ORIGINAL RESEARCH

Prospective, Single-Center, Six-Month Study of Intravitreal Ranibizumab for Macular Edema with Nonproliferative Diabetic Retinopathy: Effects on Microaneurysm Turnover and Non-Perfused Retinal Area

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Purpose: To analyze the effects on microaneurysm (MA) and perifoveal perfusion in nonproliferative diabetic retinopathy (NPDR) patients with macular edema (ME) after early intensive treatment using intravitreal ranibizumab (IVR) injections.

Patients and Methods: Prospectively, 25 eyes of 25 type 2 diabetes mellitus patients with ME were included between August 2016 and February 2019. For 6 months, patients were administered 0.5-mg IVR injections monthly. Ocular evaluation, including best-corrected visual acuity (BCVA; using the Early Treatment Diabetic Retinopathy Study chart), central retinal thickness (CRT; using optical coherence tomography), fundus photography, and fluorescein angiography, was performed for all participants. Results obtained at baseline were compared to those observed after 6 months.

Results: Mean BCVA increased significantly from 67.6 ± 3.29 letters at baseline to 76.36 ± 1.61 letters after 6 months ($P=0.002$) of IVR therapy. CRT decreased significantly from 479.12 ± 16.66 μm at baseline to 369.12 ± 13.02 μm at 6 months. Similarly, the total number of MAs decreased significantly from 5.68 ± 3.41 to 1.60 ± 1.73 ($P<0.0001$). MA turnover, calculated by adding the MA formation rate to the MA disappearance rate (both calculated as MA number/month) also decreased significantly from 6.88 ± 3.83 to 1.92 ± 1.75 after treatment ($P<0.0001$). Perifoveal non-perfused area decreased from 2.517 ± 0.456 mm^2 at baseline to 2.495 ± 0.293 mm^2 at 6 months, but the results were not statistically significant ($P=0.954$).

Conclusion: Treatment with early intensive IVR therapy in NPDR patients with ME not only improved BCVA and CRT but also decreased MA turnover. However, in the study period of 6 months, IVR therapy did not show significant improvement in perifoveal non-perfused area.

Keywords: nonproliferative diabetic retinopathy, macular edema, ranibizumab, early intensive treatment, microaneurysm

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Introduction

Increased prevalence of diabetes mellitus has roused interest in its ophthalmic sequelae such as diabetic retinopathy (DR) and diabetic macular edema (DME).[1] DR and DME are major causes of visual impairment in millions of patients worldwide,[2] and there are numerous ongoing studies on the efficacy of various

treatment modalities for these conditions. While multiple causes of retinopathy have been implicated, vascular endothelial growth factor (VEGF) seems to play an important role in the pathogenesis of these conditions.[3,4] DR, in both proliferative and nonproliferative stages, shows an increase in VEGF production,[5] which causes structural changes in the retinal vessels, leading to subsequent increase in vessel permeability and angiogenesis, and ultimately, visual impairment.[6]

Panretinal laser photocoagulation (PRP) and focal laser treatment have played important roles in preventing the progression of DR and in alleviating DME. However, PRP can cause permanent impairment of peripheral vision, night blindness, and deterioration of DME. Further, it cannot be performed in certain patients with concomitant cataract or vitreous hemorrhage.[7] Focal laser treatment can also be utilized in selected patients only.[8] To address these shortcomings, anti-VEGF therapy has been proposed for the management of both DR and DME.[9] Recently, intravitreal, injectable, anti-VEGF antibody has been reported to delay the progression and ameliorate the severity of both DR and DME.[10–13]

Results of multi-center, randomized-controlled clinical trials such as RIDE, RISE, and RESOLVE studies have shown the efficacy of intravitreal ranibizumab (IVR) injection monotherapy in the treatment of DME, based on markers such as best-corrected visual acuity (BCVA) and central retinal thickness (CRT).[14–17] Furthermore, many recent studies have shown that the formation and disappearance of microaneurysms (MAs), as well as the extent of perifoveal non-perfused area, can also be used as markers to predict the progression and prognosis of DME.[18–25]

In this prospective study, we evaluated the efficacy of IVR therapy by assessing the response to monthly administration of IVR injections for 6 months in nonproliferative diabetic retinopathy (NPDR) patients with DME. Changes in BCVA and CRT, as compared to the baseline, were evaluated monthly, for 6 months. Accompanying markers such as changes in total number of MAs, MA turnover, and extent of perifoveal non-perfused area were also analyzed and compared to their pre-treatment values.

