• Clinical Research •

Effect of aflibercept combined with triamcinolone acetonide on aqueous humor growth factor and inflammatory mediators in diabetic macular edema

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Abstract

● **AIM:** To investigate the efficacy of aflibercept combined with sub-tenon injection of triamcinolone acetonide (TA) in treating diabetic macular edema (DME) and to examine changes in growth factors and inflammatory mediator levels in aqueous humor after injection.

● **METHODS:** Totally 67 DME patients (67 eyes) and 30 cataract patients (32 eyes) were enrolled as the DME group and the control group, respectively. The DME group was divided into the aflibercept group (34 cases) and the aflibercept combined with TA group (combined group, 33 cases). The aqueous humor of both groups was collected during the study period. The aqueous levels of vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1β (IL-1β) were detected using a microsphere suspension array technology (Luminex 200TM). Aqueous cytokines, best-corrected visual acuity (BCVA), central macular thickness (CMT), and complications before and after treatment were compared between the aflibercept group and combined group.

● **RESULTS:** The concentrations of VEGF, MCP-1, IL-6, and IL-8 in the aqueous humor were significantly higher in the DME group than those of the control group (all $P<0.01$). After 1mo of surgery, the concentrations of VEGF, MCP-1, IL-6, and IL-8 in the aqueous humor were significantly lower in the combined group than those of the aflibercept group (all $P<0.01$). The BCVA and CMT values of the two groups were statistically different after 1 and 2mo of treatment ($P<0.01$). However, the difference was not statistically significant after 3mo of treatment ($P>0.05$).

● **CONCLUSION:** The cytokines VEGF, MCP-1, IL-6, and IL-8 in the aqueous humor of DME patients are significantly increased. Aflibercept and aflibercept combined with TA have good efficacy in DME patients, can effectively reduce CMT, improve the patient's vision, and have high safety. Aflibercept combined with TA can quickly down-regulate the aqueous humor cytokines and help to relieve macular edema rapidly. However, the long-term efficacy is comparable to that of aflibercept alone.

● **KEYWORDS:** diabetic macular edema; aqueous humor; cytokines; aflibercept; triamcinolone acetonide

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INTRODUCTION

Diabetic macular edema (DME) is a critical complication of diabetic retinopathy (DR) and the leading cause of visual impairment in DR patients[1-2]. DR is caused by the breakdown of the blood-retinal barrier (BRB) and subsequent increase in vascular permeability[3]. Although vascular endothelial growth factor (VEGF) is known to play a role in the development of DME, the pathological process leading to DME is complex and not solely caused by one factor. Inflammatory factors are closely related to the occurrence and development of DME. The upregulation of inflammatory cytokines and pro-inflammatory mediators is considered a key factor in the retinal vascular injury associated with DR, leading to BRB breakdown and the occurrence and development of DME[4-5].

Before researchers elucidated the role of VEGF in DME, treatment options for DME included retinal laser photocoagulation, vitrectomy, and steroids, but the results were unsatisfactory[6-7].

The successful development of anti-VEGF drugs (such as aflibercept, conbercept, and ranibizumab) has provided doctors with drugs to treat DME and improve patient vision. However, some patients still do not respond to anti-VEGF treatment or develop DME again after anti-VEGF treatment[8-9]. Cytokines in the aqueous humor are produced by the retina and enter the aqueous humor circulation through multiple pathways, which can reflect their expression in ocular tissues to some extent. Elevated levels of inflammatory cytokines in the eyes of DME patients suggest that inflammation may be related to the pathogenesis of DME. This study aims to explore the quality and concentration of multiple cytokines in the aqueous humor of DME patients and further observe the efficacy of apremilast and triamcinolone acetonide (TA)[10] with ranibizumab in treating DME patients and their effects on aqueous humor cytokines, in order to guide the diagnosis and treatment of DME in clinical practice.

