

Protective effects of piperine on the retina of mice with streptozotocin-induced diabetes by suppressing HIF-1/VEGFA pathway and promoting PEDF expression

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Abstract

• **AIM:** To evaluate the protective mechanisms of piperine in the retina of mice with streptozotocin-induced diabetes.

• **METHODS:** In experiments *in vitro*, stimulation by chemical hypoxia was established in ARPE-19 cells. Then, the expression of hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor A (VEGFA), and pigment epithelium-derived factor (PEDF) was assessed at the mRNA and protein levels. In experiments *in vivo*, diabetes mellitus was established by intraperitoneally injecting 150 mg/kg streptozotocin once. After 3wk of the onset of diabetes, 15 mg/kg piperine was intraperitoneally injected once daily for 1 or 3wk. Then, the retinal morphology and mRNA and protein expression were assessed.

• **RESULTS:** In hypoxia, 1-100 μ mol/L piperine significantly decreased the expression of VEGFA mRNA and increased the expression of PEDF mRNA without affecting HIF-1 α mRNA. Meanwhile, 100 μ mol/L piperine substantially decreased the protein level of VEGFA and increased the protein level of PEDF. The HIF-1 α protein level was also hampered by piperine. In the diabetic retina of mice, the morphological damage was alleviated by piperine. Likewise, the retinal vascular leakage was substantially decreased by piperine. Further, the protein levels of HIF-1 α and VEGFA were significantly reduced by piperine. Moreover, the level of the antiangiogenic factor of PEDF dramatically increased by piperine.

• **CONCLUSION:** Piperine may exert protective effects on the retina of mice with diabetes *via* regulating the pro-angiogenic homeostasis composed of HIF-1/VEGFA and PEDF.

• **KEYWORDS:** diabetes; diabetic retinopathy; hypoxia-inducible factor-1 α /vascular endothelial growth factor A pathway; pigment epithelium-derived factor; piperine; mice
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INTRODUCTION

Diabetes mellitus (DM), which mainly manifests as elevated blood glucose levels, has affected about 0.387 billion people globally and 92 million patients in China^[1]. Persistent blood glucose abnormalities eventually result in damage to multiple organs throughout the body. Diabetic retinopathy (DR) is one of the major microvascular complications and a great threat to the vision and quality of life of patients.

The pathogenesis of DR is complex and incompletely elucidated. Many mechanisms are involved in DR, including increased advanced glycation end products (AGEs), elevated peroxide products, activated endoplasmic reticulum stress and protein kinase C, elevated interleukin^[2], and so forth. Moreover, the elevated level of hypoxia-inducible factors-1/vascular endothelial growth factor A (HIF-1/VEGFA) is elucidated in many animal experiments and clinical researches^[3-5]. HIF-1 is made up of two subunits: HIF-1 α and HIF-1 β . HIF-1 α becomes stabilized in hyperglycemia and gets into the nucleus to further activate the expression of VEGFA^[6]. VEGFA mainly works by combining with vascular endothelial growth factor receptor 2 (VEGFR2). In DR, an abnormally elevated level of VEGFA promotes retinal vascular leakage by breaking the tight junctions of endothelial cells^[7]. Meanwhile, the reduction in the content of pigment epithelium-derived factor (PEDF) is believed to be involved in DR for the pro-angiogenic homeostasis composed of VEGFA/PEDF^[8].

Although the intraocular injection of anti-vascular endothelial growth factor (VEGF) is effective in alleviating proliferative DR (PDR) and diabetic macular edema (DME)^[9], repeated

injections are required and still has many nonresponders. Meanwhile, as an important nutrient factor for vascular and nerve survival^[10], simply blocking VEGFA may lead to side effects, such as optic nerve cells death, vascular occlusion, cardio-cerebral blood vascular accident, and arteriosclerosis. Therefore, multitarget drugs to treat DR, especially early DR, need to be urgently found.

