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ARTICLE

Sequential restoration of external limiting membrane and ellipsoid zone after intravitreal anti-VEGF therapy in diabetic macular oedema

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Abstract

Background/Objectives To study the mechanism of restoration of retinal photoreceptor ellipsoid zone (EZ), after intravitreal bevacizumab (IVB) therapy, in diabetic macular oedema (DMO).

Subjects/Methods Forty-four consecutive patients aged 40–65 years having type 2 diabetes mellitus (DM) with DMO were prospectively recruited for IVB therapy. It comprised of three doses (1.25 mg in 0.05 ml) of IVB at monthly intervals. Patients with other ocular and systemic diseases affecting retinal vessels and earlier ophthalmological interventions were excluded. Visual acuity (logMAR VA) was recorded. Spectral domain optical coherence tomography (SD-OCT) was performed pre and post intervention. Central sub-foveal thickness (CST) and grades of disorganization of retinal inner layers (DRIL), external limiting membrane (ELM) and EZ were assessed. Data were statistically analysed on SPSS software. Clinical trials registry: CTRI/2019/03/018135.

Results Mean logMAR VA decreased after IVB therapy from 1.78 ± 0.07 pre-intervention to 0.42 ± 0.05 post intervention ($p < 0.001$). Similarly, CST reduced from $354.23 \pm 15.0\,\mu m$ pre-intervention to $233.18 \pm 7.88\,\mu m$ post intervention ($p < 0.001$). Among qualitative variables, DRIL decreased from 93.2% pre-intervention to 13.6% post intervention. Likewise, global ELM disruption reduced from 81.8 to 9.1% and global EZ disruption reduced from 79.5 to 11.4%. ELM restoration preceded EZ restoration.

Conclusion Anti-VEGF therapy restores the barrier effect of ELM. It causes ELM to restore first followed by EZ restoration in DMO.

Introduction

Diabetic retinopathy (DR) affects 93 million people worldwide. Among them, 21 million have treatable form of diabetic macula oedema (DMO) [1]. Overall prevalence of DMO in DR is between 11 and 15% [1–3].

Hyperglycaemic state in diabetes mellitus (DM) results in formation of advanced glycation end products (AGEs) [4]. Formation of AGEs correlates with the glycaemic control of the patient [5]. AGEs stimulates vascular endothelial growth factor (VEGF) expression [6–8]. Multiple physiological and pathological effects i.e. angiogenesis, vascular hyperpermeability, initiation of DR-like vascular changes, antithrombotic or prothrombotic responses, and neuroprotection can be attributed to VEGF [9]. In our earlier study, an increase in the level of serum VEGF has been reported with an increase in the severity of DR [10].

Advances in spectral-domain optical coherence tomography (SD-OCT) technology have enhanced the understanding of morphological alterations in individual layers of retina and their association with various molecular mechanisms [9]. Increase in VEGF has been found to be

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related with increased central subfield thickness (CST), disruption of external limiting membrane (ELM) and ellipsoid zone (EZ) in DMO [10, 11]. Increased severity of DR has also been found to be associated with dis-organization of retinal inner layers (DRIL) [12]. Presence of DRIL has been found to correlate with EZ disruption in DMO [13]. EZ and DRIL have been found to be predictors of visual acuity (VA) in centre-involved DMO [12, 14].

Anti-VEGFs are considered as the first-line treatment for DMO. Bevacizumab is a full-length humanized monoclonal antibody of 149 kDa. Administration of intravitreal anti-VEGF agents are associated with reduction in CST and improvement in VA [15–17]. Mori et al. [9] showed restoration of the foveal photoreceptors following admin-istration of intravitreal ranibizumab in DMO.

We conducted a tertiary care, prospective, interventional study. This study was undertaken to study the mechanism of restoration of ELM and EZ after intravitreal bevacizumab (IVB) therapy in DMO for the first time.

Materials and methods

The study authors confirm adherence to the doctrines of the Declaration of Helsinki. This study was conducted after approval by Institutional Ethics Committee of King Geor-ge's Medical University, Lucknow, India. A written, informed, voluntary consent was obtained from all the study subjects. This study was registered with Clinical Trial Registry of India bearing the registration number: CTRI/2019/03/018135.

