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Improved conjunctival microcirculation in diabetic retinopathy patients with MTHFR polymorphisms after Ocufolin™ Administration

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ABSTRACT

Purpose: To investigate conjunctival microvascular responses in patients with mild diabetic retinopathy (MDR) and methylenetetrahydrofolate reductase (MTHFR) polymorphisms (D + PM) after administration of Ocufolin™, a medical food containing 900 μg L-methylfolate (levomefolate calcium or [6S]-5-methyltetrahydrofolic acid, calcium salt), methylcobalamin, and other ingredients.

Methods: Eight D + PM patients received Ocufolin™ for six months (6 M). Bulbar conjunctival microvasculature and microcirculation metrics, including vessel diameter (D), axial blood flow velocity (Va), cross-sectional blood flow velocity (Vs), flow rate (Q), and vessel density (VD, Dbox), were measured at baseline, 4 M, and 6 M.

Results: The mean age was 54 ± 7 years. No significant demographic differences were found. Conjunctival microcirculation, measured as Va, Vs, and Q was significantly increased at 4 M and 6 M, compared to baseline. Va was 0.44 ± 0.10 mm/s, 0.58 ± 0.13 mm/s, 0.59 ± 0.13 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.01$). Similarly, Vs was 0.31 ± 0.07 mm/s, 0.40 ± 0.09 mm/s, 0.41 ± 0.09 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.05$). Q was 107.8 ± 49.4 pl/s, 178.0 ± 125.8 pl/s, 163.3 ± 85.8 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.05$). The VD at 6 M was significantly higher than that at baseline ($P = 0.017$). Changes of D were positively correlated with changes of Va, Q, and VD. Effects of MTHFR and haptoglobin polymorphisms on the improvements of conjunctival microcirculation and microvasculature were found.

Conclusions: Ocufolin™ supplementation improves conjunctival microcirculation in patients with diabetic retinopathy and common folate polymorphisms.

1. Introduction

The bulbar conjunctiva is a vascularized mucus membrane covering the outer surface of the eye (Khansari et al., 2018). The conjunctival microvasculature includes branching arterioles, venules, and capillaries (Wang et al., 2016). The bulbar conjunctival microvasculature can be easily accessed and is suitable for in vivo imaging under various physiological and pathological conditions, including ocular, systemic, and cerebral diseases, such as dry eye (Chen et al., 2017a, 2017b; Chen et al., 2018), diabetes(Cheung et al., 2001; Cheung et al., 2009), Alzheimer's disease (Smith et al., 2009) and sickle cell anemia (Cheung et al., 2010).

Microvascular complications lead to end-organ damage in patients with diabetes mellitus (DM). Alterations of vessel integrity and morphology, coupled with capillary loss are classic signs of diabetic retinopathy (Chen et al., 2017a, 2017b; Devaraj et al., 2007; Kim et al., 2016; Klein et al., 2004; Kristinsson et al., 1997; Nesper et al., 2017). Besides retinal vasculature abnormalities, conjunctival microvascular abnormalities due to diabetes have also been reported (Owen et al., 2005; Owen et al., 2008; To et al., 2011). Dilations of large vessels and loss of conjunctival capillaries have been reported with DM (Owen et al., 2005; Owen et al., 2008). Using computer-assisted intra-vital microscopy, Cheung et al. reported enlarged diameters and uneven distributions of the conjunctival vessels in patients with DM (Cheung et al., 2001). To et al. found that morphometric changes in the vessels which were presented earlier in the bulbar conjunctiva than in the retina (To et al., 2011).

Microvascular abnormalities in DM (i.e., diabetic nephropathy, retinopathy, and macular edema) have been associated with hyperhomocysteinemia (Hhcy). Hhcy-associated vascular abnormalities

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include endothelial dysfunction, vascular wall malformations, loss of extracellular matrix collagen, and disruption of the blood-brain barrier (BBB), found in rodents and humans (Moore et al., 2001; Shindler, 2009). Elevated plasma homocysteine (Hcy) often occurs in carriers of the methylenetetrahydrofolate reductase (MTHFR) polymorphisms (Fekih-Mrissa et al., 2017; Santana et al., 2019; Tawfik et al., 2019) MTHFR is an enzyme involved in the remethylation of Hcy in the methionine cycle and catalyzes the reduction reaction of 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate. The MTHFR mutation at nucleotide 677 (C677T) results in the substitution of valine for alanine, while a mutation at nucleotide 1298 (A1298C) leads to a replacement of alanine for glutamine. These dysfunctional polymorphisms are reported to associate with reduced enzyme activity, resulting in a deficiency in the remethylation process and lead to elevated plasma Hcy (Frosst et al., 1995; Weisberg et al., 1998).

Ocufolin™ is a medical food designed to reduce ischemia in patients with common MTHFR polymorphisms (Brown, 2016), which has various nutrients to optimize critical metabolic pathways with vitamins and co-factors for methylation, reducing Hcy, increasing blood flow, reducing ischemia, and reducing oxidative stress in the mitochondria (Majumder et al., 2017). Each capsule of Ocufolin™ contains L-methylfolate 900 μg, vitamin C (ascorbic acid) 33.3 mg, vitamin D (as cholecalciferol) 1500 IU, vitamin E natural complex (as alpha, beta, gamma, and delta tocopherols) 7.5 IU, vitamin B1 (as thiamine hydrochloride) 1.5 mg, vitamin B2 (riboflavin) 10 mg, vitamin B6 (as pyridoxal 5′-phosphate) 3 mg, vitamin B12 (as methylcobalamin) 500 μg, pantothenic acid (as calcium-D-pantothenate) 5 mg, zinc (as zinc oxide) 26.6 mg, selenium (as L-selenomethionine) 20 μg, copper (as cupric oxide) 0.667 μg, *n*-acetyl cysteine 180 mg, lutein 3.35 mg, and zeaxanthin 700 μg. Our previous observations in a case series of diabetic retinopathy patients treated with Ocufolin™ or a similar formulation (Eyefolate™), demonstrated improvement in retinopathy, even in longstanding cases (Wang et al., 2019a, 2019b). However, the effects of Ocufolin™ on the bulbar conjunctival microvasculature and microcirculation metrics in mild diabetic retinopathy patients with MTHFR polymorphisms (D + PM) correlated with visual acuity has not been explored.