Piperine, a pungent alkaloid extracted from black pepper, has a variety of pharmacological activities^[11-14]. It inhibits the migration and angiogenesis of human umbilical vein endothelial cells induced by collagen. Remarkably, it may partly reverse insulin resistance in the high-fat mouse model^[15]. Further, as a lipid-soluble substance with aromatic rings, piperine has the capacity to penetrate the blood-retinal barrier (BRB). The present study aimed to explore the effects and potential mechanisms of systematically applied piperine in mice with diabetes.

MATERIALS AND METHODS

Ethical Approval All the experimental procedures were approved by the Ethics Committee of Xiangya Medical College of Central South University and in agreement with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the use of animals in ophthalmic and vision research.

Animals C57BL/6 male mice were purchased from Shanghai Sippr-Bikai Laboratory Animal Co. Ltd. (License number: SCXK2013-0016). All mice were kept in specific-pathogen-free (SPF) experimental animal facility with 12h dark-light cycle at a room temperature of ~23°C.

Cell Cultures Human retinal pigment epithelial cell line (ARPE-19) was purchased from Guangzhou Cellcook Biology (Guangzhou, China) with short tandem repeat (STR) authentication and without mycoplasma contamination. The cells were grown in Dulbecco's modified Eagle medium (DMEM)-F12 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; v/v; Gibco), 1% penicillin/streptomycin (v/v; Gibco) at 37°C in a humidified 5% CO₂ incubator. The cells were incubated in 10-cm culture dishes for proliferation, and medium change was changed every 2d. Subsequently, a sufficient number of cells were seeded in specific culture dishes for further experiments. For chemical hypoxia stimulation, the cells were pretreated with various concentrations of piperine (Sigma, WI, USA) for 21h and then co-cultured with 100 μmol/L cobalt chloride (CoCl₂; Sigma) for 3h. The compounds of other equilibrium liquids were purchased from China Sinopharm Co. Ltd. (Shanghai, China).

Cytotoxicity Assay Cell counting kit-8 (CCK-8; 7Sea Biotech, Shanghai, China) was used to investigate the cytotoxic effect of piperine and CoCl₂ following the manufacturer's protocol. Briefly, 5×10³ cells/well were seeded in 96-well

plates and treated with piperine or CoCl₂ at concentrations of 0-200 μmol/L or 0-1000 μmol/L at 37°C in the 5% CO₂ incubator for 24 and 48h, respectively. After incubation, 10 μL of CCK-8 solution was added to each well, and the cells continued to grow in the incubator for 1h. The optical density (OD) value was measured using a microplate reader (Thermo Fisher Scientific, CA, USA) at 450 nm, and a row of a cell-free medium was measured as a background. Cytotoxicity was calculated using the following formula: $[(OD_{durg} - OD_{background}) / (OD_{solvent} - OD_{background})] \times 100\%$.

Real-time Polymerase Chain Reaction Total RNA from ARPE-19 cells or mouse retina was separated using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer's protocols. Then, 1 μg total RNA was reverse transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time polymerase chain reaction (PCR) was performed using the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) and Fast SYBR[®] Green Master Mix (Thermo Fisher Scientific) was used for quantification with specific primers with a 20-μL mixture. The list of primers is shown in Table 1. The thermal cycling conditions were as follows: DNA polymerase activation for 20s at 95°C, followed by 40 cycles of denaturation and annealing/extension at 95°C for 3s and 65°C for 30s, respectively. The specificity of amplification was measured by melting-curve analysis. Beta-actin was used for normalizing the expression of the targeted mRNA, and the competitive C_t (2^{-ΔΔC_t}) method was used to calculate the relative expression of genes.

Western Blot Analysis The Western blot analysis was performed by standard Western blotting methods. Briefly, the cells or mouse retina was placed in sodium dodecyl sulfate (SDS) lysis buffer with protease inhibitor cocktail (Sigma; 1:100). The protein samples were crushed using an ultrasonic pulverizer, and protein concentrations were quantified using a bicinchoninic acid (BCA) protein quantitative kit (Multisciences Biotech, Hangzhou, China). Then, 35-40 μg total proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, MA, USA). After blocking with 5% skimmed milk for 1h at room temperature, the membranes were incubated at 4°C overnight with the following specific antibodies (1:1000): rabbit HIF-1α (#14179, Cell Signaling Technology, MA, USA), VEGFA (#ab46154, Abcam, Cambridge, England), PEDF (#07-280, Millipore), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #MAB374, Millipore), and mouse β-actin antibody (#CW0096M, CWbiotech, Beijing, China). After washing with 0.1% TritonX-100 in phosphate buffer saline (PBST) thrice, the membranes were incubated at room