Materials and Methods
Study Design
This was an interventional, prospective, single-center study conducted at Wonkwang University Hospital from August 2016 to February 2019.

All patients included in the study provided signed, informed consents prior to enrollment. The consent statement included the purpose and method of the study as well as information on reasonable expected benefits along with potential risks and side effects.

The study protocol was approved by the Institutional Review Board of the Wonkwang University Hospital (Approval no: WKUH 201,602-CTDG-005). The clinical trial has been registered on ClinicalTrials.gov (ID: NCT02834663). This study abided by the principles defined in the Declaration of Helsinki throughout the trial.

Method
All patients underwent the following baseline ophthalmological examinations and investigations: i) Assessment of BCVA using the Early Treatment Diabetic Retinopathy Study (ETDRS) chart, ii) measurement of intraocular pressure, iii) optical coherence tomography (OCT) (Spectralis OCT®: Heidelberg engineering, Heidelberg, Germany), iv) fundus photography (Kowa nonmyd 7®: Kowa American Corporation, Kowa, USA), and v) fluorescein angiography (FA) (SpectralisHRA2®: Heidelberg engineering, Heidelberg, Germany).

Patients were administered 0.5-mg IVR injections monthly for 6 months. At each visit, the above-mentioned ophthalmological tests (except FA) were repeated. FA was performed at baseline and was repeated after 3 and 6 months of treatment.

Enrollment Criteria
NPDR patients aged >40 years, having type 2 diabetes mellitus, with BCVA of the study eye being ≥20/200 (Snellen equivalent using ETDRS testing), and having CRT of ≥300 μm on OCT were included in this study. Patients with proliferative diabetic retinopathy (PDR), previous history of vitreoretinal surgery, post-cataract operation status (≤4 months before participation in this study), uncontrolled hypertension, or glaucoma were excluded from the study. Patients who had received prior treatment with anti-VEGF drugs, intraocular corticosteroids, and/or retinal laser application were also excluded. If both eyes met the study inclusion criteria, only the more severely affected eye was selected for treatment, based on BCVA.

Assessment of Treatment Efficacy
Patients were evaluated for changes in BCVA and CRT of the treated eye, from the start of the study to 6 months, until trial completion.

The MAs and perifoveal non-perfused areas in individual retinas were evaluated at 6 months using fundus photography and FA imaging. The Retmarker (version 1.0.2 by Retmarker Ltd, Coimbra, Portugal) software was used for automatic measurement and analysis of changes in number and extent of MAs on fundus photographs and to calculate the total number and turnover of MAs. It includes a co-registration algorithm that allows comparison within the same retinal location between different visits for the same eye. MA turnover was calculated by adding the MA formation rate (number of new MAs detected/month) to the MA disappearance rate (number of MAs resolved/month) (Figure 1).[20–22] Perifoveal non-perfused area was analyzed using the arteriovenous phase in the FA image (from each of the three examinations) when perifoveal capillary network was best visualized, that is, approximately 30 seconds after injection, and was estimated by connecting the blood vessels instead of the branches of superior and inferior temporal venules within 30 degrees. Subsequently, the ImageJ software (version 1.25a 23/04/2018 by ImageJ, USA) was used for scaling each image by 16 pixels, down to 200 μm. Thereafter, the contrast and sensitivity of all photographs were converted to the maximum values to compare the perfused and non-perfused areas. The raw red-green-blue (RGB) images were then converted to 8-bit images, with the threshold set for optimal visualization of the non-perfused area. Thus, FA images that needed analysis were converted to raw RGB images, the program automatically converted the thresholds, and one retina specialist assessed the image for accuracy. The same threshold was applied to all images of the same patient. Subsequently, particle analysis was performed to calculate the total perifoveal non-perfused area (Figure 2).

Statistical Analysis

The SPSS Statistics 18 (IBM, Armonk, NY, USA) software was used for data analysis. The repeated measures analysis of variance with Bonferroni correction was used to analyze continuous outcome measures, including BCVA, CRT, total number of MAs, MA formation rate, MA disappearance rate, MA turnover, and perifoveal non-perfused area, before and after completion of 6 months of IVR therapy. A P-value <0.05 was considered statistically significant.