Microvasculature and microcirculation can be imaged with methods such as computer-assisted microscopy (Cheung et al., 2001) and functional slit-lamp bio-microscopy (FSLB) (Chen et al., 2017a, 2017b; Chen et al., 2018; Deng et al., 2016; Jiang et al., 2014; Liu et al., 2019; Shi et al., 2019; Shu et al., 2019). FSLB is a quick, easily accessible, and non-invasive modality to evaluate the conjunctival microvascular structure and flow under physiological and pathological conditions (Chen et al., 2017a, 2017b; Chen et al., 2018; Deng et al., 2016; Jiang et al., 2014; Liu et al., 2019; Shi et al., 2019; Shu et al., 2019). FSLB is a slit lamp mounted with high-speed digital camera, which is capable of imaging of small vessels at high magnification and measuring their blood flow parameters. It measures vessel diameter, blood flow velocity (BFV), and blood flow rate (BFR) in real-time (Shu et al., 2019). In our previous studies, FSLB has been validated for analysis of the changes of hemodynamics and branching complexity in conjunctival vessels in healthy subjects (Liu et al., 2019; Shi et al., 2019), patients with dry eye (Chen et al., 2017a, 2017b; Chen et al., 2018), and contact lens wearers (Deng et al., 2016; Jiang et al., 2014).

The goal of the present study was to characterize conjunctival microvasculature and microcirculation markers using FSLB in patients with D + PM in response to the intake of Ocufolin™ for six months.

2. Methods

2.1. Study design, setting, and population

The study design, recruitment, screening, tests, medical food administration, and imaging protocol were reviewed and approved by the institutional review board (IRB) at the University of Miami. Twenty-seven diabetic patients were recruited to screen MTHFR polymorphisms and haptoglobin (HP) genotypes from Bascom Palmer Eye Institute, the University of Miami, from August 2017 to January 2020. Excluding not eligible individuals, eight mild diabetic retinopathy patients with MTHFR polymorphisms (D + PM) entered in the medical food cohort. MDR was diagnosed by a retinal specialist (JT). The diagnoses were made according to the American Academy of Ophthalmology Retina/Vitreous Panel and the American Diabetes Association (ADA) criteria (Chamberlain et al., 2016).

The patients received Ocufolin™ for six months. The dosages were: 1) in the first week, one capsule orally with breakfast per day; 2) in the second week, two capsules with breakfast per day; 3) thereafter, three capsules with breakfast per day until the final visit. The patients with D + PM were imaged at baseline, the end of the 4th month (4 M), and the end of the 6th month (6 M). The visit window was ± 7 days. At these time points, the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were measured.

2.2. Conjunctival imaging using FSLB

FSLB has been well described previously (Chen et al., 2017a, 2017b; Chen et al., 2018; Hu et al., 2018; Jiang et al., 2014). Briefly, the imaging modality was modified from a traditional slit-lamp by mounting a high-speed digital camera with special magnification capability, Movie Crop Function (MCF). The MCF enables the addition of a 7× magnification, which, combined with the slit-lamp magnification of 30×, yields high magnification ideal for imaging erythrocyte cluster motion at 60 frames per second (fps). In addition, the imaging system can be set to a low magnification (15×) for imaging the microvasculature of the bulbar conjunctiva on the temporal side of the eye. The calibrated field of view (FOV) of this setting was 15.74 × 10.50 mm on the conjunctiva.

To measure the mean BFV and BFR, six different locations of the bulbar conjunctiva approximately 1 mm away from the limbus were chosen and recorded. The FOV was 0.9 × 0.7 mm. The measurement was taken on conjunctival venules only since the majority of the conjunctival vessels are venules. Repeat imaging of the same vessels over time is challenging. We followed an optimized protocol which we developed and have previously published (Wang et al., 2019a, 2019b). At the baseline visit, a photo with a large field of view of 15.74 × 10.50 mm^2 was acquired on the temporal conjunctiva. Six small fields containing target vessels were marked. The temporal limbus was used as a reference for relocating the marked fields for follow-up imaging. This strategy enabled imaging of the same or similar vessels at the same locations from visit to visit, resulting in good repeatability of the measurements, as documented previously (Wang et al., 2019a, 2019b). Custom software was developed and validated to process the video clips and measure BFV and BFR (Fig. 1) (Chen et al., 2017a, 2017b; Chen et al., 2018). The detailed image processing procedures have been reported previously (Chen et al., 2017a, 2017b; Chen et al., 2018).

To measure vessel density of the conjunctival microvasculature, custom software (Mathworks, Inc., Natick, MA) was also developed to extract the vessels using a series of image processing procedures as described in previous publications (Fig. 2) (Chen et al., 2018; Jiang et al., 2014). Vessel density (VD) was quantified as Dbox using fractal analysis with box-counting (Chen et al., 2018; Jiang et al., 2014).Vascular metrics included vessel diameter (D), axial blood flow velocity (Va), cross-sectional blood flow velocity (Vs), flow rate (Q), and VD.

