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ORIGINAL ARTICLE

Short-Term Impact of Glycaemic Control and Intravitreal Ranibizumab Treatment on Serum Cytokine Levels and Diabetic Macular Edema in Patients with Unregulated Blood Glucose

Kan Şekeri Regüle Olmayan Hastalarda Kısa Dönem Glisemik Kontrol Ve Intravitreal Ranibizumab Tedavisinin Serum Sitokin Düzeyleri ve Diyabetik Maküler Ödem Üzerine Etkisi

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ABSTRACT

Objective: To evaluate the short-term effect of glycaemic control and intravitreal ranibizumab treatment on diabetic macular edema (DME) and to assess the correlation between HbA1c and certain serum cytokines.
Design: A prospective study of 43 participants with HbA1c levels exceeding 53 mmol/mol (7%) and with DME, as detected by spectral domain optical coherence tomography (SDOCT).
Subjects: Participants were grouped according to their initial best corrected distance visual acuity (BCVA). Participants whose BCVA worse than +0.1 LogMAR (Group 1, n = 21) were treated with three monthly doses of intravitreal ranibizumab (0.5 mg) injections, and participants whose visual acuity equal to or better than +0.1 LogMAR (Group 2, n = 22) were followed without treatment.
Methods: Serum cytokine levels, including interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1(MCP-1), and vascular endothelial growth factor (VEGF) were analysed at the beginning and at the end of 3 months, using enzyme-linked immunosorbent assays (ELISA).
Results: A significant decrease in macular thicknesses (except for one quadrant) was observed in Group 1. Changes in serum cytokine levels were not correlated with HbA1c decrease. Serum VEGF level was significantly increased in Group 1, despite the intravitreal treatment.
Conclusion: Short-term glycaemic control alone had limited value in the treatment of DME. The therapeutic effect of intravitreal treatment on DME supports the role of the local cytokine milieu in the pathophysiology.

Keywords: Diabetes Mellitus, Type 2, Glycaemic Control, Glycated Haemoglobin A1c, Macular Edema, Serum Cytokines, Vascular Endothelial Growth Factor

ÖZ

Amaç: Glisemik kontrol ve intravitreal ranibizumab tedavisinin diyabetik maküla ödemi (DMÖ) üzerindeki kısa vadeli etkisini ve HbA1c ile belirli serum sitokinleri arasındaki korelasyonu değerlendirmek.
Tasarım: Prospektif çalışmaya HbA1ç seviyeleri 53 mmol/mol (%7) üzerinde ve spektral domain optik koherens tomografi (SDOKT) ile DMÖ tespit edilmiş 43 katılımcı dahil edildi.
Katılımcılar: Katılımcılar, başlangıç en iyi düzeltilmiş uzak görme keskinliklerine (EDGK) göre gruplandırıldı. EDGK seviyesi +0.1 LogMAR altında olan katılımcılar (Grup 1, n=21) üç doz aylık intravitreal ranibizumab (0,5 mg) enjeksiyonu ile tedavi edilirken, EDGK seviyesi +0.1 LogMAR veya üzerinde olan katılımcılar (Grup 2, n=22) intravitreal tedavi uygulanmadan takip edildi.
Yöntem: İnterlökin (IL)-1β, IL-6, IL-8, tümör nekroz faktörü-alfa (TNF-α), monosit kemoatraktan protein-1(MCP-1) ve vasküler endotelyal büyüme faktörü (VEGF) dahil olmak üzere serum sitokin seviyeleri, enzime bağlı immünosorbent testi (ELISA) kullanılarak 3 aylık takibin başında ve sonunda analiz edildi.
Bulgular: Grup 1'in maküla kalınlıklarında (bir kadran hariç) anlamlı azalma gözlendi. Serum sitokin seviyelerindeki değişiklikler HbA1c düşüşü ile korele bulunmadı. Grup 1'de intravitreal tedaviye rağmen serum VEGF düzeyi anlamlı olarak yükseldi.
Sonuç: Kısa süreli glisemik kontrolün tek başına DMÖ tedavisindeki yeri sınırlıdır. İntravitreal tedavinin DMÖ üzerindeki terapötik etkisi, lokal sitokin ortamının patofizyolojideki rolünü desteklemektedir.