Table 1 Primers used in real-time PCR

Primer name	Primer sequence (5'-3')	Product size (bp)
HIF-1 α sense (H)	GGCAGCAACGACACAGAAAC	176
HIF-1 α anti-sense (H)	TGATTGAGTGCAGGGTCAGC	
VEGFA sense (H)	CGAAACCATGAACTTTCTGC	302
VEGFA anti-sense (H)	CCTCAGTGGGCACACACTCC	
PEDF sense (H)	TATGACCTGTACCGGGTGC GA	70
PEDF anti-sense (H)	CCACACTGAGAGGAGACAGGAGC	
β -actin sense (H)	GGCATGGGTCAGAAGGATT	128
β -actin anti-sense (H)	TGGTGCCAGATTTTCTCCA	
VEGFR2 sense (M)	GCCAACTGAGCAGGAGAGTGTG	103
VEGFR2 anti-sense (M)	GTGGACCGATGTTGCCTGTGAG	
PEDF sense (M)	CCAGCATTGGACCTCTGTGT	235
PEDF anti-sense (M)	GTCCCTCTGGGTAGGTAGCA	
β -actin sense (M)	GTGCTATGTTGCTCTAGACTTCG	174
β -actin anti-sense (M)	ATGCCACAGGATTCCATAACC	

HIF-1 α : Hypoxia-inducible factor-1 α ; VEGFA: Vascular endothelial growth factor A; PEDF: Pigment epithelium-derived factor; VEGFR2: Vascular endothelial growth factor receptor 2; H: Human; M: Mouse.

temperature for 1h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (#CW0103S, CWbiotech) or anti-mouse (#ZB-5305, ZSGB-Bio, Beijin, China) IgG (1:10000). Then, the blots were washed with 0.1% PBST thrice and incubated with Western blotting luminescent solution (Millipore). The densities of bands were observed by the Gel Doc 1000 imaging analysis system (Bio-Rad, CA, USA) and analyzed by Image J software (National Institute of Health, <http://rsb.info.nih.gov/ij/>). The integrated option density (IOD) of each band was calculated and normalized by β -actin (relative IOD, RIOD).

Diabetic Mouse Model Six-week-old C57BL/6 male mice (about 20 g) were fasted overnight for about 12h. Then, 150 mg/kg streptozotocin (STZ; Sigma) freshly prepared in 100 mmol/L citrate buffer (pH 4.5) was intraperitoneally injected once for establishing the diabetic mouse model. Mice with random blood glucose levels greater than 19 mmol/L, polyuria, magersucht, and glucosuria were considered diabetic (mice with STZ-induced diabetes)^[16]. After 3wk of the onset of diabetes, 15 mg/kg piperine prepared in 3% (v/v) polyethylene glycol and Tween 80 was intraperitoneally injected once daily for 1 or 3wk. Age-matched mice without and with diabetes (intraperitoneally injected solvent) were regarded as normal and diabetic control, respectively. Therefore, the mice were randomized to six groups: NC 4W (normal control group for 4wk), DM 4W (diabetic control group for 4wk), PIP (piperine) 1W+DM 4W (piperine treatment for 1wk in mice with DM for 4wk), NC 6W, DM 6W, and PIP 3W+DM 6W. Insulin (2 units) was injected subcutaneously twice a week to reduce the mortality of mice with diabetes.

Hematoxylin and Eosin Staining Mouse eyeballs were enucleated, fixed with 4% paraformaldehyde for 24h,

dehydrated in ethanol, and embedded in paraffin. The eyeballs were cut to 4 μ m and stained with hematoxylin and eosin (Servicebio, Wuhan, China) following the manufacturer's protocol. Only the sections through the posterior eye segment were chosen for further experiments and analysis. The posterior segments were the sections when the plane passed through the optic nerve or within 300 μ m from the optic head rim^[17].