2.3. Statistical analysis

IBM SPSS Statistics package for Windows (Version 25.0, IBM Corp., Armonk, NY, USA) was used to analyze the data. Generalized estimating equation (GEE) models were conducted to evaluate the variations in D + PM at different time points: Baseline, 4 M, and 6 M, and

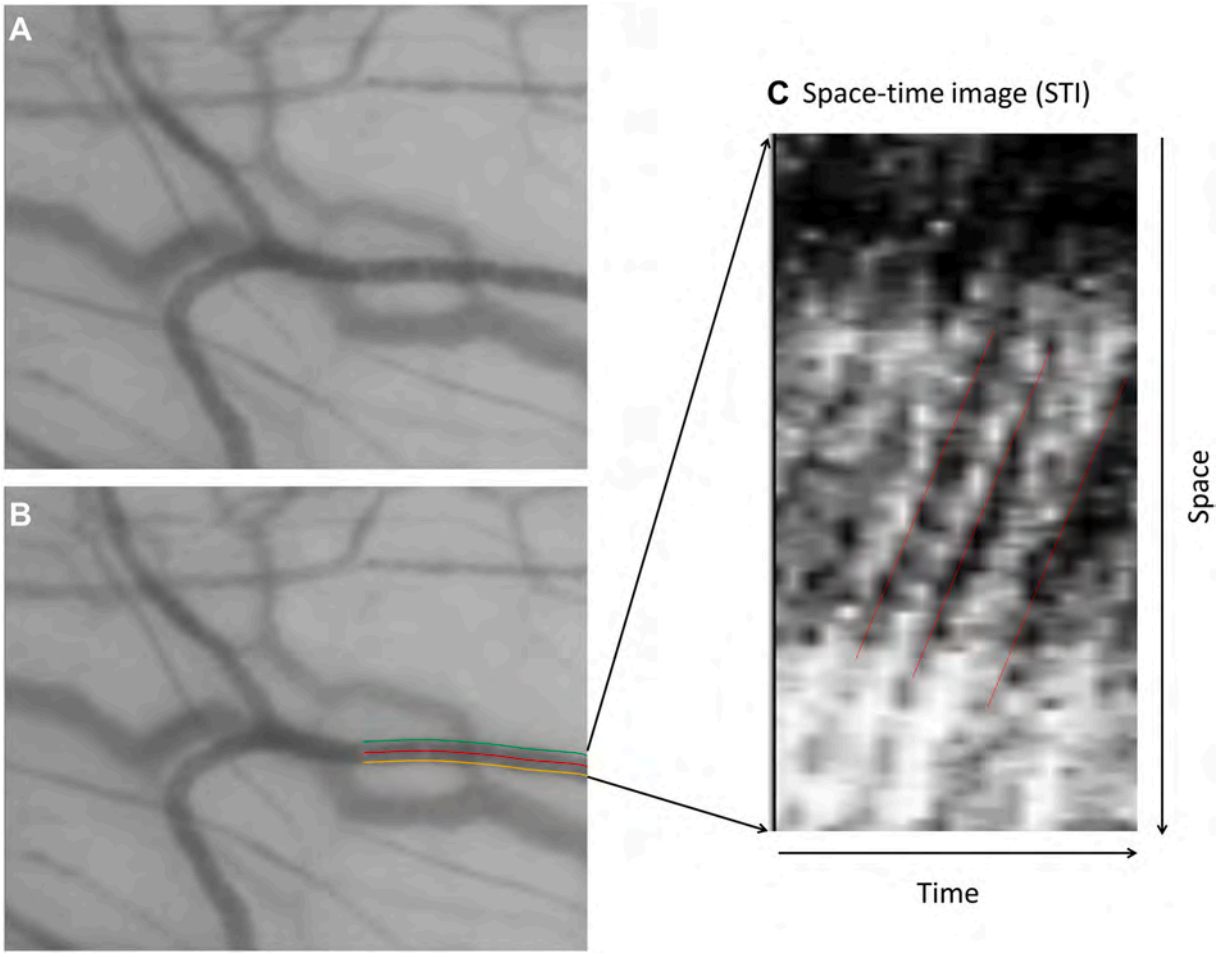

Fig. 1. Calculation of the bulbar conjunctival vascular diameter and blood flow velocity. Video clips of the bulbar conjunctiva blood flow were captured using FSLB and processed to measure blood flow velocity. (A) The first frame of the video clip was utilized for registering all frames to compensate for the eye motion. (B) Using custom software, the vascular walls were outlined and marked in green and yellow lines for measuring the vessel diameter. (C) By measuring the image intensity within the locations defined by the vascular walls, an intensity profile along the centerline (red line in image B) between these walls was generated for each frame in the video clip. Using all intensity profiles (Y-axis) of all frames over time (X-axis) in the video clips, a space-time image was obtained and used to calculate the motion of the cluster of erythrocytes over time (i.e., blood flow velocity). The slopes of the bands (i.e., moving distance over time) were manually outlined (marked in red lines in Image C) and calculated as axial blood flow velocity.

compared with NC. Eyes (left or right) and visits were set as within-subject variables in the GEE models. Vascular measurements were set as dependent variables, while age, sex, and eye were set as covariates. Pearson correlation coefficients were used to evaluate the linear correlations among changes of these variables, including BCVA, and durations of DM. Statistical difference was considered as $P < 0.05$.