Results

Baseline Characteristics

Totally, 25 eyes of 25 patients (13 males, 12 females; mean age, 63.80±11.45 years; range, 41–80 years) were

Figure 1 Comparison of new and disappeared microaneurysms on fundus photograph for detecting diabetic retinopathy using Retmarker (version 1.0.2 by Retmarker Ltd, Coimbra, Portugal) automated software.
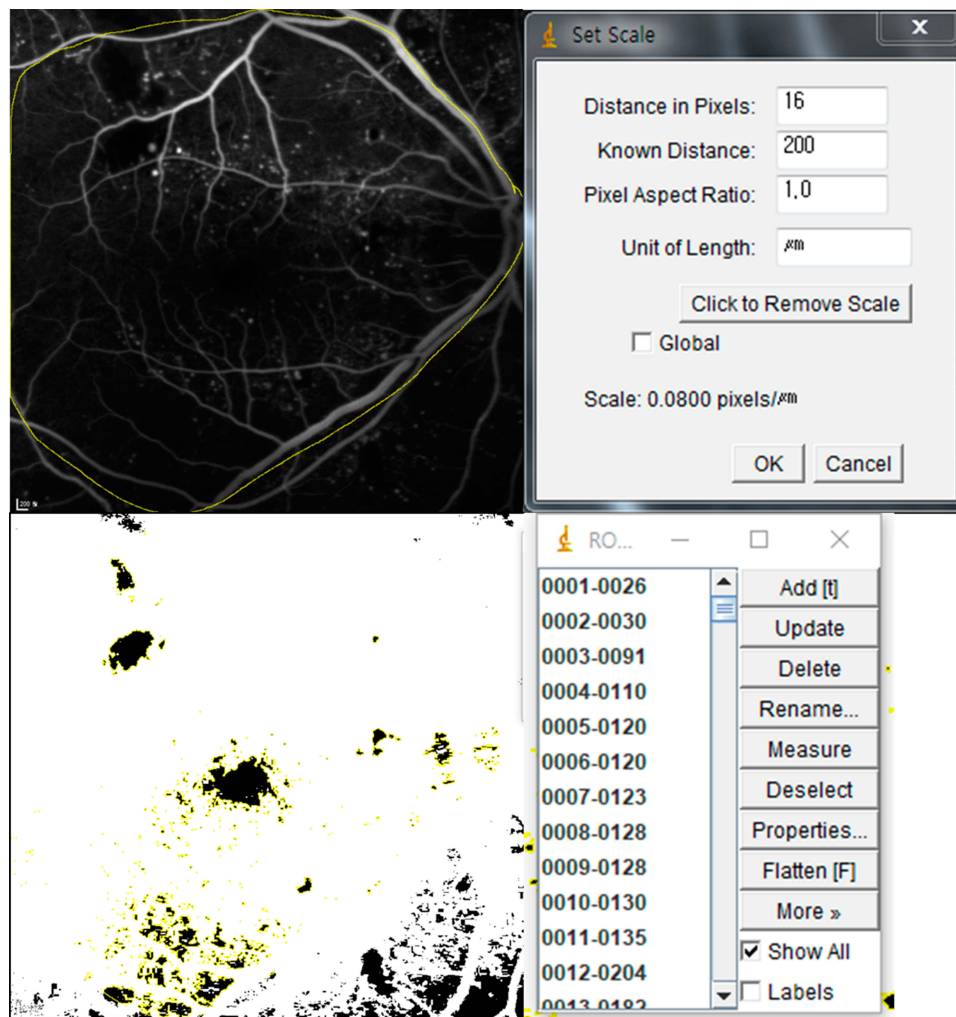
Figure 2 Comparison of changes in non-perfused retinal area estimated using perifoveal fluorescein angiography. The angiography image is analyzed using ImageJ software (version 1.52a 23/04/2018 by ImageJ, National Institutes of Health, Wayne Rasband, Bethesda, Maryland, USA), which is an automated system used for accurate image analysis (distance in pixels: 16, known distance: 200μm, pixel aspect ratio: 1.0).

included in the study. Baseline characteristics of all study patients are summarized in Table 1.

BCVA & CRT Outcomes

The mean BCVA (ETDRS letters) at baseline was 67.6 ±3.29 letters, which significantly increased to 76.36±1.61 letters after administration of six monthly IVR injections ($P<0.0001$) (Figure 3).