SUBJECTS AND METHODS

Ethical Approval This prospective study was performed from January 2023 to June 2023 at Shenzhen Aier Eye Hospital and approved by the Ethics Committee of Shenzhen Aier Eye Hospital (2023-KY014-01). The study abided by the Declaration of Helsinki, and all patients signed informed consents.

Subjects A total of 67 DME patients (67 eyes) and 30 cataract patients (32 eyes) who visited Shenzhen Aier Eye Hospital Affiliated with Jinan University from January 2023 to June 2023 were enrolled as the DME group and the control group, respectively. The DME group was divided into the aflibercept group (34 cases) and the aflibercept combined with TA group (33 cases) according to the random number table method, and received intravitreal injection of aflibercept or intravitreal injection of aflibercept combined with sub-tenon's injection of TA.

Inclusion criteria for the DME group: 1) Patients who meet the diagnostic criteria for type 2 diabetes and the clinical diagnosis and treatment guidelines for DR in China (2022) -revised based on evidence-based medicine[11]; 2) Central macular thickness (CMT) measured by optical coherence tomography is greater than 300 μm; 3) Patients with good general health and stable fasting blood glucose and glycosylated hemoglobin levels; 4) Patients who require anti-VEGF treatment and have not received anti-VEGF treatment or intraocular corticosteroid injection treatment within the past 3mo. Exclusion criteria for the DME group: the results of aqueous humor cytokine detection and clinical and imaging data of patients who meet the following conditions are not included in the statistics: 1) Patients with other ocular vascular and inflammatory diseases, including retinal artery and vein occlusion, age-related macular degeneration, polypoidal choroidal vasculopathy, and uveitis; 2) Patients with an anterior chamber depth of less than 2.5 mm and an endothelial cell count of less than 2000/mm^2; 3) Patients with corneal lesions, including pterygium exceeding 2 mm beyond the corneal edge and corneal neovascularization; 4) Patients with severe organic diseases, such as severe heart disease, liver and kidney dysfunction, and tumors; 5) Aqueous humor samples that are insufficient in quantity and result in inaccurate test results. Inclusion criteria for the control group: Non-diabetic patients who require phacoemulsification and intraocular lens implantation surgery due to lens opacity that affects their daily life. Exclusion criteria for the control group: Patients who meet the following conditions are not included in the statistics: 1) Patients with diabetes or abnormal blood glucose; 2) Patients with other ocular diseases, including retinal artery and vein occlusion, age-related macular degeneration, high myopia, polypoidal choroidal vasculopathy, glaucoma, and uveitis; 3) Patients with a history of vitrectomy surgery; 4) Patients who have undergone retinal laser photocoagulation within the past 3mo; 5) Patients who have received anti-VEGF treatment or intraocular corticosteroid injection treatment within the past 3mo; other exclusion criteria 2) to 5) are the same as those for the DME group.

Test Indicators Ophthalmological examinations were performed on all patients 1d before and 1, 2, 3mo after surgery, including: 1) Intraocular pressure (IOP) was measured using a noncontact tonometer; 2) BCVA was measured by refraction, and the results were converted into the logarithm of the minimum angle of resolution (logMAR) visual acuity for recording; 3) Anterior segment was examined using slit lamp; 4) The fundus was observed using a 90 D lens; 5) Fundus segment was examined using optical coherence tomography.