3. Results

3.1. Study population and baseline clinical characteristics

The detailed baseline characteristics were reported in Table 1. Sixteen eyes of 8 patients with D + PM were imaged. The majority were male (75.0%), and 62.5% of the patients had hypertension. The average duration of diabetes and HbA1c was 14.5 ± 7.3 years and 7.6 ± 0.9%, respectively. All the patients carried one or two MTHFR mutations, C677T or A1298C. Four patients carried HP1-1/1-2 genotypes, and two patients carried HP2-2 genotypes. Two patients did not have HP genotype results due to the poor specimen quality.

3.2. Effect of Ocufolin™ on patients with MDR

Conjunctival microcirculation, measured as Va, Vs, and Q was significantly increased at 4 M and 6 M, compared to baseline (Fig. 3, $P < 0.05$). Va was 0.44 ± 0.10 mm/s, 0.58 ± 0.13 mm/s, 0.59 ± 0.13 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.01$). Similarly, Vs was 0.31 ± 0.07 mm/s, 0.40 ± 0.09 mm/s, 0.41 ± 0.09 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.05$). Q was 107.8 ± 49.4 pl/s, 178.0 ± 125.8 pl/s, 163.3 ± 85.8 mm/s in baseline, 4 M, and 6 M, respectively ($P < 0.05$).

No significant changes in SBP, DBP, MAP, or HR were observed at visits during the Ocufolin™ intake period (Table 2).

There were no significant differences in vascular diameter, D, among visits (Fig. 3, $P > 0.05$). The VD at 6 M (1.638 ± 0.042 Dbox) was significantly higher than that at baseline (1.618 ± 0.053 Dbox) ($P = 0.017$).

Effects of MTHFR and haptoglobin (HP) polymorphisms on the improvements of conjunctival microcirculation and microvasculature were seen during the Ocufolin™ administration (Fig. 4). Va and Vs at 6 M were significantly increased over time at all genotypes subgroups (all $P < 0.05$). D and Q at 6 M in patients with HP2–2 genotype were decreased when compared to baseline. D, Q, and VD at 6 M in patients with A1298C, C677T, and A1298C, and HP1-1/1-2 were increased significantly over time (all $P < 0.05$).

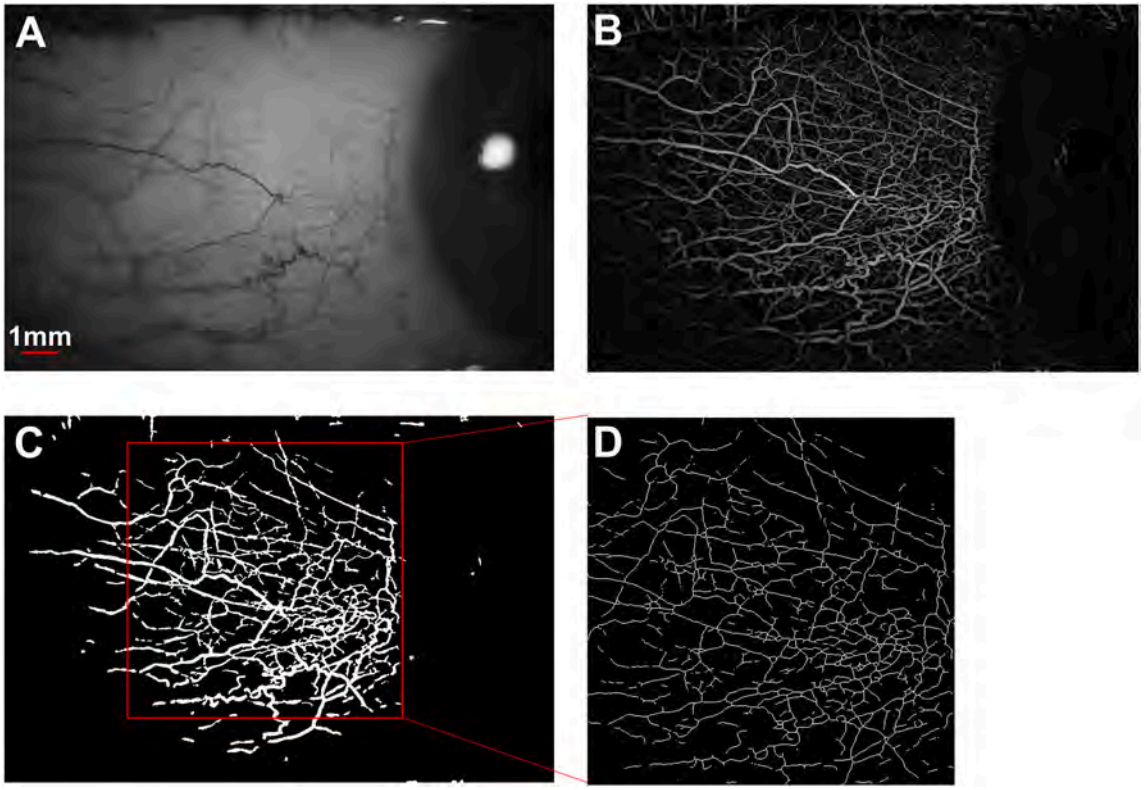

Fig. 2. Image processing to extract the microvascular network. (A) The raw image with an image size of 5184 × 3456 pixels was resized to 1024 × 683 pixels. (B) Microvascular map after a series of morphological opening operations. (C) Segmented vessels using a binary process. (D) Skeletonized image acquired from the cropped images 512 × 512 pixels with a field of view 7.87 × 7.87 mm, which was for fractal analysis to obtain vessel density (VD) using box-counting.

Table 1
Demographics of the patients with MDR at baseline and normal subjects.