Anahtar Kelimeler: Diyabetes Mellitus, Tip 2, Glisemik Kontrol, Glikolize Hemoglobin A1c, Maküler Ödem, Serum Sitokinleri, Vasküler Endotelyal Büyüme Faktörü

Introduction

Diabetes is a chronic metabolic disease. Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes. (1) Diabetic macular edema (DME) is the most important cause of vision loss in DR. (2) Agents against vascular endothelial growth factor (VEGF), which is a key inflammatory mediator in the pathogenesis of macular edema, are used for the treatment of this condition. (3) Intravitreal anti-VEGF treatment is the first-line regimen for suitable patients with DME (Euretina guidelines). (4) However anti-VEGF molecules have temporary effects (5) and anti-VEGF drugs can not stop this inflammatory process complete-

ly.
Although the pathogenesis of DR has not been clearly elucidated, the role of retinal inflammation in the aetiology is emphasized. Previous studies have described upregulation of inflammatory mediators other than VEGF in the serum, vitreous, and aqueous humour, as well as their proportional changes with the severity of DR. (6-8) It has been proposed that drugs against these specific inflammatory cytokines could be used for treatment, either alone or in combination with anti-VEGF agents. (9)

Moreover, glycaemic control can impact the response to anti-VEGF treatment used in the management of DME. (10) The importance of HbA1c in glycaemic control has been reported repeatedly, including in the Diabetes Control and Complications Trial (DCCT) and The United Kingdom Prospective Diabetes Study (UKPDS). (11, 12)

In this study we aimed to evaluate the short-term effects of glycaemic control and intravitreal ranibizumab treatment on serum cytokine levels and diabetic macular edema in patients with unregulated blood glucose.

Material and Methods

Ethics

This prospective study was approved by The Research Ethics Committee of Necmettin Erbakan University (2014/98, 09/07/2014) and adhered to the tenets of the Helsinki Declaration. Written informed consent was obtained from all participants.

Participants

Fifty-five participants with type 2 diabetes mellitus, with HbA1c levels exceeding 53 mmol/mol (7%), and with DME detected by spectral domain optical coherence tomography (SDOCT) were enrolled. For participants in whom both eyes were eligible for the study, the right eye of each participant was selected.

Individuals with any previous ocular surgery or trauma, any active ocular infection or uveitis, any systemic disease other than diabetes mellitus and hypertension, taking any medication that included corticosteroids or anti-inflammatory drugs, retinal detachment, active neovascularisation, or vitreous haemorrhage were excluded. Twelve participants were excluded during the study based on these exclusion criteria.

All participants consulted the Endocrinology and Metabolism Clinic for glycaemic regulation at the initial examination. Lifestyle recommendations, including diet and exercise, and the medications they are currently using have been revised for each participant to achieve glycaemic improvement in terms of HbA1c levels during 3-month follow up. Insulin was added to the treatment in those who did not receive insulin therapy. In those on insulin therapy, a new dose adjustment was made according to weight and/or metformin was added to their existing insulin therapy.

Ophthalmologic examination and intravitreal treatment

All participants underwent complete ophthalmological examination, including SDOCT (Spectralis®, Heidelberg Engineering, Heidelberg, Germany). Macular thicknesses were measured according to the nine Early Treatment Diabetic Retinopathy Study (ETDRS)-grid defined regions for all participants.

The included participants were divided into two groups according to their best corrected distance visual acuity (BCVA). BCVA was tested by ETDRS Chart 2 in LogMAR (Log of Minimum Angle of Resolution) sizes for testing at 4 meters and for participants with reduced vision at 4 meters, and testing distance was reduced to 1 meter. Group 1 (n = 21) participants whose BCVA worse than +0.1 LogMAR were treated with three monthly injections of intravitreal ranibizumab (0.5 mg) (baseline, month 1, month 2). Ranibizumab at a dose of 0.5 mg in 0.05 ml was injected using a 30-gauge needle under sterile conditions. Group 2 (n = 22) participants whose visual acuity equal to or better than +0.1 LogMAR were followed up without any ocular treatment.

Complete ophthalmologic examination and SDOCT measurements were repeated at baseline, in the first and second month, and at the end of the third month (1 month after the third intravitreal injection for participants in Group 1).

Sampling and laboratory analysis

This study was supported by Necmettin Erbakan University Scientific Research Projects Department (Project No: 141518017).

