



# Effects of repeated intravitreal bevacizumab administration on anterior segment parameters and limbal stem cells

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## ABSTRACT

**Background:** Macular edema (ME) is fluid accumulation in the macula caused by vascular leakage. Repeated intravitreal bevacizumab (IVB) injections are extensively used to treat ME of different origins, are well tolerated, and have few side effects. This study evaluated the effects of repeated IVB injections on the anterior segment parameters and limbal stem cells (LSCs) in eyes with ME.

**Methods:** This before–after study involved patients with ME of different causes who underwent repeated IVB injections at the Imam Khomeini Ophthalmology Center in Kermanshah, Iran. Before and after repeated IVB injections, anterior segment parameters were measured using anterior segment optical coherence tomography, and the LSCs were assessed using impression cytology.

**Results:** We enrolled 42 eyes of 42 patients with a mean (standard deviation [SD]) age of 59.6 (7.6) years, of whom 25 (59.5%) were men and 17 (40.5%) were women. The underlying diseases included diabetic ME in 30 eyes (71.4%), central (5 [11.9%]) or branch (3 [7.1%]) retinal vein occlusion, and choroidal neovascularization in 4 eyes (9.5%). The right eye was affected in 22 (52.4%) participants. The mean (SD) number of IVB injections was 4.3 (1.3). After repeated injections, the mean central corneal thickness (CCT) increased, whereas the mean anterior chamber angle (ACA) and anterior chamber depth (ACD) decreased (all  $P < 0.001$ ). Three patients developed LSC deficiency after repeated IVB injections for diabetic ME.

**Conclusions:** We observed a significant increase in the mean CCT and a decrease in the mean ACA and ACD after repeated IVB injections in our series. Three patients developed LSC deficiency after repeated IVB injections for diabetic ME management. The observed effect on LSC may cast doubt on the safety of repeated IVB injections; however, this finding must be verified in multicenter clinical trials with longer follow-up periods and larger study samples.

## KEYWORDS

Avastin, bevacizumab-awwb, optical coherence tomography, corneal thickness measurement, anterior chambers, limbal stem cell, limbal stem cell deficiency

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## INTRODUCTION

Macular edema (ME) is fluid accumulation in the macula caused by vascular leakage [1, 2]. It is most frequently a complication of diabetes mellitus (DM). The most common cause of vision loss due to diabetic retinopathy is diabetic macular edema (DME) [2], which is associated with disruption of the inner and outer blood–retinal barriers [3]. More rarely, ME occurs following retinal vein occlusion (RVO), choroidal neovascularization (CNV), and uveitis [4].

Multiple treatment modalities with variable success rates are available for managing ME, such as topical and systemic steroids, oral and topical nonsteroidal anti-inflammatory drugs, intravitreal anti-vascular endothelial growth factors (anti-VEGF) such as bevacizumab, and laser photocoagulation [5]. Bevacizumab is a human anti-VEGF monoclonal antibody that is used in cancer treatment [6].

Repeated intravitreal bevacizumab (IVB) injections (bevacizumab 1.25 mg/0.05 mL) are well tolerated, have few side effects, and are extensively used to treat ME caused by diabetic retinopathy, RVO, and CNV due to age-related macular degeneration (AMD) [7]. Mounting evidence suggests that IVB can decrease ME [8–10]. It improves vision in patients with ME secondary to branch or central RVO [11, 12].

Although there have been numerous studies on the effect of intravitreal injections on the ocular structures, especially the posterior segment, assessment of changes in the anterior segment has been limited [13, 14]. Most investigations have benefited from new imaging techniques, particularly optical coherence tomography (OCT) [15]. OCT is a non-contact, two-dimensional imaging technology that generates cross-sectional images of biological tissues using low-coherence interferometry [15, 16]. Anterior segment OCT (AS-OCT) was first introduced in 1994 and commercially used in 2001 [17, 18]. Currently, with the remarkable advances in AS-OCT technology, this tool is widely used to evaluate anterior segment structures [15].