Treatment Method The selected patients in the aflibercept group were treated with intravitreal injection. Three days before the operation, levofloxacin eye drops were applied to the affected eye, 1-2 drops per time, every 4h, to prevent infection. During the surgery, it was conducted under completely sterile conditions, with compound tropicamide for dilation, and surface anesthesia with proparacaine hydrochloride eye drops. Routine disinfection and draping were performed. The pars plana was punctured vertically into the vitreous cavity. A slow injection of 0.05 mL of aflibercept was administered once a month, and after the injection, antibiotic eye ointment was applied. The patient was instructed to rest with closed eyes, and the medication was administered once a month thereafter. When aflibercept was combined with TA, the TA injection was fully centrifuged 2h before the operation and 0.4 mL of the supernatant was taken with a 1 mL syringe. The remaining liquid was shaken in a shaker for 1min, 0.3 mL (20 mg) of the mixed solution was extracted, and the syringe was pulled back

until air was seen. The injection needle was replaced, pushed forward until the needle tip had a drop of liquid, and reserved. After surface anesthesia of the operative eye, the conjunctiva was cut parallel to the corneal margin at the midpoint between the inferior rectus and lateral rectus muscles, and the incision was about 1 mm long. The Tenon's capsule was opened after being pulled out, exposing the underlying sclera. The injection needle was inserted along the scleral surface eye arc towards the optic nerve direction, with a depth of about 3/4 of the needle length. The cotton swab was pressed against the needle hole, and TA was slowly injected while retreating. After the needle was pulled out, the cotton swab was pressed for about 30s. Aflibercept 0.05 mL was injected once a month, and after the injection, antibiotic eye ointment was applied. The patient was instructed to rest with closed eyes. Both groups were treated continuously for 3mo.

Aqueous Humor Collection DME group aqueous humor collection: Before intravitreal injection, a disposable 1 mL insulin syringe was used to carefully puncture the anterior chamber at the 2 o'clock position from the corneal margin, without touching the lens, iris, or corneal endothelium, and extracted about 50 μL of aqueous humor. The sample was immediately stored in a liquid nitrogen tank for later use.

Control group aqueous humor collection: In the operating room under sterile conditions, routine eye disinfection and draping were performed. During phacoemulsification surgery, a 15-degree puncture knife was used to make a side incision at the 2 o'clock position. A disposable 1 mL insulin syringe was used to enter the anterior chamber through the side incision, avoiding contact with the lens, iris, or corneal endothelium, and 50 μL of aqueous humor was extracted and immediately stored in a sterile aqueous humor tube in liquid nitrogen for later use.

Aqueous Humor Cytokine Test The intraoperative aqueous humor was collected in a sterile tube and immediately stored in a liquid nitrogen tank at -196℃. The samples were sent to Beijing GiantMed Medical Laboratory Co., Ltd. for testing using the Luminex 200 technology. The assay kit used was the Human Premixed Multianalyte Kit, a pre-mixed multi-analysis assay kit. The samples were subjected to incubation, antibody incubation, and color development steps, all of which were strictly performed according to the instructions. The samples and standard samples were tested using the microsphere suspension array technology detection instrument, and the sample concentration was automatically calculated.

Statistical Analysis All data were analyzed using SPSS 26.0 statistical software. The measurement data were tested for normality using the Shapiro-Wilk test. Data that followed a normal distribution were expressed as mean±SD. Independent sample *t*-tests were used to compare the differences between two groups. Counting data were expressed as *n* (%) and the differences between groups were compared using the Chi-square test. A *P*-value of less than 0.05 was considered statistically significant.

Table 1 Baseline clinical features in patients

Groups	No. of eyes	Gender (female/male)	Age (y)
DME group	67	40/27	57.8±5.1
Control group	32	15/17	65.7±4.8
χ^2/t		2.79	0.12
P		0.627	0.994
Aflibercept group	34	18/16	57.4±4.1
Aflibercept with TA group	33	22/11	57.9±2.4
χ^2/t		1.33	0.12
P		0.25	0.73

DME: Diabetic macular edema; TA: Triamcinolone acetonide.

RESULTS

There was no statistically significant difference in patient gender, age, or other demographic characteristics between the DME group and the control group, as well as between the aflibercept group and the aflibercept combined with TA group (Table 1).