HbA1c and serum cytokine levels, including interleukin 1-β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-a), monocyte chemoattractant protein-1 (MCP-1), and VEGF, were analysed at baseline and at the end of the 3 months. Blood samples were obtained by venous puncture. Samples were centrifuged at 4000 × g, at 4°C, for 10 minutes (Hettich Rotina 46R, Hettich Zentrifugen, Tuttlingen, Germany). The harvested serum was stored at -80°C (New Brunswick U570, New Brunswick Scientific, New Jersey, USA).

IL1-β, IL-6, IL-8, TNF-a, and MCP-1 levels (Boster Biological Technology, CA, USA) and VEGF levels (eBioscience Bender Medsystems, Vienna, Austria) were measured using enzyme-linked immunosorbent assays (ELISA), using a Bio Tek ELx50 microplate washer (BioTek Instruments, Winooski, VT, USA) and Bio-Rad microplate reader xMark (Bio-Rad Laboratories, Hercules, CA, USA). Concentrations were calculated from

absorbance according to calibration graphs.

Statistical analysis

Data were analysed using SPSS v. 17.0 for Windows (Chicago, IL, USA) software. Basic descriptive statistics were generated for all data and were reported as frequency and percentage, mean ± standard deviation, and median (interquartile range), as appropriate. The Pearson χ2 test was used to compare the categorical parameters. Normal distribution of continuous variables was confirmed with the Shapiro–Wilk test. Independent-samples t-tests, for normally distributed data, and Mann–Whitney U-tests, for non-normally distributed data, were used to compare parameters between two groups. The Wilcoxon signed-rank test and paired samples t-test were used to compare the serum cytokine levels. Pearson's correlation coefficient, for parametric data, and Spearman's correlation coefficient, for non-parametric data, were used for correlation analyses. For all evaluations, a P value of less than 0.05 was considered statistically significant.

Results

Demographic findings of the participants are summarized in Table 1. There was no statistically significant difference between the two groups in terms of age, duration of type 2 diabetes mellitus, sex, BMI, accompanying hypertension, smoking history, and baseline HbA1c levels. There was a predominance of women in both groups. In Group 1, about half, and in Group 2, about two-thirds of participants were receiving both oral antidiabetic drugs and insulin. This difference was not statistically significant (p=0.315). The mean BCVA was 0.49 ± 0.74 LogMAR in Group 1 and 0.05 ± 1.04 LogMAR in Group 2 (p < 0.001). The mean baseline central macular thickness of participants in Group 1 was 551.95 ± 122.22 µm and 315.31± 65.89 µm in Group 2, which was also statistically significantly different (p < 0.001) (Table 1).

The mean baseline HbA1c level in Group 1 decreased from 71 mmol/mol (8.68 ± 1.17%) to 68 mmol/mol (8.33 ± 0.99%) at the end of the third month, while that in Group 2 decreased from an initial 80 mmol/mol (9.51 ± 1.87%) to 70 mmol/mol (8.52±1.66%) at the end of the third month. The change in HbA1c levels in both groups were statistically significant (p < 0.001 and p = 0.001, respectively) (Figure 1a, Figure 1b).
The mean central macular thickness of participants in Group 1 was 407.42 ± 126.15 µm at the end of 3 months. Macular thickness changes between the baseline and the end of the 3-month follow-up in participants in Group 1 were statistically significant (p < 0.05) in all quadrants except for MT-5 (inner inferior subfield: p = 0.078). The mean central macular thickness of participants in Group 2 was 314.50 ± 70.41 µm at the end of 3 months. There was no statistically significant change in macular thicknesses in participants in Group 2 (p=0.929, p=0.070, p=0.053, p=0.109, p=0.744, p=0.510, p=0.808, p=0.747, p=0.872, respectively) (Figure 2).

No significant correlation was found between the change in HbA1c levels and the change in serum cytokine levels, including IL-1β, IL-6, IL-8, TNF-a, MCP-1, and VEGF, when all participants, participants in Group 1, and in Group 2 were evaluated separately.
We did not observe any correlation between baseline HbA1c and VEGF levels in Group 1 (p = 0.237) or Group 2 (p = 0.803). Based on all participants and in Group 2, the 3-month change in HbA1c was negatively correlated with serum IL-6 changes; however, these correlations were not statistically significant (p = 0.072, p = 0.098, respectively) (Table 2).
There was a statistically significant increase in VEGF levels in participants in Group 1 between the initial examination and the end of the third month (p = 0.035) (Table 3).