Stem cells constitute a small undifferentiated cell population within a tissue, with special characteristics such as proliferative capacity, self-maintenance, production of active progenitor cells, tissue repair after injury, and flexibility [19]. The main function of limbal stem cells (LSCs) is to repair the corneal surface epithelium and maintain ocular surface integrity. Damage to the LSCs can occur following repeated surgical trauma, and the resulting corneal conjunctivalization has been reported after multiple IVB injections [20].

The present study investigated the effects of repeated IVB administration on anterior segment parameters and evaluated the effects of this treatment modality on LSCs.

## METHODS

This before–after study recruited patients with a diagnosis of ME of different causes who received repeated IVB administration from January to June 2021 at the Imam Khomeini Ophthalmology Center in Kermanshah, Iran. The study was approved by the local ethical committee of the Kermanshah University of Medical Sciences and complied with the tenets of the Declaration of Helsinki. Informed consent to participate was obtained from all patients.

We recruited all eligible candidates for repeat IVB injections during the study period. These patients were treated with IVB for center-involved DME, RVO with cystoid ME, or CNV associated with AMD, based on clinical examination combined with the OCT and fluorescein angiography findings. The inclusion criteria were the presence of ME, candidacy for at least 3 anti-VEGF injections, and no history of intravitreal injections. We excluded patients with proven LSC deficiency (LSCD) based on clinical examinations or baseline impression cytology, history of pterygium surgery or anterior segment surgery, chemical or thermal burns, autoimmune ocular surface disease (such as Stevens–Johnson syndrome), any acquired or congenital ocular diseases with LSCD, and pregnancy or lactation.

The diagnostic criteria for DM included fasting blood glucose  $\geq 126$  mg/dL, random blood glucose  $\geq 200$  mg/dL, or glycosylated hemoglobin (HbA1c)  $\geq 6.5\%$  [21]; for hypertension, a systolic blood pressure  $\geq 140$  mmHg and/or diastolic blood pressure  $\geq 90$  mmHg in the office or clinic [22]; and for hyperlipidemia, low-density lipoprotein cholesterol level  $\geq 190$  mg/dL [23]. RVO or AMD was diagnosed based on the characteristic clinical findings and confirmed using OCT and fluorescein angiography [24, 25].

Eligible participants underwent a complete ophthalmological examination before the first injection and 3 months after the last IVB injection. Examination included measurement of best-corrected distance visual acuity using a Snellen chart (Nikon Chart Projector NP-3S; Nikon Inc., Melville, NY, USA); intraocular pressure measurement using the Goldmann applanation tonometer (AT900, Haag-Streit, Koeniz, Switzerland); undilated and dilated slit-lamp biomicroscopy (Photo-Slit Lamp BX 900; Haag-Streit); measurement of anterior segment parameters including central corneal thickness (CCT), anterior chamber angle (ACA), and anterior

chamber depth (ACD) using OCT (Optovue OCT; Optovue Inc., Fremont, CA, USA); and LSC evaluation using impression cytology [26, 27].

IVB was administered under aseptic conditions in the operating room. With the patient in the supine position, topical anesthesia was achieved using 0.5% tetracaine (Anestocaine, Sina Darou Co., Tehran, Iran). The periocular skin was disinfected using povidone-iodine 10% and covered with towels, and a sterile eyelid speculum was used to separate the eyelids. The conjunctival sac was irrigated with 5% povidone-iodine solution. A 30-gauge tuberculin needle was inserted into the pars plana, which was 3.5 and 4.0 mm behind the superior temporal corneal limbus in pseudophakic and phakic eyes, respectively, and bevacizumab 1.25 mg/0.05 mL (Avastin®, Genentech, San Francisco, CA, USA) was injected into the vitreous body. The puncture opening was compressed with a sterile cotton swab during injection to avoid drug backflow, and tactile postoperative intraocular pressure was observed. This was followed by the administration of topical antibiotic prophylaxis and instructions on postoperative complication warning signs [28].