Patients

Study patients comprised of Type 2 DM. A total of 44 consecutive patients of DMO between aged of 40–65 were included. Patients who did not give consent or had any other ocular or systemic disease affecting retinal vessels were excluded from the study. Patients who had received pre-vious intravitreal injections, lasers or surgical interventions were also omitted from the study. Sample size was calcu-lated according to Charan Biswas formula-

$$n = \frac{Z_{1-a/2}^2 p(1-p)}{d^2}$$

Data collection

Patient's age and sex were documented. All study subjects underwent detailed ophthalmological examination including fundus evaluation using slit lamp biomicroscope, and indirect ophthalmoscope. Fluorescein angiography and spectral domain optical coherence tomography (SD-OCT)

examinations were also performed. Baseline data were recorded. The outcome measures of the study were logMAR VA and OCT parameters (CST, DRIL, ELM and EZ). CST was the quantitative parameter whereas DRIL, ELM and EZ were the qualitative parameters. The outcome measures were assessed at pre-intervention (baseline) and at monthly intervals after every dose of IVB (after first, second and third dose of IVB). SD-OCT parameter, CST, was assessed in micrometre (μm) whereas the qualitative parameters were assessed according to their grades of severity. In cases with bilateral involvement, eye with more severe DMO was included. The patients were administered three doses of IVB (1.25 mg/0.05 mL) at monthly intervals in the affected eye.

Spectral domain optical coherence tomography

The Cirrus HD-OCT (Carl Zeiss Meditech, Inc., CA, USA) was used. A macular cube (128 × 512) with hor-izontal and vertical 5-line raster scans, fovea-centred, was acquired.

Image Interpretation

The baseline SD-OCT image of each patient was compared with subsequent OCT images after injections. CST, grading of DRIL, and ELM and EZ disruption were noted.

DRIL was defined as the failure to ascertain any of four inner layers of the retina, namely, the ganglion cell layer-inner plexiform layer (GCL-IPL) complex, inner nuclear layer (INL) and outer plexiform layer (OPL) boundaries [12–14]. DRIL was graded as Grade 0: absence of DRIL, and Grade 1 presence of DRIL.

The ELM was demarcated as first hyper-reflective band, representative of the junctional complex between the glial Müller cells and photoreceptor cells [9]. ELM disruption was graded as grade 0: ELM intact, grade 1: focal disruption of ELM with localized sub-foveal involvement and grade 2: global disruption of ELM with generalized involvement within macular cube.

The EZ was demarcated as the hyper-reflective band below the ELM which clinically represents the photo-receptor integrity [9, 10]. Disruption of EZ was graded as grade 0: intact EZ, grade 1: focal disruption of EZ indi-cating localized sub-foveal involvement and grade 2: global disruption of EZ indicating generalized involvement within macular cube.

Collection of data was done at monthly interval. Pre and post intervention data, after third dose of IVB, were ana-lysed. SD-OCT assessment of DRIL, ELM and EZ was done by two independent observers masked to the pre- or post-intervention status. Inter-observer correlation was computed by Spearman correlation coefficient.

Data analysis

Continuous data were depicted as mean ± SE (standard error of the mean). Discrete (categorical) data were depicted in numbers (n) and percentages (%). Continuous two independent groups were compared by Student's t test. Continuous groups were compared by analysis of variance. and the significance of mean difference within (intra) and between (inter) the groups was done by Newman–Keuls post hoc test. This was performed after ascertaining normality by Shapiro–Wilk's test and homogeneity of variance between groups by Levene's test. Categorical groups were compared using chi-square (χ^2) test. Pearson correlation analysis was done to assess association between the variables. A two-tailed ($\alpha = 2$) $p < 0.05$ was considered statistically significant. Analyses were performed on SPSS software (Windows version 17.0 was used).

Results

The demographic characteristics of patients are summarized in Table 1. Qualitative variables before and after intervention are summarized in Table 2.

It was observed that mean logMAR VA decreased significantly after IVB regimen from 1.78 ± 0.07 at baseline to 0.42 ± 0.05 ($p < 0.001$). Similar trend was observed in CST which decreased significantly from pre-treatment level of 354.23 ± 15.0 to 233.18 ± 7.88 post intervention ($p < 0.001$). SD-OCT assessment of DRIL, ELM, EZ was done. Interobserver correlation was computed as 0.9. Amongst qualitative variables, DRIL was evident in majority of patients (93.2%) pre-intervention, which reduced to 13.6% after the IVB regimen. Similarly, global ELM disruption reduced from 81.8% pre-intervention to 61.4 % after first dose, 20.5% after second dose and finally 9.1% after third dose and global EZ disruption reduced from 79.5% pre-intervention to 25% after first dose, 18.4% after second dose and subsequently to 11.4% after IVB regimen ($p < 0.001$). It was observed that in the eyes in which ELM was restored, EZ was also restored

Table 1 Demographic characteristics (Mean ± SE) of patients ($n = 44$).