	D + PM
Subjects	8
Eyes	16
Sex (male/female)	6:2
Age (years)	58 ± 7
Duration of diabetes (years)	15 ± 7
Hypertension (n, %)	5 (62.5%)
HbA1C (%)	6.9 ± 0.8
Hcy (μmol/L)	12.2 ± 4.2
MTHFR mutation	C677T/A1298C
HP genotype	HP1-1/1-2/2-2

D + PM, mild diabetic retinopathy patients with methylenetetrahydrofolate reductase polymorphisms; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; bpm, beats per minute; HbA1c, glycosylated hemoglobin; Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; HP, haptoglobin.

3.3. Correlations between vascular metrics and BCVA in patients with D + PM

The changes of Va and Q at 6 M were positively related to the change of D (Fig. 5, $P < 0.05$). However, the changes of Vs and VD at 6 M were not related to the changes in D ($P > 0.05$). There were also no significant correlations between the duration of DM with the changes in measured vascular metrics. There were no significant correlations between the duration of DM and the changes of measured vascular metrics (r range −0.595 to −0.063, $P > 0.05$).

4. Discussion

This study represents a first attempt to objectively quantify the alterations of bulbar conjunctival microvasculature and microcirculation in response to the intake of Ocufolin™, a medical food for patients with mild non-proliferative diabetic retinopathy (MDR) and MTHFR polymorphisms (D + PM). The key findings are the improvement of conjunctival microvasculature and microcirculation markers (increased Vs, Va, Q, and VD). Due to its noninvasively accessible characteristics, the bulbar conjunctiva provides a unique site with which to evaluate microangiopathy changes in vascular diseases (Chen et al., 2017a, 2017b; Karanam et al., 2019). Conjunctival vessels are easily recognizable, and measurements of the vascular responses (i.e., alterations in microvasculature and microcirculation) can be easily performed, as in the present and previous studies (Chen et al., 2017a, 2017b; Chen et al., 2018; Deng et al., 2016; Jiang et al., 2014; Liu et al., 2019; Shi et al., 2019; Shu et al., 2019). The typical microvascular abnormalities can be distinguished easily (Shu et al., 2019). Thus, using FSLB to obtain quantitative measurements of the conjunctival microcirculation and microvasculature could serve as an important, objective, and complementary approach to assess treatment effects in diabetics, especially for the treatments targeting the vascular system, such as the Ocufolin™.

Diabetes and homocysteine metabolism are impacted by nutritional deficiencies and common MTHFR genetic polymorphisms affecting vitamin, mineral uptake, and metabolism, which can result in capillary dropout, large vascular morphometric changes, and ischemia. Food supplementation with vitamins, minerals, and nutraceuticals is a safe, inexpensive, and simple way to address risk factors and drivers of visual vascular disorders, including DR (Moore et al., 2001; Shindler, 2009).

Ocufolin™ is an antioxidant-rich, vitamin complex medical food designed to target the ischemic consequences of reduced function polymorphisms of the MTHFR C677TT homozygous state and the MTHFR C677T/A1298C compound heterozygous state (Wang et al.,