Impression cytology was used to detect LSCD by observing conjunctival goblet cells on the corneal surface [29]. Following the administration of 0.5% tetracaine eye drops (Anestocaine, Sina Daru), cellulose acetate filter paper (Sartorius Stedim Biotech GmbH, Goettingen, Germany) was placed on the patient's central cornea for 10 s, after which a tissue sample was obtained. The samples were then fixed in 96% ethanol and stained with hematoxylin and eosin. The slides were placed in xylol solution for 24 h to separate the paper from the slide. Finally, the stained slides were analyzed and photographed under a Nikon YS2-T light microscope (Nikon, Japan) at 100× magnification by an expert pathologist (Figure 1).

IBM SPSS Statistics for Windows (version 20.0; IBM Corp., Armonk, NY, USA) was used for data entry and analysis. Normality of the data distribution was determined using the Kolmogorov–Smirnov test. Descriptive and inferential statistics were used to analyze the data. Descriptive statistical data are presented as frequencies and percentages or means and standard deviations (SDs). Pearson's rank correlation was used when applicable. An independent *t*-test was used to compare quantitative variables. Moreover, the chi-square or Fisher's exact test was used to compare qualitative variables between groups when applicable. To compare the CCT, ACA, and ACD before and after repeated IVB administration, paired *t*-tests or repeated measures analysis of variance (ANOVA) was performed. When ANOVA revealed a statistically significant difference between the study groups, we proceeded with further analysis using a Tukey post-hoc test for multiple pairwise comparisons between groups. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

Ultimately, 42 eyes of 42 patients with a mean (SD) age of 59.6 (7.6) years (range: 38–72) were included. The study sample consisted of 25 (59.5%) men with a mean (SD) age of 60.4 (6.4) years and 17 (40.5%) women with a mean (SD) age of 58.4 (9.2) years. The underlying diseases included DM in 30 eyes (71.4%), central RVO in 5 eyes (11.9%), branch RVO in 3 eyes (7.1%), and AMD in 4 eyes (9.5%). The right eye was affected in 22 (52.4%) participants. The mean (SD) number of IVB injections was 4.3 (1.3) (range: 3–8).



**Figure 1.** This histological specimen was obtained from the cornea of the first patient 3 months after the last intravitreal administration of bevacizumab 1.25 mg/0.05 mL (Avastin®, Genentech, San Francisco, CA, USA) for managing diabetic macular edema in his right eye. The patient was a 70-year-old man with a history of type 2 diabetes mellitus who received 6 intravitreal bevacizumab injections. The sample represents a positive impression cytology indicated by the presence of conjunctival goblet cells (green pointer) in the corneal epithelium. The specimen was stained with hematoxylin and eosin and analyzed and photographed under a Nikon YS2-T light microscope (Nikon, Japan) at 100× magnification by an expert pathologist.

We observed no significant difference between the variables of sex, underlying disease, and laterality of the involved eye and the mean difference CCT, ACA, and ACD (all  $P > 0.05$ ) (Table 1). There were no significant correlations between age and CCT ( $r = -0.12$ ;  $P = 0.430$ ), ACA ( $r = -0.07$ ;  $P = 0.630$ ), or ACD ( $r = +0.01$ ;  $P = 0.950$ ). Similarly, there was no significant correlation of sex with CCT or sex and age with ACA or ACV.

We observed a statistically significant increase in mean CCT and decrease in mean ACA and ACD after repeated IVB administration (all  $P < 0.001$ ) (Table 2). Table 3 compares the mean values of the anterior segment parameters between eyes treated with 3, 4, 5, and  $\geq 6$  IVB injections. The mean ACD decreased significantly with an increasing number of injections ( $P < 0.05$ ), whereas the CCT and ACA remained unchanged (both  $P > 0.05$ ) (Table 3). Multiple pairwise comparisons revealed a significant difference in mean ACD in eyes with 3 versus 4 injections ( $P = 0.025$ ), 3 versus 5 injections ( $P < 0.001$ ), 3 versus  $\geq 6$  injections ( $P < 0.001$ ), 4 versus  $\geq 6$  injections ( $P < 0.001$ ), and 5 versus  $\geq 6$  injections ( $P < 0.001$ ); however, ACD was not significantly different between eyes with 4 versus 5 injections ( $P = 0.137$ ).