The associated mean CRT measured at baseline was 479.12±16.66 μm, which significantly decreased to 369.12 ±13.02 μm after completion of the treatment regimen ($P<0.0001$) (Figure 4). Considering the definition of improved CRT as a ≥25% reduction in macular edema, 20/25 eyes included in the study demonstrated ≥25% reduction in macular edema. Of the 20 eyes, 14 showed improvement in CRT with administration of ≤3 IVR

injections, and 3 eyes demonstrated decrease in CRT after administration of ≥4 IVR doses. The remaining 3 eyes showed fluctuating decreases and increases in CRT during the course of treatment, but finally showed definitive improvement after administration of all 6 IVR injections (Figure 5).

Changes in MA Turnover and Perifoveal Non-Perfused Area

The total mean number of MAs before IVR injection was 5.68±3.41, which significantly decreased to 1.60 ±1.73 after administration of six monthly IVR injections ($P<0.0001$) (Figure 6). The MA turnover after the first IVR injection was 6.88±3.83, which significantly reduced to 1.92±1.75 after 6 months ($P<0.0001$) (Figure 7).

Table 1 Baseline Characteristics of All Study Patients

Variable	Data
Total (eye, %)	25
Male	13 (52%)
Female	12 (48%)
Age (years)	
Mean ± SD	63.80 ± 11.45
Range	41–80
Duration of DM (year)	12.04 ± 7.475
HbA1c (%)	
Mean ± SD	8.27 ± 2.11
Range	5.9–13
BCVA (ETDRS letters)	
Mean ± SD	67.60 ± 3.29
Range	10–84
CRT (µm)	
Mean ± SD	479.12 ± 16.66
Range	300–624
Total microaneurysms (number)	
Mean ± SD	5.68 ± 3.41
Range	13.00
Perifoveal non-perfused area (mm^2)	
Mean ± SD	2.517 ± 0.456
Range	0.41–10.86

Note: The values are presented as mean±SD and range unless otherwise indicated.
Abbreviations: SD, standard deviation; BCVA, best-corrected visual acuity; CRT, central retinal thickness; DM, diabetic mellitus; ETDRS, Early Treatment Diabetic Retinopathy Study; HbA1c, glycosylated hemoglobin.

The mean perifoveal non-perfused area measured across three FA tests was 2.517±0.456 mm^2 at baseline, which after administration of three, monthly IVR injections, decreased to 2.156±0.387 mm^2 and after six, monthly IVR injections, increased to 2.495±0.293 mm^2. Compared to baseline, after administration of six, monthly IVR injections, perifoveal non-perfused area showed a mild decrease, though the difference was not statistically significant (P=0.221) (Figure 8) (Table 2).

Safety Outcomes

No ocular or systemic adverse events associated with IVR injections were observed in any participant during the study period.

Discussion

In this study, we prospectively analyzed the outcomes following administration of monthly IVR injections for 6 months in NPDR patients with DME. The patients showed visual improvement by 8.76 letters, after completing the study-treatment protocol, as compared to baseline as well as demonstrated a 105.8-µm reduction in CRT. These results were similar to those of other studies that analyzed the use of anti-VEGF drugs for DR.[14–17] Further, we found that 80% patients showed improvements in CRT after administration of monthly IVR injections for 6 months. An interesting finding was that among these patients, 70% showed improvement in CRT with administration of ≤3 IVR injections and 30% demonstrated improvements in CRT after administration of ≤6 IVR injections. In other words, we imply that during the treatment of NPDR patients with DME, administration of all 6 injections may not be necessary and in some cases, 3 injections could prove to be quite effective.

MAs develop in the early stages of DR, and the total number as well as turnover rate are known to play an important role in identifying and defining the disease cycle. Furthermore, MAs can be a predictive factor for the occurrence of complications that may cause visual impairment by worsening retinopathy or ME.[18,21,26] Formation and disappearance of MAs is a dynamic activity, of which MA disappearance occurs mostly due to platelet and fibrin thrombi, is an irreversible process, and can indicate the occurrence of retinal capillary occlusion and vascular damage. However, it could result in the restructuring of retinal vasculature and capillary-flow bypass.[27,28] Therefore, not only the formation of MAs but also their disappearance should be considered important during the evaluation of MA turnover.