Variables	Mean ± SD
Age (years)	54.36 ± 0.80
Female	20
Male	24
HbA1c (%)	6.10 ± 0.06
Fasting blood sugar (mg/dl)	89.00 ± 1.48
Post-prandial blood sugar (mg/dl)	128.50 ± 1.84
Serum urea (mg/dl)	30.38 ± 1.23
Serum creatinine (mg/dl)	1.50 ± 0.04

after treatment. This was associated with corresponding decrease in logMAR VA. Figures 1, 2 shows complete restoration of ELM with partial restoration of EZ denoting that ELM restores first after anti-VEGF therapy.

Correlation of biochemical parameters, VA and OCT parameters before and after intervention, was done using Pearson correlation analysis. Significant positive correlation was found between CST and VA ($r = 0.65$), and this correlation increased after intervention suggesting the beneficial effect of IVB therapy ($r = 0.93$, $p < 0.001$).

Discussion

DRIL is defined as the failure to identify any of the boundaries of GCL-IPL complex, INL and OPL. Also, DRIL has been found to be a predictor of VA [14].

The EZ clinically defines the photoreceptor integrity. The biological EZ consists mainly of mitochondria. This enables higher levels of energy consumption within the photoreceptors. Focal or global absence of the EZ on SD-OCT corresponds to the reduced reflectivity or anatomic absence of the EZ. Dysfunction of mitochondria in the foveal photoreceptors results in reduced VA in DMO [9].

The ELM is defined as the junctional complex between the glial Müller cells and photoreceptor cells. It serves as a barrier against macromolecules [9]. Damage in the glial Müller cells might contribute to both ELM disruption as well as inner retinal thickening. The disrupted barrier properties in the ELM might dysregulate the fluid dynamics, concomitantly allowing accumulation of intraretinal or subretinal fluids [9].

In the present study, IVB therapy was associated with significant improvement in DRIL and restoration of ELM and EZ leading to decrease in logMAR VA.

Comyn et al. and Wells et al. found that there is significant reduction of CST and CAT in patients of DMO after anti-VEGF treatment [18, 19]. We also observed that CST significantly decreased after IVB therapy. Aiello et al. [20], Nguyen et al. [21] and Pelosini et al. [22] observed that the magnitude of CST reduction in several treatment regimens was associated with a better VA outcome. Our study also demonstrated similar findings.

We also observed that improvement in SD-OCT qualitative parameters comprising of DRIL, ELM and EZ were evident following intervention. Sun et al. suggested DRIL to be a strong predictor of VA in eyes with DMO. Therefore, DRIL can be used as a biomarker for predicting future VA [14]. We observed that improvement in VA was more evident in patients associated with improvement of DRIL. This suggests that restoration of DRIL is directly related to improvement in VA. DRIL has also been shown to correlate with EZ disruption [13]. DRIL is thought to correlate to

Table 2 Qualitative variables before and after Interventions ($n = 44$).

Variables	Total numbers (%) before intervention	Total numbers (%) after First Dose	Total numbers (%) after second dose	Total numbers (%) after intervention
DRIL				
Absent (Grade 0)	3 (6.8)	6 (13.6)	30 (68.2)	38 (86.4)
Present (Grade 1)	41 (93.2)	38 (86.4)	14 (31.8)	6 (13.6)
ELM				
No Disruption (Grade 0)	0 (0.0)	5 (11.4)	29 (65.9)	37 (84.1)
Focal Disruption (Grade 1)	8 (18.2)	12 (27.3)	6 (13.6)	3 (6.8)
Global Disruption (Grade 2)	36 (81.8)	27 (61.4)	9 (20.5)	4 (9.1)
EZ				
No Disruption (Grade 0)	2 (4.5)	3 (6.8)	22 (50.0)	35 (79.5)
Focal Disruption (Grade 1)	7 (15.9)	30 (68.2)	14 (31.6)	4 (9.1)
Global Disruption (Grade 2)	35 (79.5)	11 (25.0)	8 (18.4)	5 (11.4)
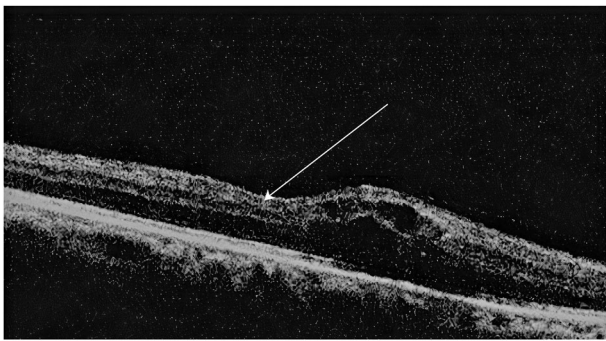
Fig. 1 SD-OCT showing DRIL with disruption of ELM and EZ. (Arrow marking DRIL).
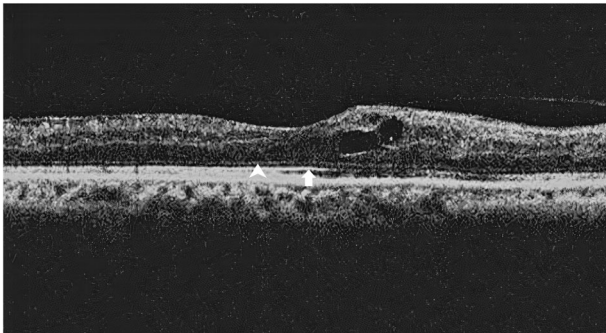
Fig. 2 SD-OCT showing partial restoration of DRIL and complete restoration of ELM with EZ in the process of restoration following third dose of intravitreal anti-VEGF (Bevacizumab). (Arrowhead marking ELM and arrow marking EZ).