The aqueous humor cytokine levels of patients in the DME group and control group were compared. The levels of VEGF, monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and IL-8 in the aqueous humor of the DME group were significantly higher than those in the control group, and the differences were statistically significant (all $P<0.01$; Table 2). However, there was no statistically significant difference in the levels of IL-1β between the two groups ($P>0.05$; Table 2).

One month after surgery, the levels of VEGF, MCP-1, IL-6, and IL-8 in the aqueous humor of the aflibercept with TA group were significantly lower than those in the aflibercept group, and the differences were statistically significant (all $P<0.01$; Table 3). However, there was no statistically significant difference in the levels of IL-1β between the two groups ($P>0.05$; Table 3).

After 1 and 2mo of treatment, there was a statistically significant difference in BCVA (logMAR) between the two groups ($P<0.05$; Table 4). However, after 3mo of treatment, there was no statistically significant difference in the comparison between the two groups ($P>0.05$; Table 4).

Before treatment, there was no statistically significant difference in CMT values between the two groups ($P>0.05$; Table 5). After 1 and 2mo of treatment, there was a statistically significant difference in CMT values between the two groups ($P<0.01$; Table 5), but after 3mo of treatment, there was no statistically significant difference in the comparison between the two groups ($P>0.05$; Table 5).

Table 2 Aqueous humor cytokines were compared between the control group and the DME group mean±SD, pg/mL

Groups	No. of eyes	Different concentrations of cytokines in aqueous humor				
		VEGF	MCP-1	IL-6	IL-8	IL-1β
Control group	32	56.22±18.24	884.37±35.29	7.21±3.16	8.93±2.97	0.38±0.04
DME group	67	147.79±21.02	2218.94±39.15	84.16±3.91	42.43±2.65	0.37±0.06
t		-13.76	-14.95	-34.97	-16.33	0.74
P		<0.01	<0.01	<0.01	<0.01	0.46

DME: Diabetic macular edema; VEGF: Vascular endothelial growth factor; MCP: Monocyte chemoattractant protein; IL: Interleukin.

Table 3 Aqueous humor cytokines were compared between the aflibercept group and the aflibercept with TA group mean±SD, pg/mL

Groups	No. of eyes	Different concentrations of cytokines in aqueous humor				
		VEGF	MCP-1	IL-6	IL-8	IL-1β
Aflibercept group	34	24.28±1.16	1740.81±37.66	39.91±4.28	23.59±3.35	0.36±0.06
Aflibercept with TA group	33	4.03±0.97	1558.63±35.82	18.7±3.97	12.09±3.17	0.38±0.04
t		89.04	14.72	22.27	15.05	-1.67
P		<0.01	<0.01	<0.01	<0.01	0.10

TA: Triamcinolone acetonide; VEGF: Vascular endothelial growth factor; MCP: Monocyte chemoattractant protein; IL: Interleukin.

Table 4 Comparison of BCVA (logMAR) before and after treatment between the two groups mean±SD

Groups	No. of eyes	Before treatment	1mo	2mo	3mo
Aflibercept group	34	0.76±0.25	0.49±0.23	0.43±0.26	0.35±0.19
Aflibercept with TA group	33	0.74±0.27	0.36±0.18	0.32±0.23	0.34±0.21
t		0.24	2.13	2.08	0.26
P		0.81	0.04	0.04	0.80

BCVA: Best-corrected visual acuity; TA: Triamcinolone acetonide.

Table 5 Comparison of CMT before and after treatment between the two groups mean±SD, μm

Groups	No. of eyes	Before treatment	1mo	2mo	3mo
Aflibercept group	34	410.23±58.42	371±23.21	336±25.95	293±12.67
Aflibercept with TA group	33	407.18±58.17	354±21.46	319±23.72	295±13.29
t		0.43	5.06	3.16	-0.70
P		0.67	<0.01	<0.01	0.49

CMT: Central macular thickness; TA: Triamcinolone acetonide.

