduction

Retinal angiomatous proliferation (RAP) was firstly described by Yannuzzi et al. as a distinct form of neovascularage-related macular degeneration (nAMD). (Yannuzzi et al., 2001).

According to the anatomic classification, RAP is defined as "type 3 neovascularization". (Freund et al., 2008). The peculiar characteristic of RAP is that it consists of two different neovascular foci, one originating in the deep retina and the other within the choroid. (Yannuzzi et al., 2008). Usually, the neovascular network originates in the deep retina and extends to choroidal neovessels through vascular anastomosis. (Freund et al., 2008). Natural course of RAP is different compared with other forms of nAMD, featuring a rapid progression to advanced stages and poor visual outcomes, especially in cases of inadequate treatment or delayed diagnosis. (Viola et al., 2009).

Intravitreal anti-vascular endothelial growth factor (anti-VEGF) therapy has become the first line treatment for nAMDand for RAP lesions as well. (Tsai et al., 2017; Reibaldi et al., 2020). On the one hand, some authors showed that RAP lesions could be characterized by a worse response to intravitreal treatment compared with other forms of nAMD. (Tsai et al., 2017). On the other hand, recent evidence demonstrated that anti-VEGF therapy can provide positive outcomes in RAP eyes, comparable with other types of nAMD (Browning et al., 2019) or even better. (Invernizzi et al., 2019). However, RAP treatment based on intravitreal anti-VEGF therapy alone could prove challenging because of frequent relapses of exudative activity and partial response to this therapy. (Viola et al., 2009). Additionally, in some cases, a more intense intravitreal anti-VEGF treatment could be required. (Rouvas et al., 2012; Gharbiya et al., 2014; Inoue et al., 2014).

On this basis, intravitreal anti-VEGF therapy has been used in combination with photodynamic therapy (PDT) in attempt to achieve a better control of RAP lesions. (Saito et al., 2010; Saito et al., 2012; Saito et al., 2013; Malamos et al., 2018). This combined approach seems to provide promising outcomes in terms of visual gain and macular thickness reduction. (Saito et al., 2010; Saito et al., 2012; Saito et al., 2013; Malamos et al., 2018). However, there is limited evidence as to whether intravitreal anti-VEGF therapy combined with PDT could provide better results compared with intravitreal anti-VEGF therapy alone.

The purpose of the present systematic review with meta-analysis was to collect available evidence on intravitreal anti-VEGF therapy alone or combined with PDT in RAP eyes and to assess whether combining anti-VEGF therapy with PDT could have a synergic effect and lead to better functional and anatomical outcomes.

2 Materials and methods

2.1 Literature search methods

The study was conducted according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) group (PRISMA checklist available in Supplementary Table S1 as Supplementary Material). (Liberati et al., 2009)

We conducted comprehensive searches of PubMed and Embase databases, from January 2009 to 5th May 2022. The electronic search strategy included the terms "retinal angiomatous proliferation," "RAP," "type 3 neovascularization," "choroidal neovascularization," "anti-vascular endothelial growth factor," "aflibercept," "ranibizumab," "bevacizumab" and "photodynamic therapy," which were connected by using "and/or" in various combinations. Only articles published in peer-reviewed journals and in English were selected. We also screened reference lists of included studies and review articles focused on similar topics.

2.2 Eligibility criteria and outcomes of interest

The following eligibility criteria were considered: 1) to include eyes affected by retinal angiomatous proliferation that were treated with intravitreal anti-VEGF therapy alone (bevacizumab, ranibizumab and aflibercept) and/or in combination with photodynamic therapy; 2) to have a follow-up of at least 12 months; 3) to report visual and/or anatomical outcomes. Case reports and case series with less than 10 cases were excluded. Choroidal neovascular membranes different from RAP were excluded. When clarifications for study eligibility were needed, we contacted study's authors.

Eyes treated with anti-VEGF therapy alone were included in the anti-VEGF group, while eyes treated with anti-VEGF therapy combined with PDT were included in the combined group.

The primary outcome of interest was the mean change in best corrected visual acuity (BCVA) in the two groups. Mean change in central macular thickness (CMT) on optical coherence tomography (OCT) was considered as secondary outcome. The influence of the number of injections on BCVA and CMT in either group was considered a secondary outcome as well. Central macular thickness referred to the average thickness of the fovea-centered area with 1 mm diameter.

2.3 Data collection and risk of bias

Two investigators (MF and IM) evaluated independently the eligibility of identified studies. The same two investigators (MF and

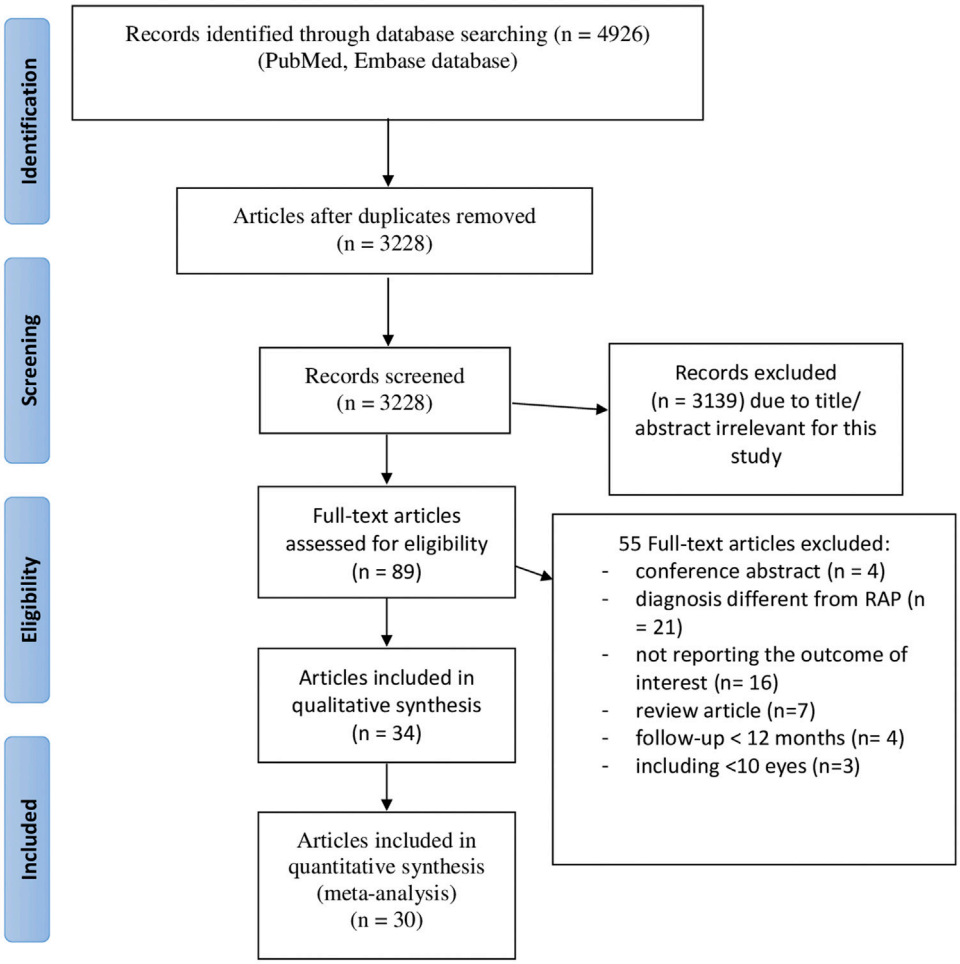

FIGURE 1
Bow diagram of study selection process.