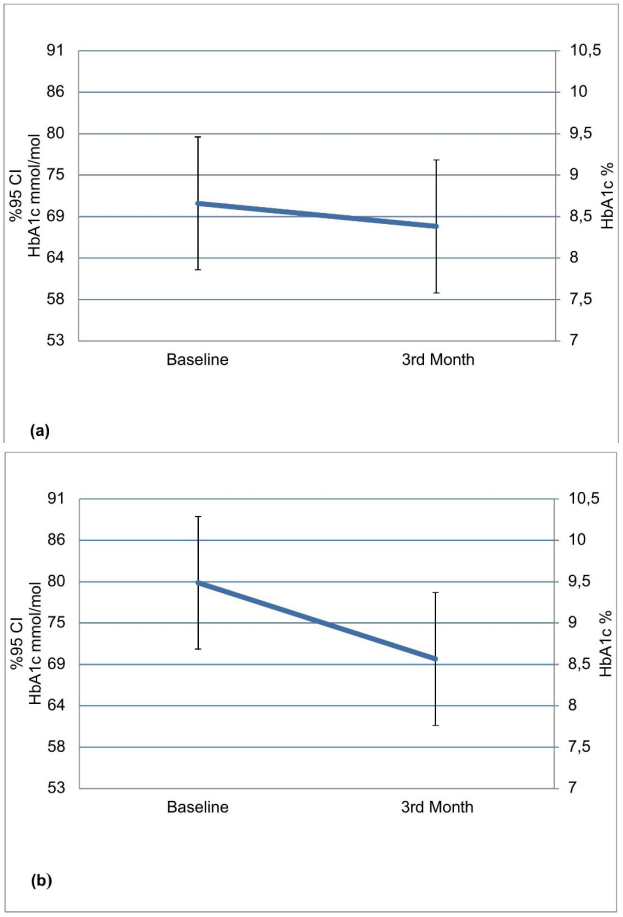

Figure 1: The three-month change in HbA1c mmol/mol (%) levels of participants.

a. The three-month change in HbA1c mmol/mol (%) levels of participants in Group 1.
b. The three-month change in HbA1c mmol/mol (%) levels of participants in Group 2.