Table 6 Comparison of incidence of complications between the two groups *n* (%)

Groups	No. of eyes	Transient elevated intraocular pressure	Foreign body sensation	Conjunctiva hemorrhage	Incidence of complications
Aflibercept group	34	0	1 (2.94)	2 (5.88)	3 (8.82)
Aflibercept with TA group	33	1 (3.03)	1 (3.03)	1 (3.03)	3 (9.09)
χ^2					0.07
P					0.79

TA: Triamcinolone acetonide.

The incidence of complications in the aflibercept group and the aflibercept combined with TA group was 8.82% and 9.09%, respectively, and the difference was not statistically significant (P>0.05; Table 6).

DISCUSSION

As research on DME continues to deepen, its occurrence and development are considered to be caused by multiple factors, among which inflammatory factors play an important role[12]. Studies have shown that in DME, hyperglycemia leads to abnormal biochemical pathways, and VEGF and various inflammatory factors promote inflammation and the development of retinal hypoxia, which are key factors in retinal vascular damage and BRB disruption[12]. A large amount of testing and analysis of intraocular fluid, such as aqueous humor or vitreous humor, from DME patients has shown significant abnormalities in the levels of VEGF and various inflammatory factors[4,13-14].

Under hypoxic conditions, the level of hypoxia-inducible factor-1α (HIF1α) increases, leading to the activation of genes that promote VEGF production, which is expressed by various retinal cells (including Müller cells) as well as lymph nodes, glial cells, retinal pigment epithelium, endothelial cells, and

pericytes[15]. Diabetes-related retinal hypoxia and inflammation lead to increased VEGF expression, and high levels of VEGF increase the expression of intercellular adhesion molecule-1 (ICAM-1), causing leukocyte stasis in retinal capillaries and damage to the BRB, leading to the occurrence and development of macular edema[12]. Therefore, VEGF is an important pathogenic factor in DME. However, VEGF must bind to VEGF receptors to activate signaling pathways and mediate its biological effects. The two VEGF receptors expressed in the retina are VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2). When VEGF binds to either of these receptors, it activates self-phosphorylation[16-17]. VEGFR-1 is mainly expressed by monocytes and macrophages, and it's signaling plays a role in white blood cell aggregation at inflammatory sites[18]. When VEGF specifically binds to VEGFR-1, it stimulates monocytes and macrophages to produce tissue factors and chemokines, thereby promoting inflammation[19]. VEGFR-2 is only expressed by endothelial cells[18]. When VEGF binds to VEGFR-2, it initiates signal transduction, increasing vascular permeability and upregulating inflammatory cytokines such as MCP-1 and ICAM-1 by activating the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in B cells. These factors and cytokines induce leukocyte chemotaxis and promote inflammatory cell adhesion to vascular endothelium, further increasing vascular permeability[20]. In addition, clinical and experimental evidence suggests that both VEGFR-1 and VEGFR-2 may affect vascular permeability during the inflammatory response[16,20]. VEGF can also act as a chemoattractant for inflammatory cells through its receptors, suggesting that in addition to increasing vascular permeability, VEGF may also promote inflammation. The results of this study showed that the quality concentration of VEGF in the DME group was significantly higher than that in the control group, and the quality concentration of VEGF in the aflibercept combined with TA group was lower than that in the aflibercept group, indicating that aflibercept combined with TA can more effectively reduce the level of VEGF in the aqueous humor of DME patients.

IL-1β is an important member of the IL-1 family and has strong pro-inflammatory activity, inducing various pro-inflammatory mediators such as cytokines and chemokines[21]. Yoshida *et al*[22] demonstrated that IL-1β is an effective inducer of IL-6 expression in cultured human Müller cells, which are considered a major source of IL-1β and other inflammatory factors in the retina. In addition to its potent pro-inflammatory ability, IL-1β and VEGF are mutually upregulated in endothelial cells and are both necessary for inducing pro-angiogenic responses, inducing angiogenesis[23]. This makes it an important target for the treatment of DME and the prevention of early DR. However, in this study, there was no significant difference in the quality concentration of IL-1β between the DME group and the control group, which is inconsistent with the above research results, and the reason for this requires further exploration.