IM) analyzed and extracted data from each included study in an independent fashion. A third investigator (VB) was involved in case of disagreement. The following items were collected from each included study: first author, publication year, country, study design, number of eyes, mean age, type of treatment, follow-up. For both the anti-VEGF group and the combined group, the following data were collected: number of eyes, mean age, naïve/non-naïve status, type of anti-VEGF drug and treatment protocol, number of injections, BCVA change, CMT change, follow-up. Information on type of PDT protocol, namely, standard verteporfin PDT, (Bressler, 2001), half-dose and half-fluence PDT, (Reibaldi et al., 2010), was collected for the combined group.

Risk of bias of randomized trials was evaluated by the means of the Cochrane collaboration tool. (Higgins, 2022). Risk of bias assessment for non-randomized studies was based on the Methodological Item for Non-Randomized Studies (MINORS) scale, (Slim et al., 2003), being a ≥9 score at low-to-moderate risk. (Fallico et al., 2020a).

2.4 Statistical analysis

For BCVA and CMT change, pooled effect size was investigated through meta-analysis and mean difference (MD) between pre- and post-treatment values was reported along with 95% Confidence Interval (95% CI). The I^2 index and the Q-statistics were used to measure and test heterogeneity across studies. When a significant heterogeneity was found ($I^2 > 50\%$ and Q-statistics $p < 0.1$), a random effect model was fitted applying the DerSimonian-Laird method. Subgroup analyses were conducted to compare BCVA and CMT outcomes between the anti-VEGF group and the combined groups. Meta-regressions were performed to assess the influence of anti-VEGF number of injections on BCVA and CMT outcomes. Results of the meta-regressions were reported as β coefficient and its standard error (SE). Publication bias was tested using the Egger's test and by visual inspection of funnel plots' symmetry. Analyses were conducted on STATA (version 17) and were two-tailed, with a level of statistical significance $\alpha < 0.05$.

3 Results

The flow diagram of the study selection is illustrated in Figure 1. Systematic search identified a total of 4,926 articles, of which 1,698 were duplicates. Titles and abstracts of the remaining 3,228 articles were reviewed for eligibility. A total of 89 articles

TABLE 1 Characteristics of included studies in the anti-VEGF monotherapy group.

Author and year	Design	Anti-VEGF drug	Eyes (n)	Gender (M/W)	Age mean (±SD, years/ range)	Follow-up (months)	Anti VEGF regimen	RAP stage (n)
Montero et al. (2009)	Retrospective	Bevacizumab	26 naive	9 /15	78 ± 8	12	3 IV monthly + PRN (followed monthly)	-14 stage 2 -12 stage 3
Engelbert et al. (2009)	Retrospective	Bevacizumab/ Ranibizumab	11 naive	3/8	85 (range, 71–92)	12	3 IV monthly + Treat and extend*	-
Atmani et al. (2010)	Prospective	Ranibizumab	29 naive	7/19	78.2 ± 6.7 (range 66-90)	12	3 IV monthly + PRN (follow up interval not specified)**	stage 2/3
Parodi et al. (2013)	Prospective randomized	Bevacizumab/ Ranibizumab	50 naive	21/29	73 ± 7.5	12	3 IV monthly + PRN (followed monthly)	Bevacizumab -14 stage1 -12 stage 2B Ranibizumab -14 stage 1 -10 stage 2B
Reche-Frutos et al. (2011)	Prospective	Ranibizumab	53 (31 naive)	16/37	81.91 ± .3	53	3 IV monthly + PRN (followed monthly)	-21 stage 2A -18 stage 2B -14 Stage 3
Rouvas et al. (2012)	Randomized controlled trial	Ranibizumab	13 naive	5/8	76.87	36	3 IV monthly + PRN -Retreatment: 3 IV monthly	-10 stage 2 -3 stage3
Shin and Yu (2014)	Prospective	Ranibizumab	31 naive	6/25	70.4 ± 6.5	24	3 IV monthly + PRN (followed monthly)	-5 stage 1 -12 stage 2 -14 stage 3
Gharbiya et al. (2014)	Prospective	Bevacizumab/ Ranibizumab	21 naive	5/14	74.5 ± 9.6	36	3 IV monthly + PRN (followed monthly)	-
Inoue et al. (2014)	Retrospective	Ranibizumab	17 naive	4/10	80.5 ± 4.7	36	3 IV monthly + PRN (followed monthly)	-1 stage 1 10stage 2 -6 stage 3
Park and Roh (2015)	Retrospective	Ranibizumab	41 naive	16/25	67.09 ± 11.76	12	3 IV monthly + PRN (followed monthly)	-8 stage 1 -17 stage 2 -16 stage 3
Cho et al. (2016)	Retrospective	Bevacizumab/ Ranibizumab	38 naive	20/18	74.3 ± 7.5	36	3 monthly IV + PRN (followed monthly)	-4 stage 1 -24 stage 2 -10 stage 3
Arias et al. (2016)	Randomized controlled trial	Ranibizumab/ Ranibizumab + PDT	10 no naive	3/7	79.5 ± 8.0	12	3 IV monthly + PRN (followed monthly)	-2 stage 1 -6 stage 2 -2 stage 3
Matsumoto et al. (2016)	Retrospective	Aflibercept	17 naive	6/11	76.9	12	3 monthly IV + Treat and extend°	-8 stage 1 -1 stage 2 without PED -7 stage 2 with PED -1 stage 3
Hemeida et al. (2010)	Retrospective	Bevacizumab/ Ranibizumab	20	6/9	85.8 ± 4.54	24	1 IV baseline + PRN (followed monthly)	-
Kim et al. (2017a)	Retrospective	Ranibizumab	42 naive	13/29	75.5 ± 5.8	12	3 IV monthly + PRN ***	-
Kim et al. (2017b)	Retrospective	Ranibizumab	38 naive	4/15	75.8 ± 7.7	12	3 IV monthly + PRN ***	-17 stage 1 -21 stage 2

(Continued on following page)

TABLE 1 (*Continued*) Characteristics of included studies in the anti-VEGF monotherapy group.