Table 1. Comparison of mean differences in AS parameters with the demographic data of participant

Parameters		CCT ( $\mu\text{m}$ ), MD $\pm$ SD	ACA (degree), MD $\pm$ SD	ACD (mm), MD $\pm$ SD
Gender	Male	5.9 $\pm$ 5.7	-0.2 $\pm$ 0.2	-0.1 $\pm$ 0.1
	Female	5.9 $\pm$ 7.2	-0.2 $\pm$ 0.2	-0.1 $\pm$ 0.1
	P-value	0.992	0.788	0.547
Underlying disease	DM	6.4 $\pm$ 6.5	-0.2 $\pm$ 0.2	-0.1 $\pm$ 0.1
	RVO	3.8 $\pm$ 4.7	-0.1 $\pm$ 0.1	-0.1 $\pm$ 0.1
	P-value	0.280	0.382	0.894
Eye involved	OD	6.0 $\pm$ 6.7	-0.2 $\pm$ 0.2	-0.1 $\pm$ 0.1
	OS	5.8 $\pm$ 5.9	-0.2 $\pm$ 0.2	-0.1 $\pm$ 0.1
	P-value	0.901	0.915	0.802

Abbreviations: AS, anterior segment; CCT, central corneal thickness;  $\mu\text{m}$ , micrometer; MD, mean difference; SD, standard deviation; ACA, anterior chamber angle; ACD, anterior chamber depth; mm, millimeter; DM, diabetes mellitus; RVO, retinal vein occlusion; OD, right eye; OS, left eye. Note: The *t*-test was used to compare the mean difference CCT, ACA, and ACD in subgroup analysis.

Table 2. Comparison of AS parameters before and after repeated IVB administration

AS parameters	Before	After	P-value
CCT ( $\mu\text{m}$ ), Mean $\pm$ SD (Range)	509.7 $\pm$ 22.7 (456 to 555)	515.7 $\pm$ 23.6 (479 to 565)	<b>&lt; 0.001</b>
ACA (degree), Mean $\pm$ SD (Range)	26.2 $\pm$ 2.5 (22.1 to 32.2)	26.1 $\pm$ 2.5 (22 to 32)	<b>&lt; 0.001</b>
ACD (mm), Mean $\pm$ SD (Range)	2.9 $\pm$ 0.2 (2.4 to 3.5)	2.8 $\pm$ 0.2 (2.1 to 3.4)	<b>&lt; 0.001</b>

Abbreviations: AS, anterior segment; IVB, intravitreal bevacizumab; CCT, central corneal thickness;  $\mu\text{m}$ , micrometer; SD, standard deviation; ACA, anterior chamber angle; ACD, anterior chamber depth; mm, millimeter; before, before treatment; After, 3-month after the last IVB injection. Note: *P*-values  $< 0.05$  are shown in bold; The paired *t*-test was used to compare the mean CCT, ACA, and ACD before and 3-month after the last IVB injection.

Table 3. Comparison of mean AS parameters before and after treatment according to number of IVB injections