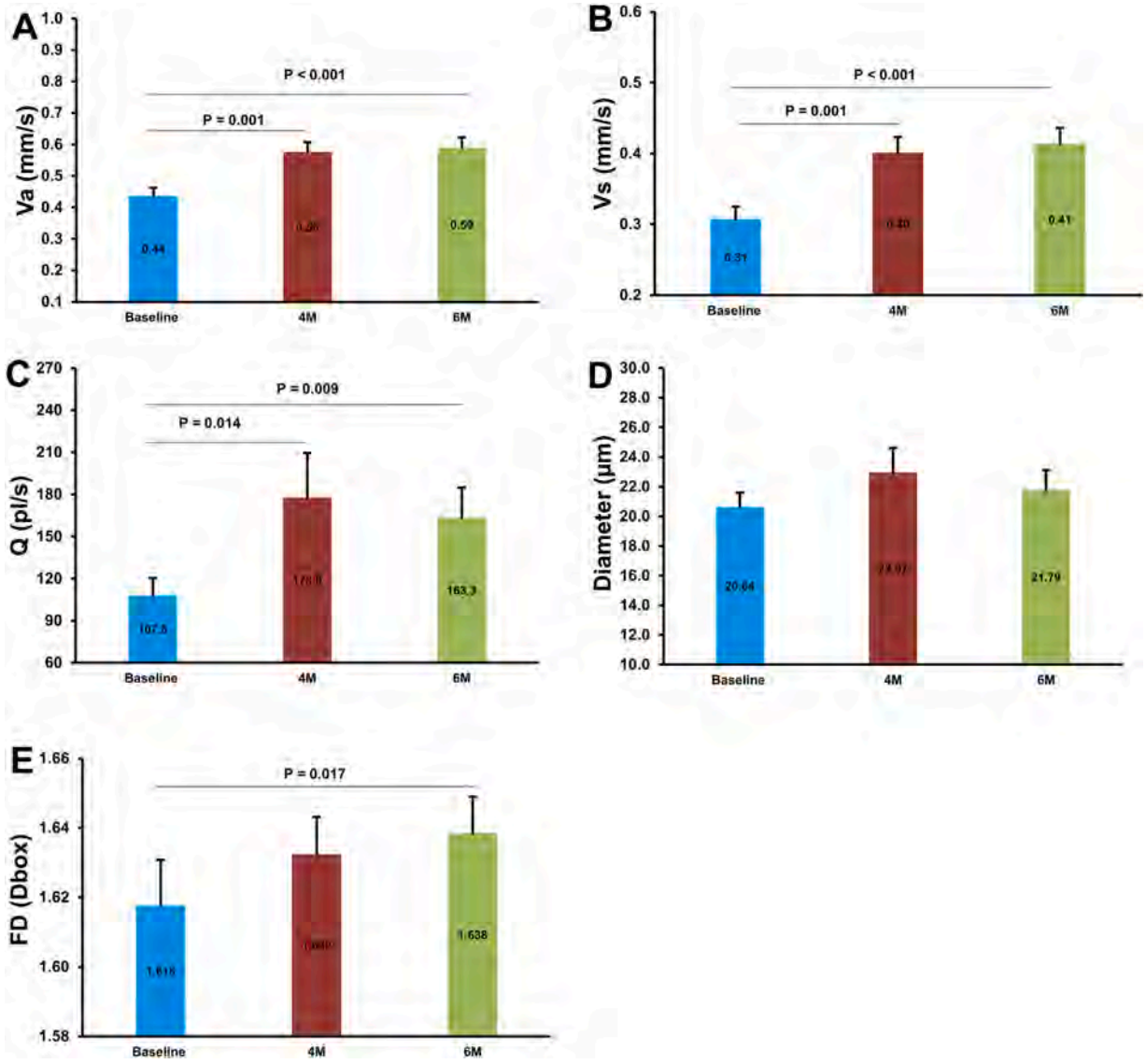

Fig. 3. The changes of conjunctival microvasculature and microcirculation after the intake of the Ocufolin™ in D + PM patients. The measurements were taken at baseline, 4 M, and 6 M after intake of the medical food. Conjunctival microcirculation measured as Va (A), Vs (B), and Q (C) in patients with D + PM were significantly increased at 4 M and 6 M, compared to baseline ($P < 0.05$). There were no significant differences in the D (D) in the D + PM group among visits ($P > 0.05$). The VD (E) at 6 M in the D + PM group was significantly higher than that at baseline ($P = 0.017$). D + PM, mild diabetic retinopathy patients with methylenetetrahydrofolate reductase polymorphisms; Va, axial velocity; Vs, cross-sectional velocity; Q, flow rate; VD, vessel density.

Table 2
Effect of Ocufolin™ on blood pressure, and HR.

Variables	Baseline	4 M	6 M	P-value
SBP, mmHg	138.3 ± 15.8	141.3 ± 26.5	134.6 ± 21.2	> 0.05
DBP, mmHg	81.8 ± 7.3	83.0 ± 12.5	81.4 ± 9.1	> 0.05
MAP, mmHg	100.6 ± 9.1	102.4 ± 16.8	99.1 ± 12	> 0.05
HR, beats/min	68.1 ± 13.2	73.3 ± 10.6	69 ± 13	> 0.05

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

2019a, 2019b). In the present study, improved vessel density and increased microcirculation occurred after Ocufolin™ administration, even at 4 M. This may be due to the optimization of the critical metabolic pathways by supplying vitamins and other components for methylation. Although the level of homocysteine was not measured at the follow-up visits, the reduction of the homocysteine may also play a role in the vascular responses.

There are some important components in Ocufolin™, which may contribute to the reversal of conjunctival blood flow. The B vitamins convert Hcy into methionine, lower blood pressure, and improve insulin resistance (Kumar et al., 2017). By removing folic acid and boosting L-methylfolate intake, Ocufolin™ increases central nervous system (CNS) absorption of L-methylfolate, lowers homocysteine, and decreases oxidative stress thus reversing the downstream metabolic effects of the MTHFR polymorphisms (Stover et al., 2017). Vitamin D further enhances folate uptake (Alam et al., 2019). Thiamine and zinc protect the cells from damage from advanced glycation end products, AGEs. *N*-acetyl cysteine increases glutathione, the main mitochondrial antioxidant, which improves glucose metabolism by reducing oxidative stress (Atkuri et al., 2007;Kowluru and Chan, 2007; Tafuri et al., 2019). Antioxidants such as vitamins C and E, further reduce oxidative stress (Mullarky and Cantley, 2015).

Haptoglobin (HP) polymorphisms have been implicated as a risk factor for cardiovascular disease (CVD) in patients with diabetic mellitus (DM). Haptoglobin is an acute-phase protein that binds to freely circulating hemoglobin, which exists as two distinct forms, HP1 and HP2. HP1 has a small molecular weight that allows it to enter the