Author and year	Design	Anti-VEGF drug	Eyes (n)	Gender (M/W)	Age mean (±SD, years/ range)	Follow-up (months)	Anti VEGF regimen	RAP stage (n)
Hata et al. (2017)	Retrospective	Ranibizumab/ Aflibercept	41 naive	16/30	82.2 ± 6.6	27.6 ± 15.5	Ranibizumab 3 IV monthly + PRN (followed monthly) Aflibercept 3 IV monthly + fixed (bimonthly IV)	-.4 stage 1 -15 stage 2 -27 stage 3
Author and year	**Design**	**Anti-VEGF drug**	**Eyes (n)**	**Gender (M/W)**	**Age mean (±SD, years/ range)**	**Follow-up (months)**	**Anti VEGF regimen**	**RAP stage (n)**
Kim et al. (2018)	Retrospective	Ranibizumab /Aflibercept	42 naive	6/34	76.3 ± 6.4	12	Ranibizumab/ Aflibercept 3 monthly IV + PRN ***	-14 stage 2 -26 stage 3
Invernizzi et al. (2019)	Prospective	Bevacizumab/ Ranibizumab /Aflibercept	157 naive	46/111	83.1 ± 6.4, (65–96)	12	§	-
Ernest et al. (2020)	Prospective	Aflibercept	14 naive	9/5	71 ± 9	12	3 monthly IV +fixed (bimonthly IV)	-7 Stage 2 -7 Stage 3
Maruyama-Inoue et al. (2019)	Retrospective	Ranibizumab/ Aflibercept	85 naïve	21/40	84.0 ± 6.7	36	68 eyes 3 monthly IV + PRN (followed monthly or bimonthly) 17 eyes Fixed monthly o bimonthly	-8 stage 1 -53 stage 2 -24 stage 3
Browning et al. (2019)	Prospective	Aflibercept	46 naïve	12/34	81.5	24	3 IV monthly + fixed (bimonthly IV)	-3 stage 1 -9 stage 2 -34 stage 3
Kim et al. (2019)	Retrospective	Ranibizumab/ Aflibercept (retreatment with Ranibizumab/ Aflibercept or Bevacizumab)	137	38/99	74.9 ± 5.9	42.4±18.9	3 IV monthly + PRN ***	-32 stage 2 -105 stage 3
Arias et al. (2020)	Prospective, multicenter trial	Aflibercept	32 naive	10/22	78.2 ± 7.7	12	3 IV monthly + Treat and Extend°°	-
Author and year	**Design**	**Anti-VEGF drug**	**Eyes (n)**	**Gender (M/W)**	**Age mean (±SD, years/ range)**	**Follow-up (months)**	**Anti VEGF regimen**	**RAP stage (n)**
Kim et al. (2020)	Retrospective	Ranibizumab/ Aflibercept Retreatment with Ranibizumab, Aflibercept or Bevacizumab	195 naive	42/153	75.7 ± 6.0	47.5 ± 20.7	3 IV monthly + PRN (followed monthly) or switch treat and extend	-43 stage 2 -152 stage 3
Kim (2020)	Retrospective	Ranibizumab/ Aflibercept Retreatment with Ranibizumab, Aflibercept or Bevacizumab	17 naive	2/15	75.4 ± 6.2	39.7 ± 10.9	3 IV monthly + PRN (followed monthly)	-2 stage 2 -15 stage 3

N: number; M/W: men/women; SD: standard deviation; IV: intravitreal; RAP: retinal angiomatous proliferation; PRN: *pro re nata*

*Treat and Extend: at least 3 monthly injections followed by continued treatment at intervals increasing by 2 weeks per visit once visual acuity was stable.

**Further treatments were given if any of the following changes applied: best corrected visual acuity (BCVA) loss of at least five letters associated with fluid within the macula as evaluated by OCT, central macular thickness (CMT) increase of at least 100 mm, and/or persistence of fluid within the macula as evaluated by OCT, new onset macular haemorrhages, persistence of leakage from the lesions on fluorescein angiography.

°In the maintenance phase, the interval of injections is extended by 2 weeks if there is no exudative change, The scheduled treatment interval is extended to a maximum of 12 weeks in the current study.

***After a loading phase, patients were scheduled to attend the hospital every 1 month to 2 months. In some of the cases without long-term recurrence, the follow-up period was extended up to 3 months at the discretion of the treating physician.

§Treatment decisions, such as the choice of drug and frequency and timing of treatment, were entirely at the discretion of the practitioner in consultation with the patient, thereby reflecting real-world practice. Only eyes that had received at least three injections in the first year of treatment were included in the study.

°°Retreatment (initially scheduled at Weeks 12–14) was extended by 2 weeks per visit (in relation to the period since the last visit) to a maximum of 12 weeks if no evidence of exudative disease activity was observed. If there were signs of exudative disease, the patient was retreated and the next visit was 4 weeks later.

received a full-text evaluation, of which 55 were excluded because they did not meet inclusion criteria. Thirty-four studies were included.

3.1 Characteristics of included studies

Of the 34 included studies, 24 reported on anti-VEGF therapy alone (Engelbert et al., 2009; Montero et al., 2009; Atmani et al., 2010; Hemeida et al., 2010; Reche-Frutos et al., 2011; Parodi et al., 2013; Gharbiya et al., 2014; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Cho et al., 2016; Matsumoto et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Hata et al., 2017; Kim et al., 2018; Browning et al., 2019; Invernizzi et al., 2019; Kim et al., 2019; Maruyama-Inoue et al., 2019; Arias et al., 2020; Ernest et al., 2020; Kim, 2020; Kim et al., 2020), 8 reported on anti-VEGF therapy combined with PDT (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Saito et al., 2012; Saito et al., 2013; Seidel et al., 2013; Saito et al., 2016; Malamos et al., 2018) and 2 studies compared anti-VEGF therapy alone *versus* combined with PDT. (Rouvas et al., 2012; Arias et al., 2016).