Immunofluorescence Analysis Paraffin-embedded sections were dewaxed and hydrated in xylene and ethanol, respectively. Then, they were immersed in 10 mmol/L sodium citrate (pH 6.0) and heated in a microwave oven for antigen retrieval. After washing with 1 \times PBS for 5min thrice, the sections were blocked with 1% bull serum albumin for 30min at room temperature and incubated overnight at 4 $^{\circ}$ C with VEGFA and PEDF antibodies mentioned in the Western blotting section. Then, the sections were washed with 1 \times PBS again and incubated with secondary antibody (fluorescein isothiocyanate labeled goat anti-mouse; Servicebio) for 50min in the dark at room temperature for the next day. After washing with 1 \times PBS, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 10min. Then, the DAPI was washed thrice with 1 \times PBS, and the sections were coverslipped with anti-fluorescence quenching sealant (#G1401; Servicebio). The immunofluorescence analysis was performed using a fluorescence microscope (Leica, Wetzlar, Germany) and Image J software (average optical density, AOD). A total of 10 different positions on each layer were tested in 3 mice.

Transmission Electron Microscopy Analysis Mouse eyeballs were enucleated, a pinhole was made in the limbus using a 29-gauge needle (BD, Oakland, USA), and then

the eyeballs were immersed in fixing solution (#G1102; Servicebio) immediately at 4°C for 4h. After washing with 0.1 mol/L phosphate buffer (PB; pH 7.4), the eyeballs were postfixed in 1% osmic acid and 0.1 mol/L PB solution at 20°C for 2h. The eyeballs were dehydrated in a graded ethanol series and 100% acetone sequentially. After infiltration and embedding in acetone and SPI-Pon 812 (SPI, West Chester, USA), the eyeballs were cut to 60-80 nm ultrathin sections using an ultramicrotome (Leica, Wetzlar, Germany) and then stained with 2% uranium acetate-saturated ethanol and lead citrate solution. Transmission electron microscopy (Hitachi, Tokyo, Japan) and Image J software were used for imaging and further analysis. A total of 3 mice and 10 different positions on Bruch's membrane in each mouse were tested.

Vascular Permeability Analysis Measurement of qualitative vascular permeability using Evans blue dye (#E2129, Sigma) in this study was in accordance with the standard operation^[18]. Briefly, the mice were anesthetized with pentobarbital sodium, and 45 mg/kg Evans blue dye was injected into the femoral vein for 2h of circulation. In qualitative experiments, the eyes were enucleated and fixed with 50% FAS (formaldehyde/acetic acid/alcohol/saline) ophthalmic fixator (Servicebio) for 2h. Then, the retinal preparation was photographed using a fluorescence microscope (Leica).

Statistical Analysis The results were expressed as means±SEM (standard error of the mean) and analyzed using SPSS 25.0 software (SPSS Inc., IL, USA). Each experiment was performed three times. The Shapiro-Wilk test was used for the normality test. Comparison among groups was analyzed using a one-way analysis of variance (ANOVA) followed by Tamane's test and the least significant difference (LSD) method. The Kruskal-Wallis test was used to analyze the data that did not conform to the normal distribution. $P<0.05$ indicated a statistically significant difference.

RESULTS

Effects of Piperine on ARPE-19 Cells in CoCl₂-induced Hypoxia The cell viability was tested using CCK-8 to verify the toxicity of CoCl₂ and piperine. The cellular viability remained stable when the cells were co-cultured with various concentrations of CoCl₂ or piperine for 24h. However, the cell viability was markedly inhibited when the cells were co-cultured with CoCl₂ (100-1000 μmol/L) but not piperine for 48h. Given the availability of various experimental schemes^[19-21], the efficacy of hypoxia cellular model was detected by measuring the expression of HIF-1α and VEGFA. When co-cultured with 100 μmol/L CoCl₂ for 3h, the intracellular protein content of HIF-1α reached the peak, as detected using Western blot analysis. Meanwhile, the expression of VEGFA mRNA significantly increased. Hence, co-culture with 100 μmol/L CoCl₂ for 3h was set