MA counts and MA turnover are good markers for DR progression, and high MA turnover is a risk factor for the development of clinically significant macular edema (CSME) requiring photocoagulation. Further, MA turnover has been shown to be more reliable than MA counts.[18,20] Leicht et al,[29] reported that high MA turnover can result in a higher risk of developing CSME and worsening DR without DME. They also suggested that anti-VEGF therapy using IVR could delay the progression to diabetic fundus and reverse existing fundus changes to restore normality. Limited research exists regarding the effects of VEGF inhibitors such as ranibizumab on MA turnover in NPDR patients with DME, and no prospective study has evaluated the efficacy of intensive treatment using ranibizumab, which has been performed in our study. This is the strength of our study. In this study, we prospectively observed that the total number, formation rate, disappearance rate, and overall turnover of MAs, each reduced
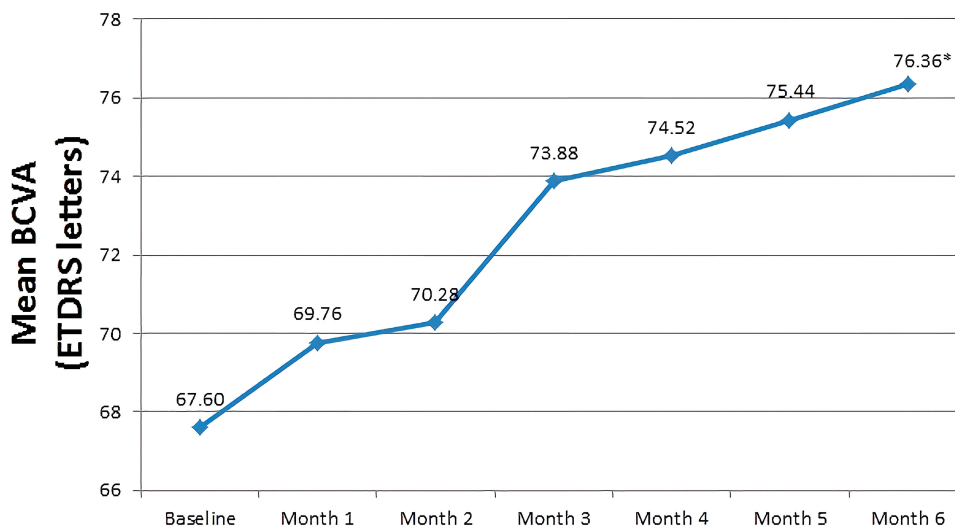
Figure 3 Mean best-corrected visual acuity (using Early Treatment Diabetic Retinopathy Study chart) over the six-month study period. *Statistically significance is P < 0.05 with baseline.
Abbreviations: BCVA, best-corrected visual acuity; ETDRS, Early Treatment Diabetic Retinopathy Study.
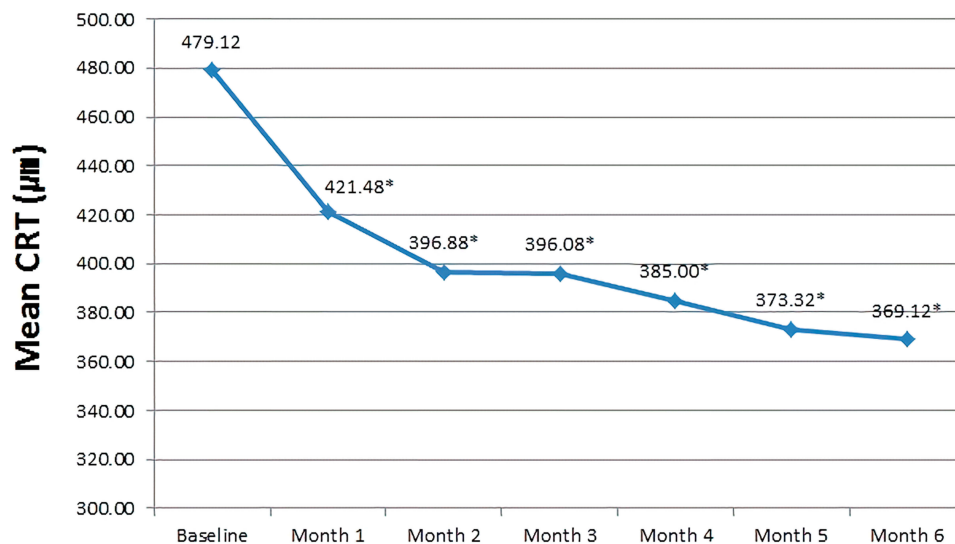
Figure 4 Mean central retinal thickness over the six-month study period. *Statistically significance is $P < 0.05$ with baseline.
Abbreviation: CRT, central retinal thickness.

significantly after administration of six monthly IVR injections. Our results showed that these MA-related parameters could be used as appropriate markers for evaluating the progression of retinopathy and that IVR injections could delay the progression of DR.