3.1.1 Anti-VEGF group

Cohorts from 26 studies were included in the anti-VEGF group, with a total of 1,221 eyes. Characteristics of included studies are shown in Table 1. Publication year ranged from 2009 to 2020. Of 26 studies, 15 were retrospective, (Engelbert et al., 2009; Montero et al., 2009; Hemeida et al., 2010; Inoue et al., 2014; Park and Roh, 2015; Cho et al., 2016; Matsumoto et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Hata et al., 2017; Kim et al., 2018; Kim et al., 2019; Maruyama-Inoue et al., 2019; Kim, 2020; Kim et al., 2020), 8 were prospective, (Atmani et al., 2010; Reche-Frutos et al., 2011; Gharbiya et al., 2014; Shin and Yu, 2014; Browning et al., 2019; Invernizzi et al., 2019; Arias et al., 2020; Ernest et al., 2020), and 3 were randomized trials. (Rouvas et al., 2012; Parodi et al., 2013; Arias et al., 2016). Two randomized trials compared anti-VEGF therapy alone *versus* PDT combined with anti-VEGF therapy, (Rouvas et al., 2012; Arias et al., 2016), while Parodi et al. (Parodi et al., 2013) compared ranibizumab *versus* bevacizumab. In all studies RAP diagnosis was based on fluorescein and indocyanine green angiography. Nine studies reported on ranibizumab only, (Atmani et al., 2010; Reche-Frutos et al., 2011; Rouvas et al., 2012; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Arias et al., 2016; Kim et al., 2017a; Kim et al., 2017b), 4 studies on aflibercept only (Matsumoto et al., 2016; Browning et al., 2019; Arias et al., 2020; Ernest et al., 2020) and one study on bevacizumab only; (Montero et al., 2009); 5 studies reported on both ranibizumab and bevacizumab, (Engelbert et al., 2009; Hemeida et al., 2010; Parodi et al., 2013; Gharbiya et al., 2014; Cho et al., 2016), 3 studies on both ranibizumab and aflibercept (Hata et al., 2017; Kim et al., 2018; Maruyama-Inoue et al., 2019) and 4 studies on all three anti-VEGF agents. (Invernizzi et al., 2019; Kim et al., 2019; Kim, 2020; Kim et al., 2020). Twenty-two studies included only naïve eyes (Engelbert et al., 2009; Montero et al., 2009; Atmani et al., 2010; Rouvas et al., 2012; Parodi et al., 2013; Gharbiya et al., 2014; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Cho et al., 2016; Matsumoto et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Hata et al., 2017; Kim et al., 2018; Browning et al., 2019; Invernizzi et al., 2019; Maruyama-Inoue et al., 2019; Arias et al., 2020; Ernest et al., 2020; Kim, 2020; Kim et al., 2020), 2 studies included non-naïve eyes, (Reche-Frutos et al., 2011; Arias et al., 2016), and two studies did not provide information about previous treatment. (Hemeida et al., 2010; Kim et al., 2019). In all studies but one (Engelbert et al., 2009; Montero et al., 2009; Atmani et al., 2010; Reche-Frutos et al., 2011; Parodi et al., 2013; Gharbiya et al., 2014; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Cho et al., 2016; Matsumoto et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Hata et al., 2017; Kim et al., 2018; Browning et al., 2019; Invernizzi et al., 2019; Kim et al., 2019; Maruyama-Inoue et al., 2019; Arias et al., 2020; Ernest et al., 2020; Kim, 2020; Kim et al., 2020), a loading phase of 3 monthly injections was administered at baseline, followed by the selected regimen. Hemeida et al. (Hemeida et al., 2010) gave only one injection at baseline, which was followed by a pro re nata (PRN) protocol. A PRN was adopted in 20 trials (Montero et al., 2009; Atmani et al., 2010; Hemeida et al., 2010; Reche-Frutos et al., 2011; Rouvas et al., 2012; Parodi et al., 2013; Gharbiya et al., 2014; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Arias et al., 2016; Cho et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Kim et al., 2018; Kim et al., 2019; Maruyama-Inoue et al., 2019; Kim, 2020; Kim et al., 2020), while 3 studies used a treat and extend regimen (Engelbert et al., 2009; Matsumoto et al., 2016; Arias et al., 2020) and 2 a fixed regimen with bimonthly injections. (Browning et al., 2019; Ernest et al., 2020). Hata et al. (Hata et al., 2017) used two different treatment protocols according to the anti-VEGF agent: a PRN regimen was used in the ranibizumab arm, while a fixed bimonthly regimen was used in aflibercept arm. In 19 out of 20 studies which followed a PRN regimen, retreatment was performed with a single intravitreal injection, while in one study (Rouvas et al., 2012) retreatment consisted of 3 more monthly intravitreal injections.

Follow-up period ranged from 12 months to 48 months. Twenty-two studies (Engelbert et al., 2009; Montero et al., 2009; Atmani et al., 2010; Hemeida et al., 2010; Reche-Frutos et al., 2011; Parodi et al., 2013; Inoue et al., 2014; Shin and Yu, 2014; Park and Roh, 2015; Arias et al., 2016; Cho et al., 2016; Matsumoto et al., 2016; Kim et al., 2017a; Kim et al., 2017b; Hata et al., 2017; Kim et al., 2018; Browning et al., 2019; Invernizzi et al., 2019; Kim et al., 2019; Arias et al., 2020; Ernest et al., 2020; Kim et al., 2020) provided data on 12-month follow-up, while 4 studies (Rouvas et al., 2012; Gharbiya et al., 2014; Maruyama-Inoue et al., 2019; Kim, 2020) did not report 12-month outcomes, providing only outcomes at 24 months or longer.

3.1.2 Anti-VEGF combined with PDT

Cohorts from 10 studies were included in the combined group, with a total of 159 eyes. Characteristics of included studies are shown in Table 2. Publication year ranged from 2010 to 2018. Of these 10 studies, 5 were retrospective (Saito et al., 2010; Nakano et al., 2012; Saito et al., 2012; Saito et al., 2013; Saito et al., 2016), 3 were prospective (Lee et al., 2011; Seidel et al., 2013; Malamos et al., 2018) and two were randomized trials. (Rouvas et al., 2012; Arias et al., 2016). The two randomized trials compared anti-VEGF therapy alone *versus* PDT combined with anti-VEGF therapy. In all studies, RAP diagnosis was based on fluorescein and indocyanine green angiography. PDT was combined with intravitreal ranibizumab in 8 studies (Lee et al., 2011; Nakano et al., 2012; Rouvas et al., 2012;

TABLE 2 Characteristics of included studies in the combined group.