for the CoCl₂-induced cellular model in the subsequent experiments. In hypoxia, piperine markedly decreased the expression of VEGFA mRNA to 13% and increased the expression of PEDF mRNA to 7.5 times at most, without affecting HIF-1α mRNA (Figure 1A). Accordingly, 100 μmol/L piperine could substantially decrease the protein level of VEGFA and increase the protein level of PEDF ($P<0.05$ vs CoCl₂ group). Meanwhile, 10 μmol/L and 100 μmol/L piperine dramatically decreased the intracellular protein level of HIF-1α ($P<0.05$ vs CoCl₂ group; Figure 1B and 1C).

Body Weight and Blood Glucose Level of Mice with STZ-Induced Diabetes Each group comprised 10 mice. The modeling success rate of STZ-induced diabetes was about 85%, and mice with diabetes were grouped by the random number method. The weight decreased (NC 4W vs DM 4W vs PIP 1W+DM 4W: 25.21±0.63 vs 19.46±0.65 vs 20.36±0.55 g; NC 6W vs DM 6W vs PIP 3W+DM 6W: 26.73±0.79 vs 18.10±1.01 vs 20.04±0.40 g) and the level of random blood glucose significantly increased (NC 4W vs DM 4W vs PIP 1W+DM 4W: 7.72±0.35 vs 32.42±0.50 vs 30.44±0.62 mmol/L; NC 6W vs DM 6W vs PIP 3W+DM 6W: 8.46±0.40 vs 33.06±0.19 vs 32.62±0.49 mmol/L) after the onset of diabetes. Piperine did not affect the weight and glucose level of mice with STZ-induced diabetes (Figure 2A and 2B).

Protective Effects of Piperine on the Diabetic Retinal Morphology Clear edema was observed in the diabetic retina in the fourth week after the onset of diabetes. Meanwhile, the nuclear loss was found in the diabetic retina. Edema subsided in the sixth week of diabetes. Piperine could substantially alleviate these lesions (Figure 2C).

Protective Effects of Piperine on Bruch's Membrane Remarkable edema was noted in Bruch's membrane and retinal pigment epithelium (RPE) in the fourth week after the onset of diabetes. Meanwhile, obvious cavitation changes could be observed in Bruch's membrane, and piperine remarkably alleviated these changes. Edema was eliminated in the sixth week of diabetes, but visible cavitation changes in Bruch's membrane and reduced basal fold in RPE were observed (Figure 3A). Further thickness analysis of Bruch's membrane showed that piperine significantly reduced the thickness of Bruch's membrane in the fourth week ($P<0.001$ vs DM 4W group) but not in the sixth week of diabetes (Figure 3B).

Protective Effects of Piperine on Retinal Vascular Leakage Induced by Diabetes Evans blue was used to measure the retinal vascular leakage in this study. With the progression of DM, distention of microvessel tips and staining of vessel walls increased. On the contrary, microvascular density decreased after the onset of diabetes. Meanwhile, background fluorescence blurring caused by vessel leakage increased. After using piperine, the staining of vessel walls was alleviated