Hyperglycemia in diabetes induces high glucose concentration within the retina, eventually damaging the endothelium and pericytes of the retinal vessels. These structural damages lead to retinal ischemia and destruction of the blood-retinal barrier, causing the development of MAs, further decreasing retinal perfusion. Increased

VEGF level is known to play an important role in mediating this process.[30] However, physiologic levels of VEGF are important in maintaining retinal microvascular circulation during ischemic states.[24] Thus, although the mechanism of action of an anti-VEGF antibody such as ranibizumab on retinal vessels in DME is not yet elucidated, it may have a detrimental effect on retinal circulation.

Capillary loss is known to be a risk factor for DR progression. In a subanalysis of the RESTORE (extension) study, all treatment arms (ranibizumab monotherapy, ranibizumab plus laser combination therapy, laser monotherapy)
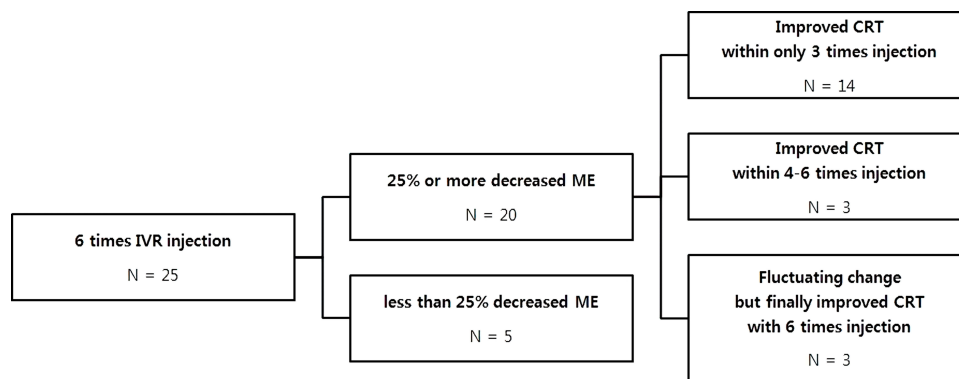
Figure 5 A flow chart showing relationship between numbers of injection and improvement in central retinal thickness.
Abbreviations: IVR, intravitreal ranibizumab; ME, macular edema; CRT, central retinal thickness.
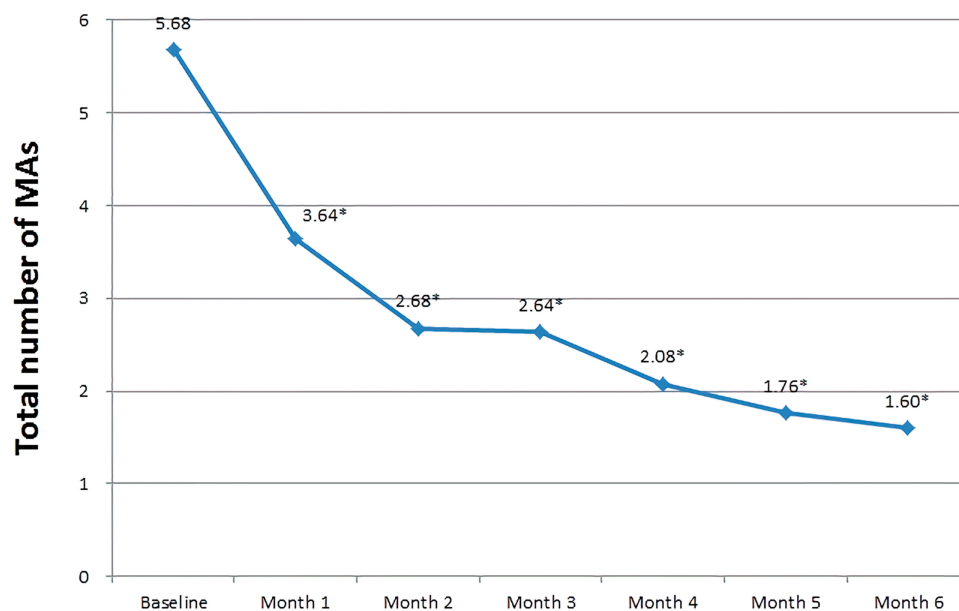