Author and year	Design	Anti-VEGF drug	Eyes (n)	Gender (M/W)	Age mean (±SD, years/range)	Follow-up (months)	Anti VEGF regimen	Stage	PDT dose/fluence	PDT regimen
Saito et al. (2010)	Retrospective	Bevacizumab	13 naive	8/3	78.3 (range 63–89)	12	baseline +PRN	-5 stage 2 without PED -6 stage 2 with PED -2 stage 3	standard dose and standard fluence	baseline + PRN
Lee et al. (2011)	Prospective	Ranibizumab	10 naive	2/7	76 (range 65–87)	12	3 IV monthly + PRN -Retreatment: 3 more monthly IV		standard dose and standard fluence	baseline + PRN
Saito et al. (2012)	Retrospective	Ranibizumab	20 naive	8/8	84.8 ± 4.8	12	3 IV monthly + PRN -Retreatment: 1 IV	-11 stage 2 without PED -7 stage 2 with PED -2 stage 3	standard dose and standard fluence	baseline + PRN
Rouvas et al. (2012)	Randomized controlled trial	Ranibizumab	13	4/9	77.12	36	3 IV monthly + PRN -Retreatment: 3 IV monthly	13 stage 2	standard dose and standard fluence	baseline + PRN
Nakano et al. (2012)	Retrospective	Ranibizumab	11 naive	4/7	80.3 ± 7.2	12	1 IV at baseline +PRN -Retreatment: 1 IV	-3 stage 1 -5 stage 2 -3 stage 3	standard dose and standard fluence	baseline + PRN
Author and year	Design	Anti-VEGF drug	Eyes (n)	Gender (m/w)	Age mean (±SD, years/range)	Follow-up (months)	Anti VEGF regimen	Stage	PDT dose/fluence	PDT regimen
Saito et al. (2013)	Retrospective	Bevacizumab/ Ranibizumab.	13 naive	7/5	77.0 ± 9.5	24	1 IV Bevacizumab at baseline + PRN -Retreatment: Before February 2009: 1 IV Bevacizumab After March 2009: 3 monthly Ranibizumab	-7 stage 1 without PED -5 stage 2 with PED -1 stage 3	Standard dose and standard fluence	Baseline + PRN

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TABLE 2 (*Continued*) Characteristics of included studies in the combined group.

Author and year	Design	Anti-VEGF drug	Eyes (n)	Gender (m/w)	Age mean (±SD, years/range)	Follow-up (months)	Anti VEGF regimen	Stage	PDT dose/fluence	PDT regimen
Seidel et al. (2013)	Prospective	Ranibizumab	15 naive	4/10	79.7 ± 4.7	12	1 IV at baseline + PRN -Retreatment: 1 IV	-7 stage 1 -4 stage 2a -4 stage 2b	Standard dose and standard fluence	Baseline only
Saito et al. (2016)	Retrospective	Ranibizumab	37 naive	12/19	82.0 ± 6.3	24	3 monthly IV + PRN -Retreatment: 1 IV	-2 stage 1 -20 stage 2 without PED; -13 stage 2 with PED - 2 stage 3	Standard dose and standard fluence	Baseline + PRN 1
Arias et al. (2016)	Randomized controlled trial	Ranibizumab	10 7 naive	6/4	79.2 ± 3.7	12	3 monthly IV +PRN -Retreatment: 1 IV (followed monthly)*	-3 stage 1 -5 stage 2 -2 stage 3	-	Baseline + PRN (followed monthly) *
Malamos et al. (2018)	Prospective	Ranibizumab	17 13 naive	-	80.7	24.7	1 IV at baseline + PRN -Retreatment: 1 IV	-3 stage 1 -4 stage 2 -8 stage 3	Half dose standard fluence	Baseline only

N, number; M/W: men/women; SD, standard deviation; IV, intravitreal; RAP, retinal angiomatous proliferation; PRN, *pro re nata*; PDT, photodynamic therapy; PED, pigmented epithelium detachment.
*All retreatments in group B consisted of combined therapy of a single intravitreal injection of ranibizumab and PDT with verteporfin. In addition, in group B, ranibizumab 0.5 mg could be administered in monotherapy as rescue therapy, if necessary.

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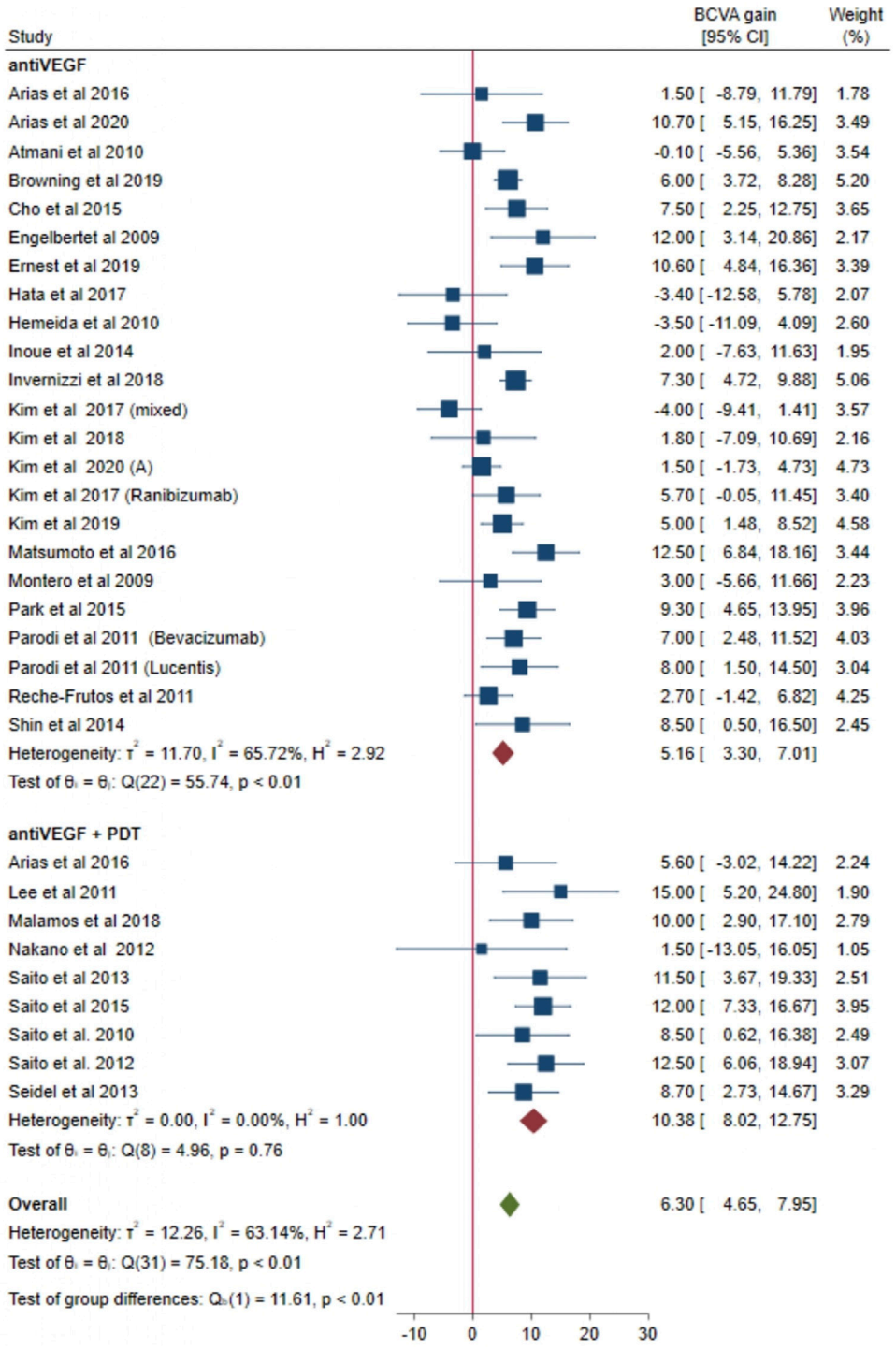

FIGURE 2
Comparison of best corrected visual acuity (BCVA) gain after 1 year of treatment with anti-VEGF alone or in combination with photodynamic therapy (PDT).