IL-6 is a widely functional and versatile inflammatory cytokine, whose signaling pathway is involved in endothelial dysfunction and vascular inflammation, playing a prominent role in the pathogenesis of inflammatory eye diseases such as DME[24]. IL-6 can promote increased vascular permeability by directly disrupting the barrier function of endothelial and epithelial cells or inducing the production of pro-permeability factors including VEGF, making it an important mediator of VEGF-mediated and inflammatory vascular leakage[25]. Clinical studies have shown that anti-VEGF drugs may not fully control inflammation in some DME patients[26]. Patients with higher initial levels of IL-6 in the aqueous humor have been found to have worse visual outcomes than those with lower initial levels of IL-6[26]. Therefore, the level of IL-6 in the aqueous humor may have prognostic and therapeutic implications, and IL-6 may be a predictive factor or therapeutic target for DME[27]. IL-8 is a pro-inflammatory chemokine that promotes the inflammatory response by activating neutrophils and T cells[28]. Additionally, IL-8 affects cell-to-cell tight junctions, and downregulation of IL-8 can increase vascular permeability[29]. In a study of DME patients, it was found that the mean level of IL-8 in patients who responded to anti-VEGF treatment was lower than that of untreated patients[4]. Therefore, the level of IL-8 in the aqueous humor may reflect the response to anti-VEGF drug treatment in DME. MCP-1, as an effective chemokine, plays an important role in the recruitment and accumulation of monocytes/macrophages, changes in retinal vascular permeability, formation of reactive oxygen species, cell damage, inflammation and angiogenesis, and the pathogenesis of DME[30]. Similar to IL-8, MCP-1 opens tight junctions by promoting the phosphorylation of tight junction-related proteins[31]. Abraham *et al*'s[32] clinical study found that the MCP-1 level in patients who responded to treatment was lower than that of untreated patients, suggesting that baseline MCP-1 may be a predictive indicator of response to anti-VEGF treatment.

The results of this study showed that the mass concentrations of IL-6, IL-8, and MCP-1 in the DME group were significantly higher than those in the control group, consistent with previous research findings. Furthermore, the mass concentrations of IL-6, IL-8, and MCP-1 in the aflibercept combined with TA group were lower than those in the aflibercept group, indicating that aflibercept combined with TA can more effectively reduce the levels of these inflammatory factors in the aqueous humor of DME patients. In addition, observation of the BCVA and CMT values in the Aflibercept combined with TA group and

the Aflibercept group showed that although the improvement in BCVA and CMT values after 1 and 2mo of treatment was better in the aflibercept combined with TA group, indicating better short-term efficacy, there was no statistically significant difference in BCVA and CMT values between the two groups after 3mo of treatment. At the same time, there was no significant difference in safety between the two groups, and no serious complications occurred. The research findings of Yu *et al*[10] demonstrate that it is effective and cost-effective to treat DME by utilizing triamcinolone as an adjunct to the combination of anti-VEGF. In comparison to their study, this research demonstrates that the aflibercept combined with TA treatment is effective in reducing the quality concentration of inflammatory cytokines in the aqueous humor of DME patients.

In summary, aflibercept combined with TA treatment can quickly reduce the body's inflammatory response, lower the mass concentrations of VEGF, IL-6, IL-8, and MCP-1 in the aqueous humor of DME patients, and improve visual acuity and reduce CMT. However, there is no significant difference in long-term treatment efficacy compared to treatment with aflibercept alone. During the treatment of DME, the increased expression of inflammatory cytokines, worsening inflammation, and development of resistance to anti-VEGF treatment suggest that anti-inflammatory treatment should be considered a key component of overall DME treatment when seeking new treatment strategies in the future.

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