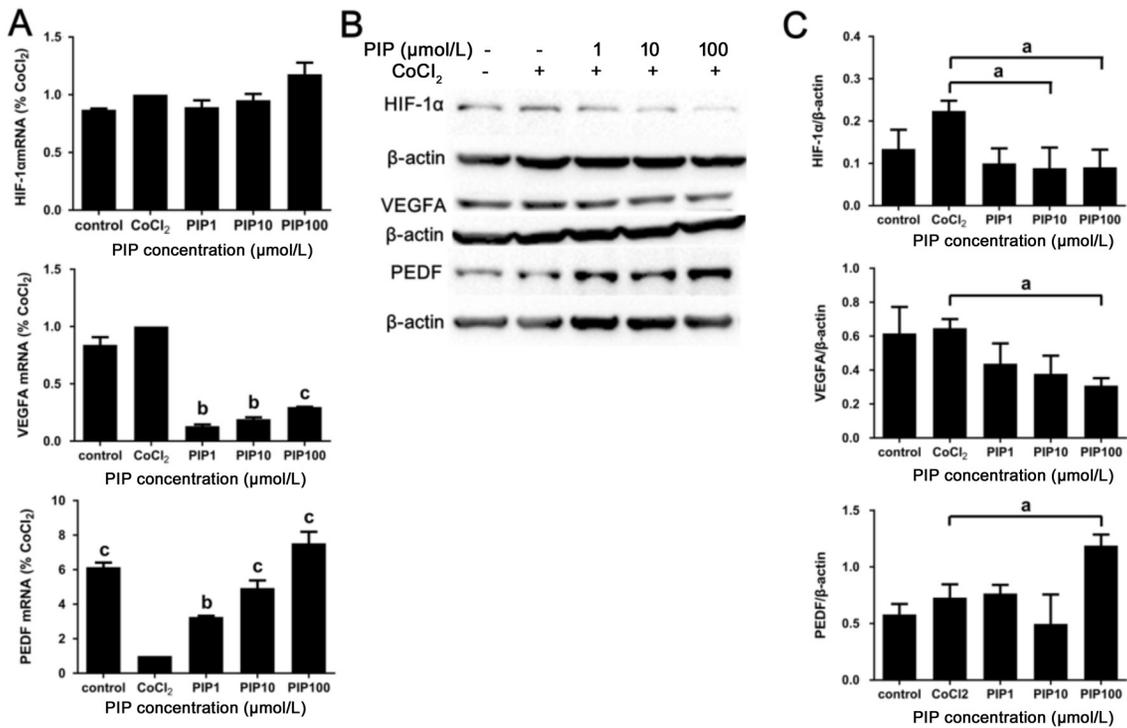


Figure 1 Effect of piperine on relevant mRNA and protein levels in hypoxic ARPE-19 cells. A: The mRNA expression of HIF-1α, VEGFA, and PEDF at different concentrations of piperine. ^b*P*<0.01 and ^c*P*<0.001 compared with the values of hypoxic control (CoCl₂ group). B: Representative photographs of protein levels under various conditions. C: Densitometry analysis of HIF-1α, VEGFA, and PEDF protein levels. ^a*P*<0.05. PIP: Piperine. The contiguous number was concentration (μmol/L). Data were expressed as mean±SEM.