Figure 6 Mean total number of microaneurysms over the six-month study period. *Statistically significance is $P < 0.05$ with baseline.
Abbreviation: MA, microaneurysm.

showed increased capillary loss, but no difference in capillary loss around fovea was reported. This shows that IVR injections do not increase the risk of aggravating central capillary perfusion.[24] Findings of the RISE and RIDE trials conducted by Ip et al,[12,13] showed that administration of IVR injections can reduce the risk of progression and severity of DR and that capillary loss within the macula is an important factor for the progression to PDR. However, IVR injections for DME lead to improved vision on their own accord, regardless of the absence of macular capillary perfusion. This suggests that the presence of retinal non-perfusion may not significantly correlate with improving vision, despite being an important risk factor for predicting the severity and progression of DR, and early intervention may

be indicated for achieving maximal therapeutic effects. Additionally, Bonnin et al,[25] showed that 3 monthly injections of anti-VEGF agents improved BCVA and CRT and reduced grades of DR severity, such as regression of red dots (hemorrhages or MAs) were observed on color photographs, but no improvement in vessel perfusion in capillary non-perfusion area was reported. Similarly, in this study, despite a decrease in perifoveal non-perfused area after 3 months of treatment and an increase after 6 months, a significant improvement in vision was observed after early intensive treatment.

A retrospective analysis of FA images from the RISE and RIDE trials performed by Campochiaro et al,[30] over a period of 3 years revealed that the area of retinal non-perfusion
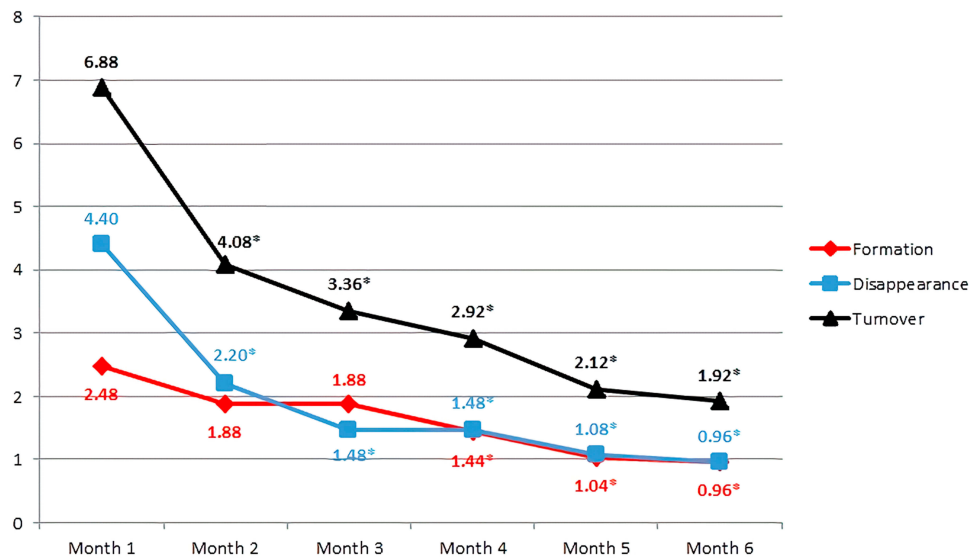
Figure 7 Mean turnover, rate of formation, and rate of disappearance of microaneurysms over the six-month study period. *Statistically significance is $P < 0.05$ with baseline.
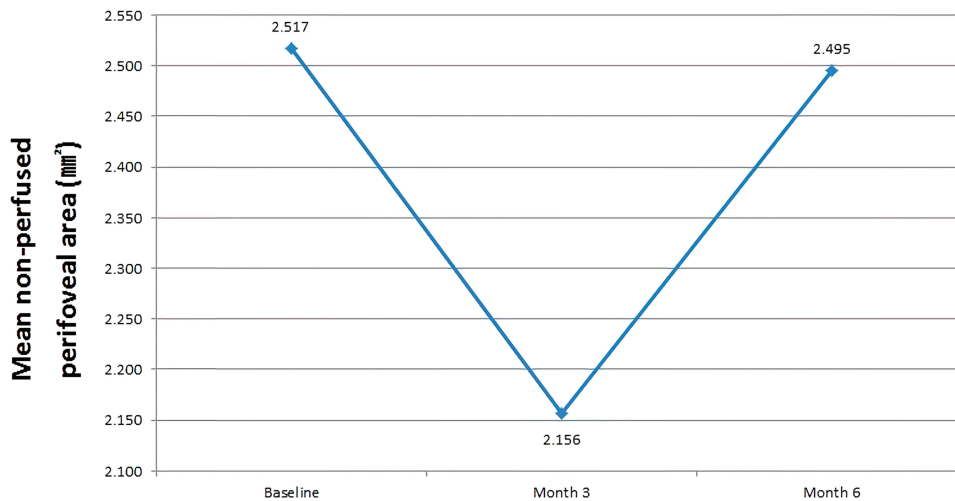