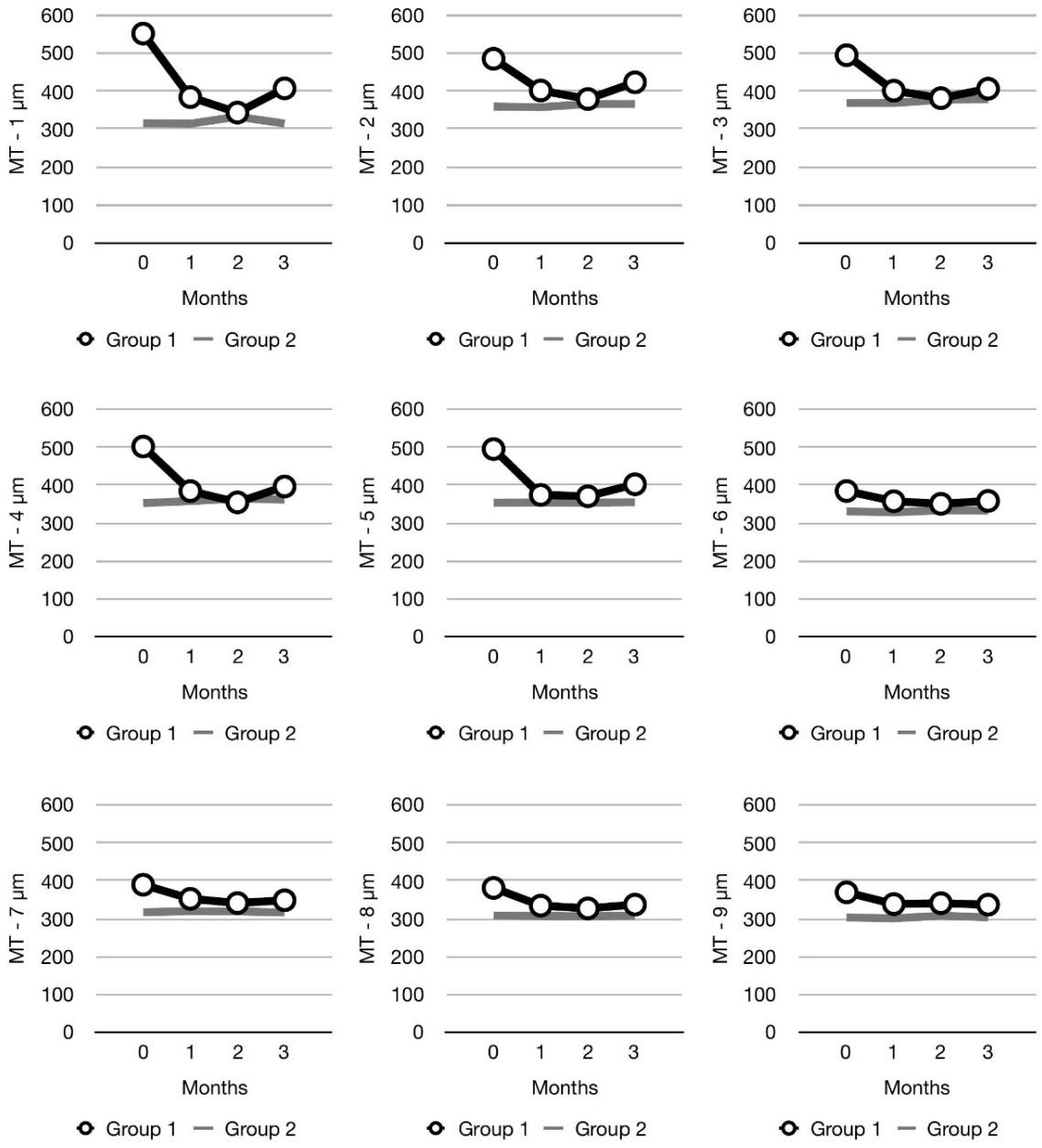

Figure 2: The three-month macular thickness changes (µm) of participants in the standard nine ETDRS subfields.

Footnote: MT-1 describes the central macula (0.5 mm radius around the fovea); MT-2, MT-3, MT-4, MT-5 describe the inner macula (region between 0.5 and 1.5 mm radius around the fovea; nasal, superior, temporal, and inferior quadrants respectively); MT-6, MT-7, MT-8, MT-9 describe the outer macula (region between 1.5 and 3.0 mm radius around the fovea; nasal, superior, temporal, and inferior quadrants respectively).
Abbreviation: ETDRS: Early Treatment Diabetic Retinopathy Study, MT: macular thickness.

Footnote: Abbreviation: CI: Confidence Interval.

Table 1: Baseline characteristics of participants

Variable	Group 1 (*n* = 21)	Group 2 (*n* = 22)	*p*-value
Age (year)*	62.09 ± 7.3	59.72 ± 9.2	0.359
T2DM duration (years)*	16.5 ± 7.8	15.6 ± 7.4	0.720
Sex, women, n (%)**	17 (81)	13 (59.1)	0.185
Body mass index (kg/m²)*	33.46 ± 6.26	31.21 ± 4.60	0.185
Hypertension [a], n (%)**	10 (47.6)	8 (36.4)	0.543
Smoking history, n (%)**	4 (19)	3 (13.6)	0.698
Medication, n (%)** (OAD + insulin)	10 (47.6)	15 (68.2)	0.315
BCVA (LogMAR)*	0.49 ± 0.74	0.05 ± 1.04	**< 0.001**
Central macular thickness (µm)*	551.95 ± 122.22	315.318 ± 65.89	**<0.001**
Haemoglobin A1c mmol/ml (%)*	71 (8.68 ± 1.17)	80 (9.51 ± 1.87)	0.090

Footnotes: Data are expressed as mean ± SD or n (%) as appropriate.
*indicates data analysed with independent samples *t*-test.
** indicates categorical parameters analysed with the Pearson χ2 test.
The bold *P* values represent statistically significant differences.
[a]Hypertension was defined as blood pressure ≥130/80 (or the use of anti-hypertensive medication).
Abbreviations: LogMAR: Log of Minimum Angle of Resolution, OAD: oral antidiabetic drug, SD: Standard deviation, T2DM: type 2 diabetes mellitus.