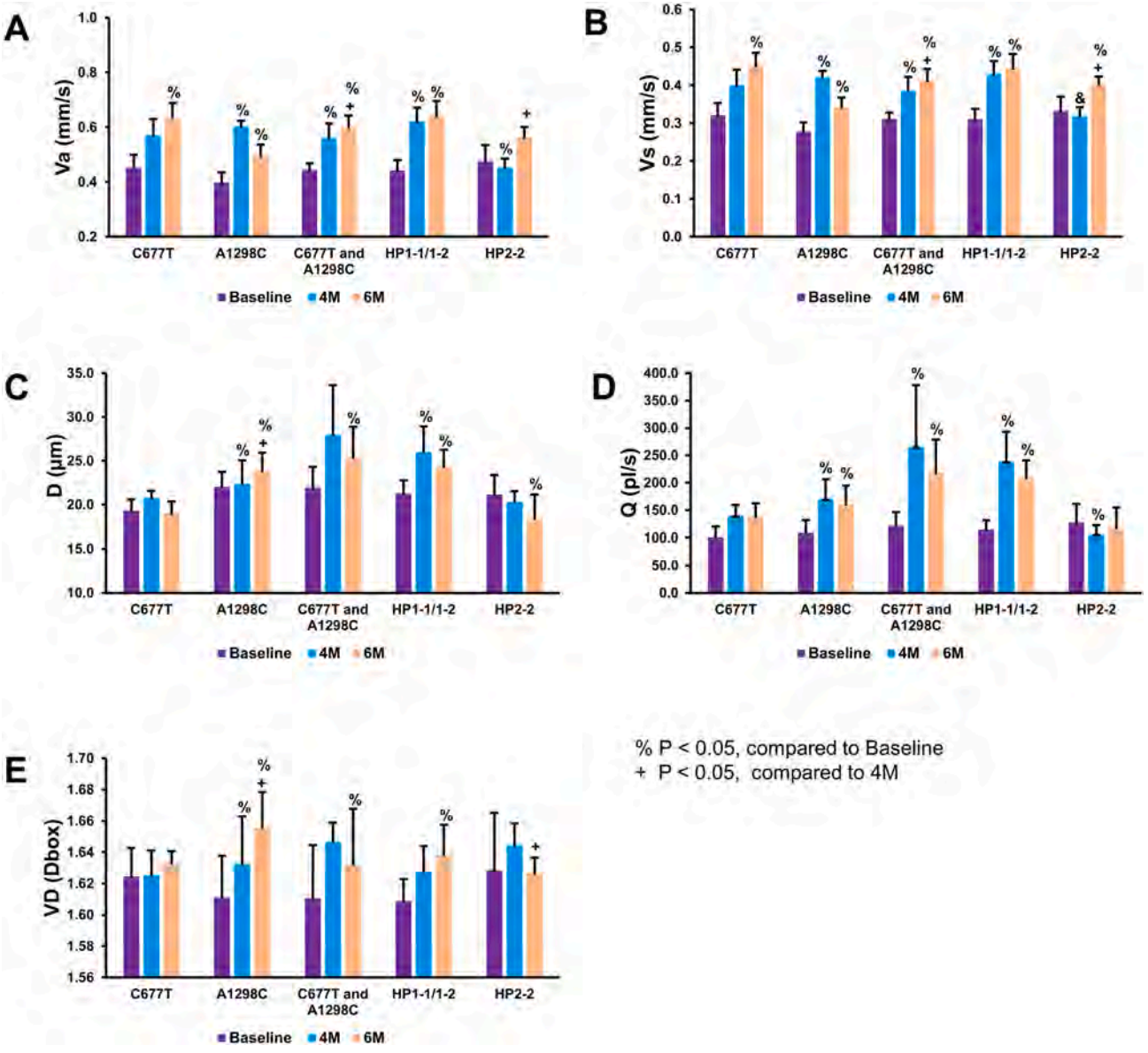

Fig. 4. The effect of MTHFR polymorphisms and HP genotypes on the improvements of conjunctival microcirculation and microvasculature in patients with D + PM during the Ocufolin™ administration. Va and Vs at 6 M were significantly increased over time at all genotypes subgroups (all $P < 0.05$). D and Q at 6 M in patients with HP2-2 genotype were decreased when compared to baseline. D, Q, and VD at 6 M in patients with A1298C, C677T, and A1298C, and HP1-1/1-2 were increased significantly over time (all $P < 0.05$). MTHFR, methylenetetrahydrofolate reductase; HP, haptoglobin; D + PM, mild diabetic retinopathy patients with methylenetetrahydrofolate reductase polymorphisms; Va, axial blood flow velocity; Vs, cross-sectional blood flow velocity; D, vessel diameter; Q, flow rate, VD, vessel density.

extravascular space. The increased size of HP2 prevents its entrance into the extravascular space and prolongs the clearance of HP2 complexes. The HP2-2 genotype predicts the highest risk for CVD in diabetes, HP1-2 predicts intermediate-risk, and HP1-1 predicts the lowest risk. Diabetic patients with HP2-2 genotype are five times more likely to have CVD than patients with HP1-1. Diabetic patients with HP1-2 are three times more likely to have CVD than patients with HP1-1. In the current study, both patients with HP2-2 genotype had CVD. The vascular metrics (VDs, VDd, VDr) at 6M slightly decreased in patients with HP2-2, which may indicate that capillary reperfusion dysfunction due to CVD patients with D + PM.

This was the first attempt to determine the conjunctiva vascular responses to the medical food in selected patients with D + PM. While the findings in this prospective study are intriguing and novel, they need to be considered in light of limitations. First, the study was conducted with small sample size. Despite only including eight patients, significant differences in microvasculature and microcirculation were documented, suggesting that the response could be sufficiently captured in these patients.

Second, we did not include symptomatic change (i.e., ocular comfort) or tear film markers (i.e., break-up time and Schirmer test), which may be important markers to evaluate the impact the ocular surface after medical food administration. Future longitudinal studies are necessary to assess tear film stability, and ocular surface comfort to address the relationship of the improvement in conjunctival blood flow with ocular surface amelioration.

Third, we did not follow up on the changes in Hcy at 4M and 6M. It is necessary to evaluate the relationships between the changes of Hcy and conjunctival blood flow, which may provide a clinical guideline for timing and dosage administration of Ocufolin™ in D + PM. Despite these limitations, this study is the first to characterize bulbar conjunctival vascular changes in response to Ocufolin™ administration in