Saito et al., 2012; Seidel et al., 2013; Arias et al., 2016; Saito et al., 2016; Malamos et al., 2018), with bevacizumab in one study (Saito et al., 2010) and with either ranibizumab or bevacizumab in another one. (Saito et al., 2013). Seven studies included only naïve eyes (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Rouvas et al., 2012; Saito et al., 2012; Saito et al., 2013; Saito et al., 2016), 3 studies included non-naïve eyes. (Seidel et al., 2013; Arias et al., 2016; Malamos et al., 2018). A loading phase of 3 monthly injections was administered in 5 studies (Lee et al., 2011; Rouvas et al., 2012; Saito et al., 2012; Arias et al., 2016; Saito et al., 2016), while a single

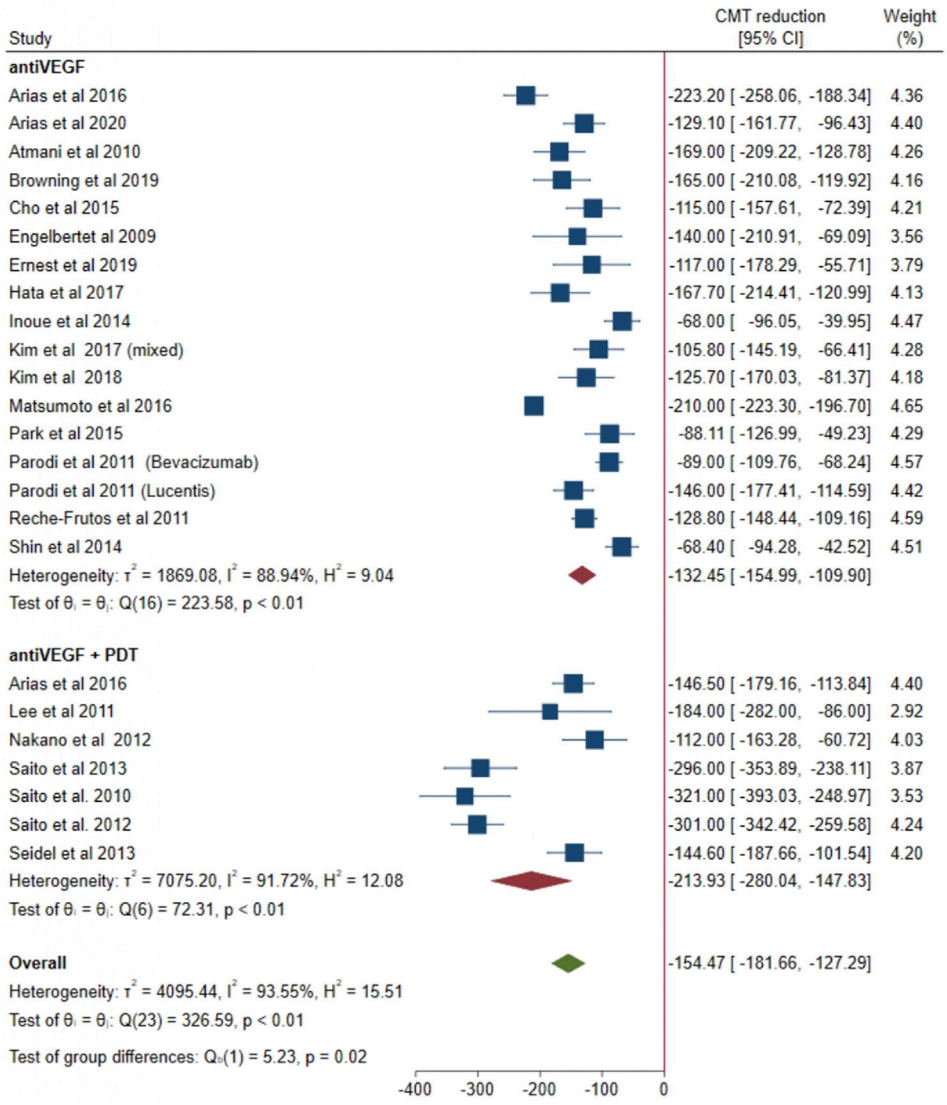

FIGURE 3
Comparison of central macular thickness (CMT) reduction after 1 year of treatment with anti-VEGF alone or in combination with photodynamic therapy (PDT).

intravitreal injection was give as loading phase in the remaining 5 studies. (Saito et al., 2010; Nakano et al., 2012; Saito et al., 2013; Seidel et al., 2013; Malamos et al., 2018). A PRN regimen was used in all studies. (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Rouvas et al., 2012; Saito et al., 2012; Saito et al., 2013; Seidel et al., 2013; Arias et al., 2016; Saito et al., 2016; Malamos et al., 2018). Retreatment was done with either 3 more 4-weekly injections (Lee et al., 2011; Rouvas et al., 2012; Saito et al., 2013) or a single intravitreal injection. (Saito et al., 2010; Nakano et al., 2012; Saito et al., 2012; Seidel et al., 2013; Arias et al., 2016; Saito et al., 2016; Malamos et al., 2018). As regards PDT, a standard-dose and standard-fluence PDT was used in 8 studies, (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Rouvas et al., 2012; Saito et al., 2012; Saito et al., 2013; Seidel et al., 2013; Saito et al., 2016), in one study a half-dose and standard-fluence PDT was performed, (Malamos et al., 2018), Arias et al. did not report information on PDT parameters. (Arias et al., 2016). In 8 studies PDT was performed both at baseline and at each retreatment (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Rouvas et al., 2012; Saito et al., 2012; Saito et al., 2013; Arias et al., 2016; Saito et al., 2016), while in 2 studies PDT was performed only at baseline. (Seidel et al., 2013; Malamos et al., 2018). Follow-up period ranged from 12 months to 38 months. All studies but one provided data on 12-month outcomes. (Saito et al., 2010; Lee et al., 2011; Nakano et al., 2012; Saito et al., 2012; Saito et al., 2013; Seidel et al., 2013; Arias et al., 2016; Saito et al., 2016; Malamos et al., 2018). Only Rouvas et al. (Rouvas et al., 2012) did not report on 12-month follow-up, providing outcomes at 36 months only.