Table 2: Correlation between the change in HbA1c levels and the change in serum cytokine levels

	ΔHbA1c (All participants)		ΔHbA1c (Group 1)		ΔHbA1c (Group 2)	
	***r*-value**	***p*-value**	***r*-value**	***p*-value**	***r*-value**	***p*-value**
ΔIL-1β	-0.102	0.514	-0.066	0.777	-0.111	0.622
ΔIL-6	0.277	0.072*	-0.227	0.323*	0.361	0.098*
ΔIL-8	0.057	0.714	0.053	0.818	0.090	0.689
ΔTNF-α	0.048	0.758	0.014	0.951	0.019	0.934
ΔMCP-1	-0.212	0.173	-0.188	0.415	-0.164	0.465
ΔVEGF	-0.097	0.537	-0.102	0.661	-0.002	0.994

Footnotes: The Pearson and Spearman's correlation analysis of the inflammatory cytokines.
***indicates data analysed with Pearson's correlation analysis.
r is the correlation coefficient. Correlation is significant at the 0.05 level.
Abbreviations: Δ: Delta, HbA1c: Haemoglobin A1c, IL-1β: Interleukin 1-β, IL-6: Interleukin-6, IL-8: Interleukin-8, MCP-1: Monocyte chemoattractant protein-1, TNF-α: Tumor necrosis factor-alpha, VEGF: Vascular endothelial growth factor.

Table 3: Baseline and third month serum cytokine levels of participants

	Group 1			Group 2		
	Baseline	**Third month**	***p*–value**	**Baseline**	**Third month**	***p*-value**
IL-1β (pg/ml)	18.70 (10.30–35.95)	14.50 (8.60–26.70)	0.794*	25.70 (11.42–47.77)	17.65 (8.30–62.47)	0,910
IL-6 (pg/ml)	15.44 ± 2.79	14.80 ± 1.92	0.813**	17.82 ± 5.46	14.55 ± 2.06	0.336**
IL-8 (pg/ml)	1.50 (0.66–12.84)	1.05 (0.64–2.43)	0.068*	1.96 (0.57–6.51)	0.95 (0.43–12.10)	0.709*
TNF-α (pg/ml)	29.00 (15.10–52.15)	27.20 (17.10–62.30)	0.852*	29,95 (15.75–77.70)	22,30 (14.77–45.10)	0.168*
MCP-1 (pg/ml)	53.80 (24.25–84.35)	60.60 (32.90–106.80)	0.170*	58.70 (34.72–83.00)	67,30 (37.02–124.62)	0.101*
VEGF (pg/ml)	402.00 (223.00–538.00)	456.00 (274.00–790.50)	**0.035***	360.50 (215.00–479.75)	332,00 (176.00–507.50)	0.709*

Footnotes: Data are expressed as mean ± SD for IL-6 and median (interquartile range) for IL-1β, IL-8, TNF-α, MCP-1, and VEGF.

*indicates data analysed with Wilcoxon signed rank test.

**indicates data analysed with Paired samples *t*-test.

The bold *P* value represents statistically significant difference.
Abbreviations: IL-1β: Interleukin 1-β, IL-6: Interleukin-6, IL-8: Interleukin-8, MCP-1: Monocyte chemoattractant protein-1, SD: Standard deviation, TNF-α: Tumor necrosis factor-alpha, VEGF: Vascular endothelial growth factor.

Discussion

In this study, we compared participants with similar demographic findings and concomitant DME. We observed a significant decrease in macular thicknesses (except for the MT-5 quadrant) in Group 1, but no significant changes found in Group 2 at the end of 3 months. Changes in serum cytokine levels did not correlate with an HbA1c decrease. Moreover, serum VEGF level was significantly increased in Group 1, despite the intravitreal treatment.

High blood pressure and duration of diabetes mellitus levels are reported as risk factors for DR. (13) In Group 1, 47.6% of subjects, and in Group 2, 36.4% of subjects had hypertension and received anti-hypertensive treatment in our study. As an indicator of glycaemic control, HbA1c levels of participants in both groups decreased significantly during the 3-month follow-up. According to previous studies, HbA1c is an important indicator of microvascular complications and of hyperglycaemia. The UKPDS reported that a decrease of 0.9% in HbA1c levels would result in a 25% decrease in microvascular complications and a 21% decrease in retinopathy. The DCCT study followed > 99% of people with type 1 diabetes mellitus for a mean of 6.5 years

and demonstrated a 35–76% reduction in retinopathy and other microvascular complications with intensive therapy (three or more daily insulin injections or insulin pump therapy guided by self-monitored glucose levels), as compared with conventional therapy. (11, 12) In our study, despite the decrease in HbA1c levels, no decrease in macular thicknesses were observed in Group 2, who only received treatment for glycaemic control. The macular thicknesses of Group 2 were already lower than that of Group 1; therefore, we may not have seen a relative macular thickness reduction in Group 2. This finding may also be explained by the short-term unsatisfactory impact of glycaemic control. Diabetes mellitus is associated with persistent retinal damage, which may be due to 'hyperglycaemic memory', which results in persistent expression of proinflammatory genes even after glycaemia is normalized. (14) A similar mechanism may be the fundamental for persisting vascular complications despite decreases in HbA1c.