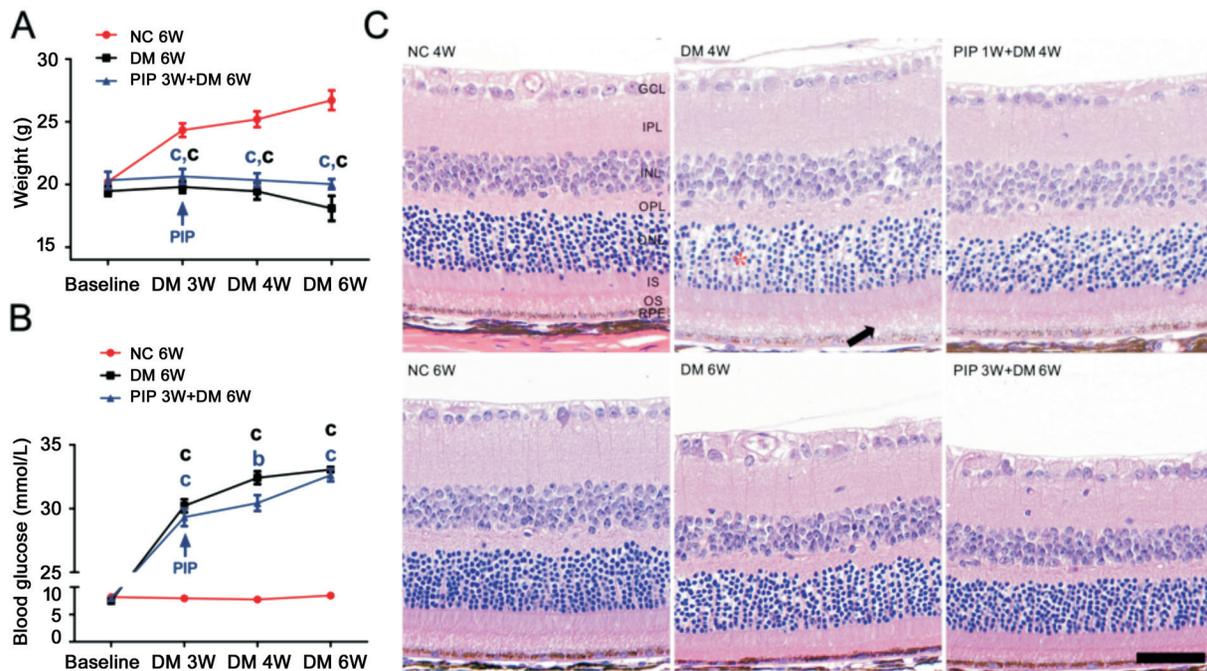


Figure 2 Effect of piperine on body weight (A), blood glucose level (B), and retinal morphology (C) in mice with diabetes mellitus (DM). ^b*P*<0.01 and ^c*P*<0.001 compared with the values in normal control (NC) mice (*n*=10 in each group). Data were expressed as mean±SEM. C: Representative photographs of hematoxylin-eosin staining in the mouse retina. NC 4W: NC mouse with clear retinal layers according to DM 4wk; DM 4W: DM 4wk of control mouse with diabetes. Nuclear loss (red asterisk) and edema (black arrow) were seen; PIP 1W+DM 4W: Mouse retina after using piperine for 1wk without obvious edema; NC 6W: NC mouse according to DM 6wk; DM 6W: DM 6wk of control mouse with diabetes; PIP 3W+DM 6W: Mouse retina after using piperine for 3wk without obvious abnormality. PIP: Piperine; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; IS: Inner segment; OS: Outer segment; RPE: Retinal pigment epithelium. Magnification, 400×; scale bar, 50 μm; *n*=3 in each group.