AS parameters	CCT ( $\mu\text{m}$ ), Mean $\pm$ SD		ACA (degree), Mean $\pm$ SD		ACD (mm), Mean $\pm$ SD	
	Before	After	Before	After	Before	After
N						
3	507.1 $\pm$ 23.3	511.7 $\pm$ 23.9	25.4 $\pm$ 1.8	25.2 $\pm$ 1.9	2.9 $\pm$ 0.2	2.8 $\pm$ 0.2
4	501.2 $\pm$ 23.0	508.1 $\pm$ 23.1	26.5 $\pm$ 2.1	26.4 $\pm$ 2.1	2.9 $\pm$ 0.3	2.8 $\pm$ 0.2
5	521.3 $\pm$ 17.3	527.8 $\pm$ 22.1	27.5 $\pm$ 3.1	27.3 $\pm$ 3.1	3.0 $\pm$ 0.2	2.9 $\pm$ 0.2
$\geq 6$	515.9 $\pm$ 23.7	521.7 $\pm$ 22.7	25.6 $\pm$ 3.0	25.5 $\pm$ 2.9	2.8 $\pm$ 0.2	2.7 $\pm$ 0.3
P-value	0.821		0.898		<b>0.048</b>	

Abbreviations: AS, anterior segment; IVB, intravitreal bevacizumab; CCT, central corneal thickness;  $\mu\text{m}$ , micrometer; SD, standard deviation; ACA, anterior chamber angle; ACD, anterior chamber depth; mm, millimeter; N, number of injections; before, before treatment; After, 3-month after the last IVB injection. Note: *P*-value  $< 0.05$  is shown in bold; Repeated measures analysis of variance was used to compare the mean CCT, ACA, and ACD before and 3-month after the last IVB injection.

LSCD was detected in 3 patients following repeated IVB administration. The first patient (Figure 1) was a 70-year-old man with a 25-year history of type 2 DM. He received 6 once-monthly IVB injections at the supratemporal pars plana for right center-involved DME. He had well-controlled DM on insulin therapy (HbA1c = 6.4%) and had no other comorbidities. His respective baseline and final values for CCT were 538  $\mu\text{m}$  and 540  $\mu\text{m}$ , for ACA were 22.1° and 22°, and for ACD were 2.9 mm and 2.9 mm.

The second patient was a 68-year-old man with a 21-year history of type 2 DM. He received 4 once-monthly IVB injections at the supratemporal pars plana for right center-involved DME. This patient had poorly controlled type 2 DM on insulin treatment (HbA1c = 9.1%) and systemic hypertension and hyperlipidemia as comorbidities. His respective baseline and final values for CCT were 495  $\mu\text{m}$  and 500  $\mu\text{m}$ , for ACA were 24.8° and 24.6°, and for ACD were 2.7 mm and 2.6 mm.

The third patient was a 62-year-old man with a 17-year history of DM. He received 8 once-monthly IVB injections at the supratemporal pars plana for left center-involved DME. This patient had poorly controlled DM on insulin treatment (HbA1c = 8.7%) and no other comorbidities. His respective baseline and final values for CCT were 475  $\mu\text{m}$  and 485  $\mu\text{m}$ , for ACA were 25.1° and 24.9°, and for ACD were 2.7 mm and 2.1 mm.

## DISCUSSION

In the present study, after repeated IVB administration into eyes with ME, mean CCT significantly increased; however, the observed change was not clinically significant [30]. On the contrary, Chiang et al. observed no significant difference in CCT after 6 months of using a single dose of IVB [31]. Moreover, in a study by Perez-Rico et al. of 52 patients with neovascular AMD, there was no significant difference in CCT before injection, 7 days after the first injection, and 6 months after the first injection [32].

Generally, CCT is an essential anterior segment parameter in the examination of patients with ocular disorders [33]. Measurement and comparison of CCT with values within the normal range can be effective for the screening, diagnosis, and treatment of ocular disorders [34]. Evidence suggests that individuals with CCT < 555  $\mu\text{m}$  are at higher risk of glaucoma relative to people with CCT > 588  $\mu\text{m}$  [35, 36]. Moreover, CCT is considered an influential factor in intraocular pressure measurement [37]. The observed CCT change in the current study may indicate continued CCT assessment in eyes with two simultaneous problems, namely glaucoma and ME requiring treatment.

We observed no significant correlation between CCT and patient age or sex. However, previous studies demonstrated that CCT in women is lowest at the beginning of the menstrual cycle, whereas it reaches maximum level at the time of ovulation and at the end of the cycle, suggesting consideration of these hormonal changes in screening candidates for refractive surgery [38, 39]. With aging, the CCT becomes thinner [40].