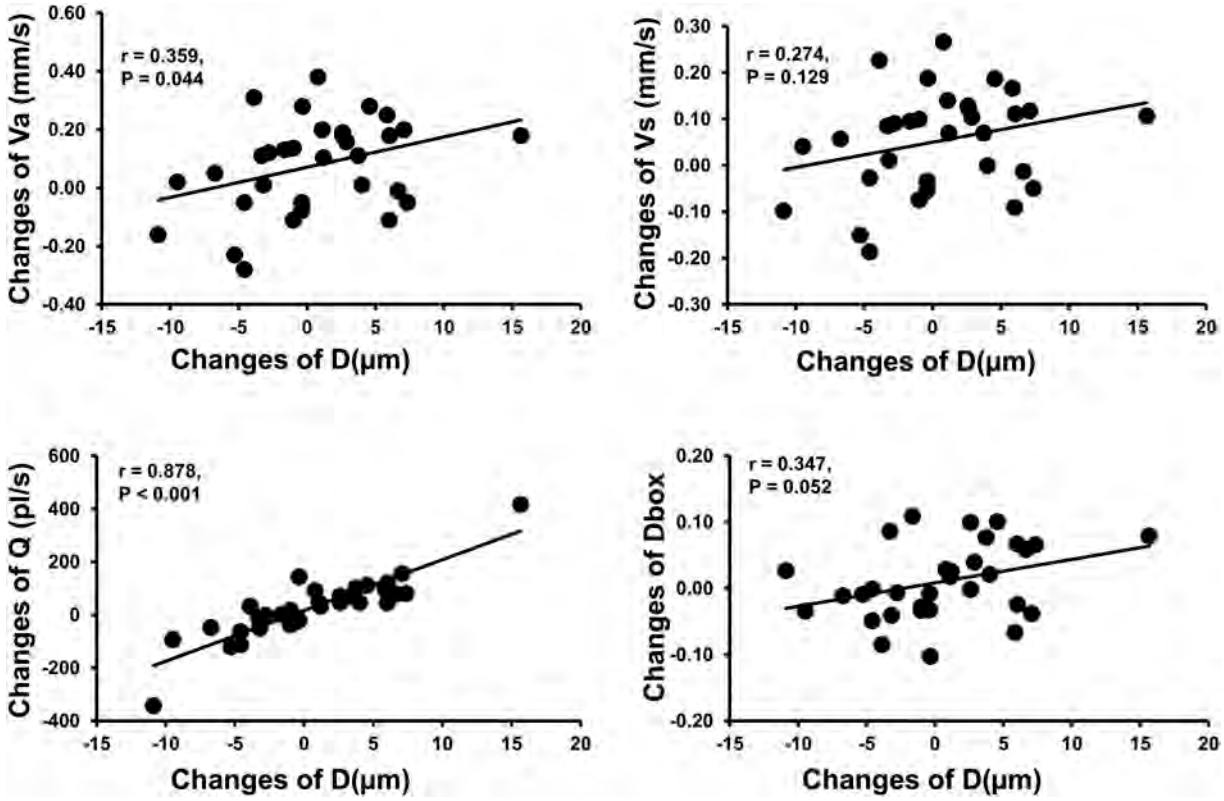

Fig. 5. Correlations among changes at 6 M after the intake of Ocufolin™ medical food. In patients with D + PM, the changes of Va and Q at 6 M were positively related to the change of D ($P < 0.05$). However, the changes of Vs and VD at 6 M were not related to the changes in D ($P > 0.05$). D + PM, mild diabetic retinopathy patients with methylenetetrahydrofolate reductase polymorphisms; D, vessel diameter; Va, axial blood flow velocity; Vs, cross-sectional blood flow velocity; Q, flow rate; VD, vessel density.

patients with D + PM.

Fourth, we did not include a study design with two doses to test the dose-effect on the conjunctival microvasculature. Folic acid has a dose-dependent effect on the reduction of blood concentrations of homocysteine, revealed from a meta-analysis of the randomized trials (Homocysteine Lowering Trialists' Collaboration, 2005). In addition, Ocufolin™ containing 900 μg L-methylfolate has been tested with a lower dose (one capsule daily) over a shorter term (3 months), and the study found a tendency toward an increased retinal blood flow (Schmidl et al., 2020). Based on the available information on the dose-effect, we designed this study with 3 capsules daily and a follow-up period of 6 months, in an attempt to show the effect on conjunctival vasculature for the first time. Future studies with at least two doses are needed to determine whether the dose-effect occurs on the conjunctival vasculature. In addition, we did not include a challenge and re-challenge approach in this study since this pilot study aimed to reveal whether there was a discernible conjunctival response. In addition, we believe it might take months or years to show visible deterioration after stopping the product, which makes it impractical for this pilot study. It may be speculated that once the endothelium heals, deterioration may be slow to recur just as the diabetic deterioration is slow to develop. Therefore, such a challenge and re-challenge approach would take a couple of years and run a real risk of comorbidities and loss of follow-up.

Lastly, Koutsiaris et al. reported that the averaged axial velocity was a function of vessel diameters by studying capillaries and pre-capillary arterioles (not venules) (Koutsiaris, 2005). It is worth noting that the diameters of microvessels mentioned were not the changes of the diameters of the same vessels, but the diameters of different vessels in the vascular system on the conjunctiva. Therefore, vessel dilation of the venules may not necessarily lead to increased axial velocity. In the present study, there were no differences in vessel diameters, although a weak relationship was found between the changes of Va and changes of D in individuals. The positive relation may indicate the increase Va co-existed with the changes of D. However, whether the changes of D had a casual effect on increased Va remained unknown.

In summary, this prospective study characterized conjunctive microvasculature and microcirculation in patients with D + PM in response to the intake of Ocufolin™, a medical food. Improvement of conjunctival microvasculature and microcirculation markers were found after 4 and 6 months of intake. Further studies with large sample sizes are needed to validate further whether the conjunctival vascular measurements can be markers for monitoring the effect of medical foods in patients with various vascular diseases.

CRediT authorship contribution statement

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LZ, JH, JT, and JW collected and analyzed the data. LZ, JH, JT, and JW interpreted the data. ZL and JW were the major contributors in writing the manuscript. All authors read and approved the final manuscript. All the authors report no conflicts of interest.

JW had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declaration of competing interest

All authors have no competing interests.

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