Figure 8 Changes in extent of mean perifoveal non-perfused area over the six-month study period.

increased from baseline over time in groups of patients who were administered IVR in concentrations of 0.3 mg, 0.5 mg, and sham (including patients crossed over to treatment with ranibizumab). However, slower changes were evident in the IVR injection group, implying that IVR injections retarded the progression of non-perfusion faster, compared with sham. Furthermore, in patients treated using IVR injections, retinal non-perfusion area decreased paradoxically until 12 months. Karst et al,[24] analyzed data from the RESTORE study for 3 years and showed that capillary loss decreased at 6 months and increased after 6 months on FA with IVR monotherapy. Although our result was not statistically significant and the study was conducted in a short period, we observed

a decrease in perifoveal non-perfused area after six monthly IVR injections, similar to the results obtained by Campochiaro et al and Karst et al.

Limitations of the current study include the small sample size (n=25 eyes), short study-duration of 6 months, and the lack of a control group. However, this study is unique, as it prospectively analyzed not only the improvement in BCVA and reduction in CRT but also changes in both MA turnover and perifoveal non-perfused area in NPDR patients with DME, following IVR therapy. Furthermore, this study employed RetmakerDR and ImageJ software programs to assess the occurrence of changes within the same retinal location, despite differing timings of examinations ensuring

Table 2 Comparative Analysis of Prognostic Ophthalmic Markers of Diabetic Retinopathy

	Before Injection	After 6 IVR Doses	P value
BCVA (ETDRS letters)	67.60 ± 3.29	76.36 ± 1.61	<0.0001*
CRT (μm)	479.12 ± 16.66	369.12 ± 13.02	<0.0001*
Total number of MAs	5.68 ± 3.41	1.60 ± 1.73	<0.0001*
MA formation rate (MA number/month)	2.48 ± 1.98	0.96 ± 1.02	<0.0001*
MA disappearance rate (MA number/month)	4.40 ± 2.66	0.96 ± 1.09	<0.0001*
MA turnover	6.88 ± 3.83	1.92 ± 1.75	<0.0001*
Perifoveal non-perfused area (mm^2)	2.517 ± 0.456	2.495 ± 0.293	0.221

Notes: MA formation rate, MA disappearance rate, MA turnover are defined as before injection and after first injection. The values are presented as mean±SD and range unless otherwise indicated. Statistically significance is $p < 0.05$ with *Repeated measures analysis of variance.
Abbreviations: IVR, intravitreal ranibizumab; BCVA, best-corrected visual acuity; CRT, central retinal thickness; MA, microaneurysm; ETDRS, Early Treatment Diabetic Retinopathy Study.

consistency and continuity of findings and enabling a more accurate analysis of the examination results.

Conclusion

After administration of early, intensive IVR injections, BCVA improved and CRT decreased in NPDR patients with ME. Further, they decreased the MA turnover as compared to baseline. However, the extent of perifoveal non-perfused area showed a mild overall decrease as compared to baseline, after 6 months. Thus, our data suggest that early intensive IVR injections can delay DR progression, but over a 6-month period, IVR therapy did not show a significant improvement in perifoveal non-perfused area. Hence, further randomized-controlled studies of longer duration are indicated to understand the impact of newer treatment strategies on the progression and prognosis of DR.

Data Sharing Statement

Available from the corresponding author on reasonable request.

Ethics and Approval

All procedures performed in this study were approved by the Institutional Review Board of the Wonkwang University Hospital and conducted according to the principles of the Declaration of Helsinki. Prior to the procedure, written informed consent was obtained from all patients. Also, potential risks and side effects were explained.

Disclosure

The authors report no conflicts of interest with respect to this work.

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