We observed that the macular thickness in Group 1, which decreased until the 2nd month, tended to increase after the 2nd month. Considering the half-life of intravitreal ranibizumab, the results might suggest that the clinical improvement in Group 1 was the result of monthly intravitreal injections, rather than that of glycaemic control. The regression of DME with anti-VEGF agents supports the role of this local effect of VEGF. (15)

Previous studies that included participants with different stages of DR with different metabolic statuses revealed some different results. (16-18) Cavusoglu et al. reported that increasing levels of serum VEGF were related to the stages of DR and there was a correlation between serum VEGF levels and HbA1c. (16) Comparing vitreous samples of participants with proliferative DR (PDR) and people without diabetes, Burgos et al. observed significantly increased vitreous VEGF levels in PDR patients. However, they reported similar serum VEGF levels in both these groups and in healthy control participants. (17) Ozturk et al. did not detect a statistically significant difference in serum VEGF levels between people with non-PDR and PDR, although VEGF levels were higher in those groups than that in healthy participants and participants with diabetes without retinopathy. (18) In our study, participants had similar demographic data and initial metabolic status in terms of the duration of the diabetes, BMI, and HbA1c levels. We did not observe any significant correlation between baseline HbA1c and VEGF levels in either group, or between the changes in HbA1c and serum cytokine levels. In Group 1, serum cytokine levels decreased, except for MCP-1 and VEGF. The increase in VEGF level was statistically significant ($p = 0.035$). Thus, despite the maintained short-term glycaemic control and three doses of anti-VEGF treatment, there was a significant increase in serum VEGF levels. A similar increase in serum and plasma VEGF levels with anti-VEGF therapy have previously been reported. Shao et al. reported significant multiphasic changes of total and free plasma VEGF in a child following intravitreal ranibizumab injections. (19) As a result of two ranibizumab injections for DME, Gnanasekaran et al. found an increase in VEGF levels in serum samples collected before and four weeks after administration. (20) Since this increase was seen only in the participants who received intravitreal injection, contrary to the hypothesis that hypoglycaemia induces the release of VEGF, it is more likely that this effect is due to an increase in VEGF synthesis, (21) and its release from the tissues into the circulation, (22) as mentioned in studies on cancer, which was the first field of use of anti-VEGF drugs.

To the best of our knowledge, no previous study had evaluated the correlation between short-term glycaemic control and serum cytokine levels in people with DME. Using SDOCT, we qualitatively evaluated the macular response to glycaemic control and treatment in participants who had demographically similar characteristics. We suggest that the therapeutic effect of anti-VEGF treatment on DME might indicate the role of the local cytokine milieu, rather than systemic cytokine levels, in the pathophysiology of the condition. These results should be taken into account when considering how to address resistant DME. Our research provides additional insight into the relationship between intravitreal anti-VEGF treatment and serum VEGF levels.
Some limitations of the study should be noted. Taking into account the available storage conditions of the serum samples, the study was designed to have a 3-month duration. The short interval of the study may result in underestimation of the change in serum cytokine levels, despite the statistically significant decrease in HbA1c levels. More significant results may be obtained in a long-term study with a larger patient population. Another limitation was the undetected effect of other organs and of subclinical infections on serum cytokine levels, and the wide range of other detectable cytokines in the serum. We also could not compare the serum levels of the cytokines, since none of the subjects consented to an aqueous or vitreous tap.

Conclusion

Short-term glycaemic control alone has limited value in the treatment of DME. Serum cytokine levels might not be directly correlated with short-term glycaemic control. There may be different clinical findings, including visual acuity and macular thickness, in people with DME who have similar HbA1c levels, BMI, age, and duration of type 2 diabetes mellitus, which suggests that factors other than demographic or metabolic parameters have an impact on disease progression.

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Declaration of Conflicting Interest

The authors declare that there is no conflict of interest.

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