regions where bipolar, horizontal, and amacrine cell synaptic connections have been disrupted, thus interrupting the transmission pathway between the ganglion cells and photoreceptors, leading to decrease in VA [12, 23]. Anti-VEGF therapy improves DRIL, thereby leading to restoration of synaptic connections and transmission of impulses from photoreceptors and resultant improvement of VA [9].

Similar significant findings were observed with regard to ELM and EZ disruption [10, 24]. Percentage disruption of EZ has been found to correlate with VA in DMO [12]. Integrity of ELM and EZ has been found to be a positive predictor for visual outcome [24–27]. Kang et al. demonstrated that the sturdiest predictor of final BCVA was the status of EZ integrity followed by status of ELM [28]. We observed that decrease in logMAR VA was more pronounced in patients which were associated with restoration of ELM and EZ. ELM restoration was observed in 84.1%, whereas, EZ restoration was seen in 79.4% after IVB.

Hareedy et al. [29] found that improvement in photoreceptor integrity took place after second and third dose of ranibizumab with improvement in VA and colour vision. They found a statistically significant relation between colour vision and EZ after the first injection, but the correlation after the second and third injections was much more significant. They also reported that improvement in BCVA significantly correlated with EZ after the second and third injection.

Achiron et al. [30] concluded that restoration of foveal photoreceptor microstructure following anti-VEGF therapy was associated with improvement in VA. A larger foveal photoreceptor microstructure defect was associated with lower VA. Patients with larger foveal photoreceptor microstructure defects at baseline had smaller VA improvements, and the improvement in foveal photoreceptor microstructure integrity was associated with VA improvement.

Chatzerelli et al. [31] also reported significant restoration of EZ photoreceptors in patients of DMO following intravitreal ranibizumab after 12 months follow-up. The improvement in EZ defect size was dependent on the pattern of DMO on SD-OCT.

Omri et al. [32] demonstrated in rat and monkey retina that ELM comprised of attachment of outer process of glial Muller cells to one another and also to inner photoreceptor segments. They also found that tight junctions (TJ) exist in

the ELM. Ultrastructural analysis suggested that TJ exist between glial Muller cells and photoreceptors. Occludin, a protein, is a key component of TJ. Occludin is organized between the glial Muller cells and the photoreceptors at the ELM. They suggested that the ELM should be considered as part of a retinal barrier that may be disrupted in various pathological conditions. During diabetic retinopathy, not only are the glial Muller cells swollen but they also lose their occludin content at the ELM level. Hence, ELM junctions could be unique regulatory targets in treatment [32]. Murukami et al. [33] highlighted in primary bovine retinal endothelial cells that VEGF treatment induced occludin phosphorylation, ubiquitination and fragmentation of TJ. They demonstrated that occludin had a significant role in regulation of barrier properties and might serve as a possible therapeutic target. We also observed in our earlier SD-OCT based study that an increase in VEGF was associated with increased severity of DR and sequential ELM and EZ disruption, highlighting that an intact ELM was a prerequisite for an intact EZ in DMO [10, 24].

Our study provides insight into the mechanism of ELM and EZ restoration after anti-VEGF therapy. Anti-VEGF therapy led to restoration of barrier effect of ELM. The ELM was found to restore first followed by EZ restoration in DMO.

Summary

What was known before

- Increase in VEGF levels is associated with ELM and EZ disruption in DMO.
- After anti-VEGF therapy, decrease in macular thickness is associated with improvement in visual acuity in DMO.
- After anti-VEGF therapy, improvement in DRIL and restoration of EZ occurs.

What this study adds

- This study for the first time highlights the mechanism of EZ restoration.
- Anti-VEGF therapy restores the barrier effect of ELM.
- ELM restores first followed by EZ restoration in DMO.
- An intact ELM is a prerequisite for an intact EZ in DMO.

Compliance with ethical standards

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