After repeated IVB injections, the mean ACA decreased from 26.2° to 26.1°. The mean difference before and after treatment was significant and could be related to the disruption of zonules [41, 42] due to repeated intravitreal injections. No significant relationship was observed between ACA and patient age or sex. The reported mean ACA in a sample of the Iranian population aged > 40 years was 34.3° [43], which exceeds the value reported in the current study; this difference could be attributed to variations in the study groups. In contrast with our results, Hashemi et al. observed a significant correlation between increasing ACA and male sex [43].

We observed a significant decrease in the mean ACD after repeated IVB administration, which might suggest disruption of zonules [41, 42] following repeated intravitreal injections. Consistent with the current results, a retrospective study of 100 patients by Arslan et al. demonstrated a significant decrease in ACD at 1 and 2 months after injection compared to that of the pre-injection phase [14]. Additionally, in a study of 70 patients by Hamidi et al., ACD after injections was significantly decreased compared to that of the pretreatment phase [13]. In contrast, in a study by Guler et al. measuring the ACD 1 month after bevacizumab injection, the mean value decreased in the eyes of 43 people 1 month after injection, although the difference was not statistically significant [44].

The present study demonstrated that repeated IVB injections increased the probability of LSCD, especially in patients with DM and older individuals. This finding was first reported by Capella et al. [20], who detected clinically significant LSCD using impression cytology in a 68-year-old man after 7 IVB injections for managing left-eye idiopathic choroidal polypoidal vasculopathy, with a notable improvement 2 months after ipsilateral limbal autograft [20]. However, previous studies have shown no long-term neurodevelopmental defects in preterm infants receiving intravitreal anti-VEGF therapy for retinopathy of prematurity [45, 46]. Considering the outcomes of our study, we believe that further evidence is required regarding the long-term effects of anti-VEGF therapy on growth and neurodevelopmental maturation.

Diabetic retinopathy is one of the most common indications for intravitreal injections [47], and 71.4% of our participants had diabetes. Diabetic keratopathy is a challenging clinical manifestation of DM. LSCs are influenced by numerous complexities that prevail systemically and locally in the corneas of patients with DM, ultimately leading to LSC depletion or loss of function. However, the precise sequence of molecular pathophysiological events leading to diabetic keratopathy is yet to be determined [48]. Ueno et al. observed a significant reduction in the number of corneal progenitor/stem cells in an animal model of type 2 DM [49]. Chen et al. observed a significant reduction in the palisades of Vogt of the limbal cornea in patients with type 2 DM compared to those of controls. The most relevant indicators of high risk for stem cell damage were DM duration, total cholesterol level, and low-density lipoprotein cholesterol level [50]. One may attribute the observed LSCD in our 3 patients to their underlying DM as a confounding factor. However, baseline impression cytology was normal in all included eyes.

The limitations of our study include its small sample size and non-homogeneous study sample in terms of the causes of ME and patient comorbidities. However, our findings of changes in anterior segment parameters and the observed effect on LSCs may cast doubt on the safety of repeated IVB injections. Our results must be verified in multicenter clinical trials with longer follow-up periods and larger study populations.

## CONCLUSIONS

Following repeated IVB administration, we observed a significant increase in the mean CCT and a decrease in the ACA and ACD. Nevertheless, there was no significant correlation between the anterior segment parameters and patient age or sex. The mean ACD, but not the CCT or ACA, varied significantly with the number of injections. Three older patients with DM developed LSCD. The observed effect on LSC may cast doubt on the safety of repeated IVB injections. We strongly believe that further studies are required to verify these preliminary findings and establish a robust conclusion.

## ETHICAL DECLARATIONS

**Ethical approval:** The study was approved by the local ethical committee of the Kermanshah University of Medical Sciences and complied with the tenets of the Declaration of Helsinki. Informed consent to participate was obtained from all patients.

**Conflict of interests:** None.

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