TABLE 3 Meta-regressions showing the effect of the number of injections on BCVA and CMT outcomes.

Outcome and treatment group	Effect of the number of injections, β (standard error)	*p*-value
BCVA		
Overall	0.16 (0.49)	0.741
anti-VEGF	0.74 (0.61)	0.216
anti-VEGF combined with PDT	0.02 (1.25)	0.984
CMT		
Overall	10.32 (8.07)	0.201
anti-VEGF	−11.34 (8.15)	0.164
anti-VEGF combined with PDT	18.16 (30.04)	0.546

Footnote: BCVA, best corrected visual acuity; CMT, central macular thickness; PDT, photodynamic therapy.

3.2 Quality assessment

Risk of bias assessment for randomized studies is illustrated in Supplementary Figures S1, S2. Random sequence generation was deemed as low risk in one trial (Parodi et al., 2013) and unclear risk in 2 trials. (Rouvas et al., 2012; Arias et al., 2016). Risk of allocation concealment bias was unclear for all randomized trials. (Rouvas et al., 2012; Parodi et al., 2013; Arias et al., 2016). Risk of both performance bias and detection bias was judged high in one trial (Rouvas et al., 2012) and unclear for the two other. (Parodi et al., 2013; Arias et al., 2016). Attrition bias was considered low in all randomized trials. (Rouvas et al., 2012; Parodi et al., 2013; Arias et al., 2016). Reporting bias was judged as low risk in 2 trials, (Parodi et al., 2013; Arias et al., 2016), while Rouvas's study (Rouvas et al., 2012) was considered as high risk because the primary outcome of the trial was not pre-specified and the outcomes of interest of the present systematic review were not reported. Risk for other bias was unclear in 2 trials, (Rouvas et al., 2012; Parodi et al., 2013), while Arias's study (Arias et al., 2016) was judged as high risk because it failed to achieve the sample size that was initially planned. The MINORS scale assessment for non-randomized studies is shown in Supplementary Table S2, with all studies achieving a ≥10 score.

The inspection of funnel plots did not allow to completely exclude the presence of publication bias, especially for the CMT outcome (Supplementary Figures S3, S4).

3.3 Best corrected visual acuity change

Data from 22 and 9 studies were pooled together to explore mean visual change at 12 months in the anti-VEGF group and in the combined group, respectively (Figure 2). Overall, considering both groups together, a mean gain of 6.30 letters was evident at 12 months (95% CI = 4.65–7.95). A high heterogeneity was found when considering both groups together (I^2 = 63.14%; $p < 0.01$). In the anti-VEGF group, a mean gain of 5.16 letters was shown at 12 months (95% CI = 3.30–7.01). A significant heterogeneity was found across studies included in the anti-VEGF group (I^2 = 65.72%; $p < 0.01$). In the combined group, a mean gain of 10.38 letters was found at 12 months (95% CI = 8.02–12.75). Heterogeneity was low in the combined group (I^2 = 0%; $p = 0.76$). Of note, visual gain in the combined group was higher compared with the anti-VEGF group (10.38 *versus* 5.16, respectively) and 95% CI of the combined group does not overlap with those of the anti-VEGF group. Accordingly, the test of group differences revealed that 12-month visual gain in the combined group was significantly greater compared with the anti-VEGF group ($p < 0.01$).

3.4 Central macular thickness change

Data from 16 and 7 studies were pooled together to explore mean CMT change at 12 months in the anti-VEGF group and in the combined group, respectively (Figure 3). Overall, considering both groups together, a mean CMT reduction of 154.47 μm was shown at 12 months (95% CI = from −181.66 to −127.29). A high heterogeneity was found when considering both groups together (I^2 = 93.55%; $p < 0.01$). In the anti-VEGF group, a mean CMT reduction of 132.45 μm was found at 12 months (95% CI = from −154.99 to −109.90). A significant heterogeneity was shown across studies included in the anti-VEGF group (I^2 = 88.94%; $p < 0.01$). In the combined group, a mean CMT reduction of 213.93 μm was evident at 12 months (95% CI = from −280.04 to −147.83). Heterogeneity was high in the combined group as well (I^2 = 91.72%; $p < 0.01$). Of note, at 12 months a greater CMT reduction was shown in the combined group compared with the anti-VEGF group (−213.93 μm *versus* −132.45 μm, respectively) and 95% CI of the combined group does not overlap with those of the anti-VEGF group. Accordingly, the test of group differences demonstrated a significantly greater CMT decrease in the combined group compared with the anti-VEGF group ($p = 0.02$).

3.5 Influence of injection number

The average number of injections over a 12-month follow-up was higher in the anti-VEGF group compared with the combined

group: a mean of 4.9 injections (95% CI = 4.2–5.6) were administered in the anti-VEGF group while a mean of 2.8 injections (95% CI = 1.3–4.4) were administered in the combined group ($p = 0.02$).

Meta-regression analyses showed no influence of injection number on visual and CMT outcomes in either group and overall considering both groups together (Table 3).

4 Discussion

This meta-analysis investigated functional and anatomical outcomes of intravitreal anti-VEGF therapy alone or combined with PDT in eyes with RAP, comparing these two different therapeutic options. In summary, our findings showed that anti-VEGF therapy combined with PDT provided a better visual gain and a greater CMT reduction compared with anti-VEGF therapy alone over a 12-month follow-up.

Treatment of RAP lesions could represent a challenge for medical retina physicians because this type of neovascular membranes may show a poor or incomplete response to traditional intravitreal anti-VEGF drugs. (Viola et al., 2009; Tsai et al., 2017). This behavior could be related to the anatomical and pathogenetic characteristics of RAP. (Ghazi and Conway, 2005; Haj Najeeb et al., 2021).

Many published studies have shown that intravitreal anti-VEGF therapy is effective in improving visual outcomes and in reducing vascular leakage and retinal oedema. (Costagliola et al., 2007; Viola et al., 2009; Tsai et al., 2017). Even if recent studies showed a better response to anti-VEGF drugs of RAP lesions when compared to other forms of neovascularization, (Browning et al., 2019; Invernizzi et al., 2019), RAP lesions, in some cases, could require an intense and prolonged treatment due to frequent recurrence of membrane activity. (Rouvas et al., 2012; Saito et al., 2012; Gharbiya et al., 2014; Inoue et al., 2014). Additionally, this type of neovascular membrane has been shown to remain active in most patients on a long-term follow-up. (Costagliola et al., 2007; Gupta et al., 2010; Tsai et al., 2017). Different anti-VEGF agents have been used for RAP treatment with different therapeutic regimens, such us fixed, as needed (pro re nata) and treat-and-extend. (Tsai et al., 2017; Fallico et al., 2020b).

In this scenario, a combined therapy with intravitreal anti-VEGF agents and verteporfin PDT could offer advantages over anti-VEGF monotherapy, slowing or completely blocking the neovascularization process. (Tsai et al., 2017).

The mechanism of action of PDT is based on the activation of verteporfin by a light source with subsequent release of free radicals in the treatment site, specifically in the choriocapillaris. (Bressler, 2001). This process leads to endothelial cell damage and choriocapillaris hypoperfusion. The treatment is highly selective and photoreceptors are spared. (Boscia et al., 2006). After the advent of anti-VEGF therapy, the role of PDT has been significantly downsized. Variations of standard PDT protocol have been introduced in order to reduce the risk of persistent choriocapillaris hypoperfusion and RPE changes. (Reibaldi et al., 2010). Currently, the most commonly adopted protocols are either half-dose PDT or half-fluence PDT, which are mainly used for the treatment of chronic central serous chorioretinopathy (CSC). (Reibaldi et al., 2010). Photodynamic therapy has also been combined with intravitreal therapy for the treatment of choroidal neovascular membranes, including RAP lesions and polypoidal choroidal vasculopathy. (Lim et al., 2020).

Type 3 neovascular membranes are considered as “high-flow” lesions. The rationale of a combined therapy (PDT plus intravitreal anti-VEGFs) lies in a synergic mechanism of action of these two therapeutic approaches. In fact, PDT could induce complete occlusion of the retinal–retinal anastomosis, while intravitreal anti-VEGF therapy could counteract the release of VEGF caused by the PDT-related hypoxia in the choriocapillaris. (Saito et al., 2010; Seidel et al., 2013).

Saito et al. demonstrated a complete occlusion of the retinal–retinal anastomosis in 89.5% of RAP cases treated with this combined therapy. (Saito et al., 2012). However, the evidence of supporting combined approach in RAP treatment is mostly based on small-sized retrospective studies. Furthermore, only two randomized trials compared intravitreal anti-VEGF therapy alone *versus* intravitreal anti-VEGF therapy combined with PDT in RAP eyes. (Rouvas et al., 2012; Arias et al., 2016). Both of these trials were small-sized with less than 15 eyes for each treatment arm. (Rouvas et al., 2012; Arias et al., 2016). Additionally, results of these randomized trials are controversial. On the one hand, Rouvas et al. did not demonstrate any improvement in visual and anatomical outcomes following combined therapy after a 3-year follow-up. (Rouvas et al., 2012). On the other hand, Arias et al. reported a better visual gain in eyes receiving combined therapy, but failed to show any statistical significance. (Arias et al., 2016).

To the best of our knowledge, no previous systematic review has compared the visual and anatomical outcome of combined therapy of anti-VEGF plus PDT *versus* intravitreal anti-VEGF therapy alone in RAP eyes. Tsai et al. (Tsai et al., 2017) performed a review focused on diagnostic and treatment options for RAP. Besides, the authors conducted a meta-analysis of 9 included studies exploring mean change in visual acuity and central foveal thickness, but no comparison between different treatment approaches was made. (Tsai et al., 2017).

Our analyses revealed a better visual improvement in eyes treated with anti-VEGF therapy combined with PDT. Mean visual gain in the combined group was was more than two-fold higher compared with the mean gain of the anti-VEGF monotherapy group. Looking at 95% confidence intervals, the minimal improvement in the combined group (7.66 letters) was yet superior to the maximum improvement in the anti-VEGF alone group (6.56 letters), confirming that the combined approach yielded better visual results.

With regard to macular thickness, the results of the two randomized trials showed a comparable final CMT between eyes treated with anti-VEGF monotherapy and eyes treated with anti-VEGF therapy combined with PDT. (Rouvas et al., 2012; Arias et al., 2016). Conversely, our analyses on 12-month CMT outcome revealed a greater reduction of macular thickness in the combined group compared to the anti-VEGF monotherapy group.

Our findings showed that combined therapy with PDT and intravitreal anti-VEGFs could yield better outcomes with a lower number of injections, thanks to their different and synergic mechanism of action.

This may also have a positive impact on the financial burden, on the anxiety of patients and reduce the risk of injection-related complications and side effects. (Reibaldi et al., 2020; Reibaldi et al., 2022).

In the present meta-analysis, combined therapy has also shown a good safety profile despite PDT being used in all studies except for one with standard dose and fluence.

In light of a widespread use of PDT at lower dose and fluence, further studies are needed to investigate efficacy and safety of a combined approach using modified PDT protocols (half dose or half fluence) for RAP lesions.

In the present meta-analysis, no study on brolucizumab was included. There is paucity of data in literature on the use of brolucizumab in the treatment of RAP lesions. Only a retrospective case series on 12 eyes showed a good short-term efficacy of brolucizumab in reducing the size of type 3 neovascular membranes. (Gigon et al., 2022).

The present study presents some limitations. First, significant heterogeneity was found among included studies. This could limit the strength of our findings. A possible reason for high heterogeneity could be the variability in clinical characteristics and treatment protocols between included studies. However, all studies based RAP diagnosis on fluorescein and indocyanine green angiography and most included studies adopted a protocol treatment based on 3 monthly injections followed by a pro re nata regimen. Second, only two randomized clinical trials were included in this systematic review, of which only one provided data included in our pooled analyses. Furthermore, quantitative analyses were carried out from tabulated data extracted from each study because no individual data was available. However, confidence intervals yielded by meta-analysis studies are more powered and more accurate compared with individual studies. (Fallico et al., 2020c; Fallico et al., 2021). Finally, we could conduct meta-analyses only on data from a 12-month follow-up because data at a longer follow-up were provided by few studies. Pooled analyses of data with a long-term follow-up could have offered further insights in this issue and help to understand whether a combined approach could maintain functional and anatomical advantages in a such long-term. In conclusion, our analyses revealed, even if with a limited evidence, that the use of a combined approach with intravitreal anti-VEGF therapy and PDT could provide better functional and anatomical outcomes in RAP treatment. Such a combined approach seems to reduce the number of anti-VEGF injections, which could be a relevant advantage for both healthcare provider and patients. Further large randomized trials are needed to corroborate these findings and to investigate the role of new anti-VEGF drugs in this scenario.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization: VB, IM, and MF methodology: AR and AL literature search: MV, NC, and CP data curation: MR, FP, and MN statistical analysis: AM, GF, MB, and RM writing original draft preparation: IM, VB, and MF writing-review and editing: all authors supervision: TA and VB. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GR declared a shared affiliation with the authors MF, AM, GF, MB, RM, AA, AR, AL, TA, and NC to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1141077/full#supplementary-material

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