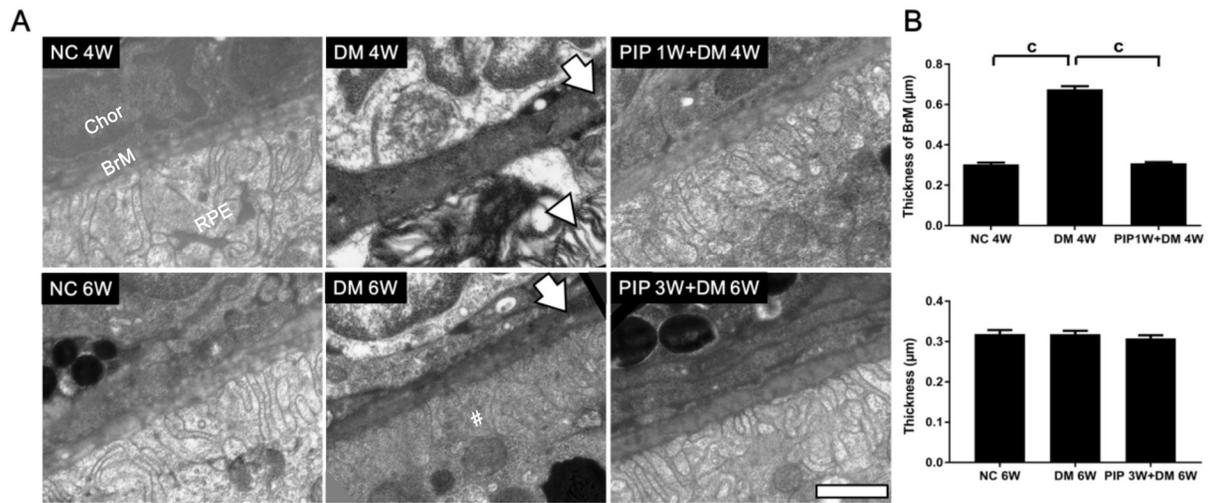


Figure 3 Effect of piperine on Bruch's membrane in the retina of mice with diabetes A: Representative photographs of the retina using transmission electron microscopy. NC 4W: Normal control (NC) mouse with a clear retinal structure according to DM 4wk; DM 4W: DM 4wk of control mouse with diabetes. Obvious cavitation changes (arrow) and edema (arrow head) were seen; PIP 1W+DM 4W: mouse retina after using piperine for 1wk without cavitation changes and edema; NC 6W: NC mouse according to DM 6wk; DM 6W: DM 6wk of control mouse with diabetes. Cavitation changes (arrow) and reduced basal fold in RPE (hash mark) were still visible; PIP 3W+DM 6W: Mouse retina after using piperine for 3wk without obvious abnormality. Chor: Choroid; BrM: Bruch's membrane; RPE: Retinal pigment epithelium; DM: Diabetes mellitus; PIP: Piperine. Magnification, 6000×; scale bar, 1 µm. B: Thickness analysis of Bruch's membrane in DM 4wk and 6wk in each group. * $P < 0.001$ compared with the values in control mice with diabetes ($n=3$ in each group). Data were expressed as mean±SEM.

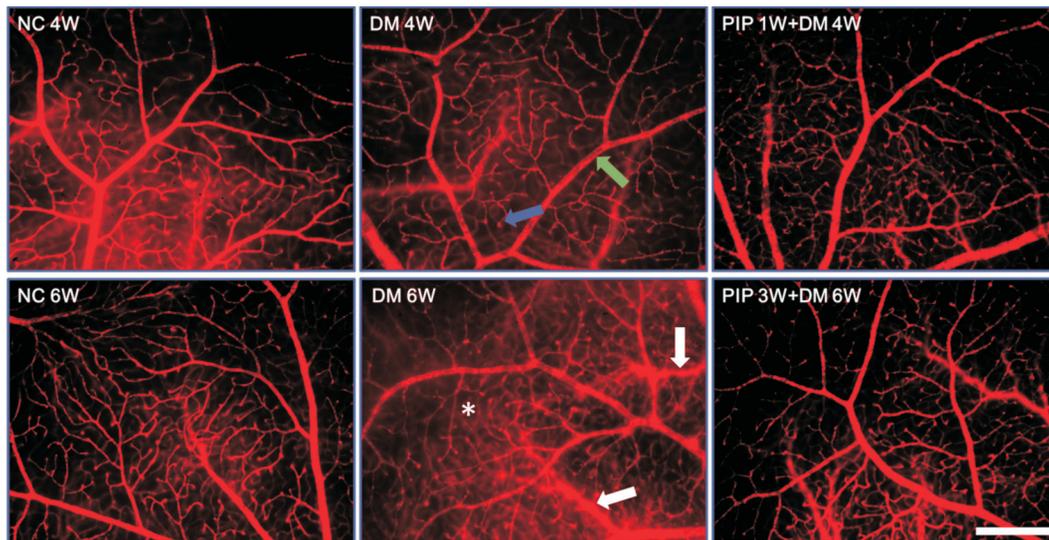


Figure 4 Representative photographs of Evans blue in the mouse retina Normal control (NC) 4wk mice with clear vascular morphology (NC 4W); DM 4wk of mouse with diabetes (DM 4W). Distention of microvessel tips (blue arrow) and staining of vessel walls (green arrow) increased compared with those in the normal group; mouse retina after using piperine for 1wk with little staining of vessel walls (PIP 1W+DM 4W); NC 6wk mice (NC 6W); DM 6wk of mouse with diabetes (DM 6W). Background fluorescence blurring (white asterisk) and vascular leakage (white arrow) were obvious; mouse retina after using piperine for 3wk without typical vascular leakage (PIP 3W+DM 6W). Magnification, 100×; scale bar, 100 µm.

without affecting microvessel tips and microvascular density (Figure 4).

Piperine Protected the Retina of Mice with Diabetes by Suppressing HIF-1α Protein In the fourth week of diabetes, HIF-1α protein level significantly increased in the retina, and piperine could decrease it notably ($P < 0.05$ vs DM 4W group).

Dispite no differences between groups in the sixth week of diabetes, the protein levels in the retina were similar to those in the fourth week (Figure 5B and 5C).

Piperine Protected the Retina of Mice with Diabetes by Suppressing the Expression of VEGFA The VEGFA protein level in the diabetic retina was substantially suppressed by

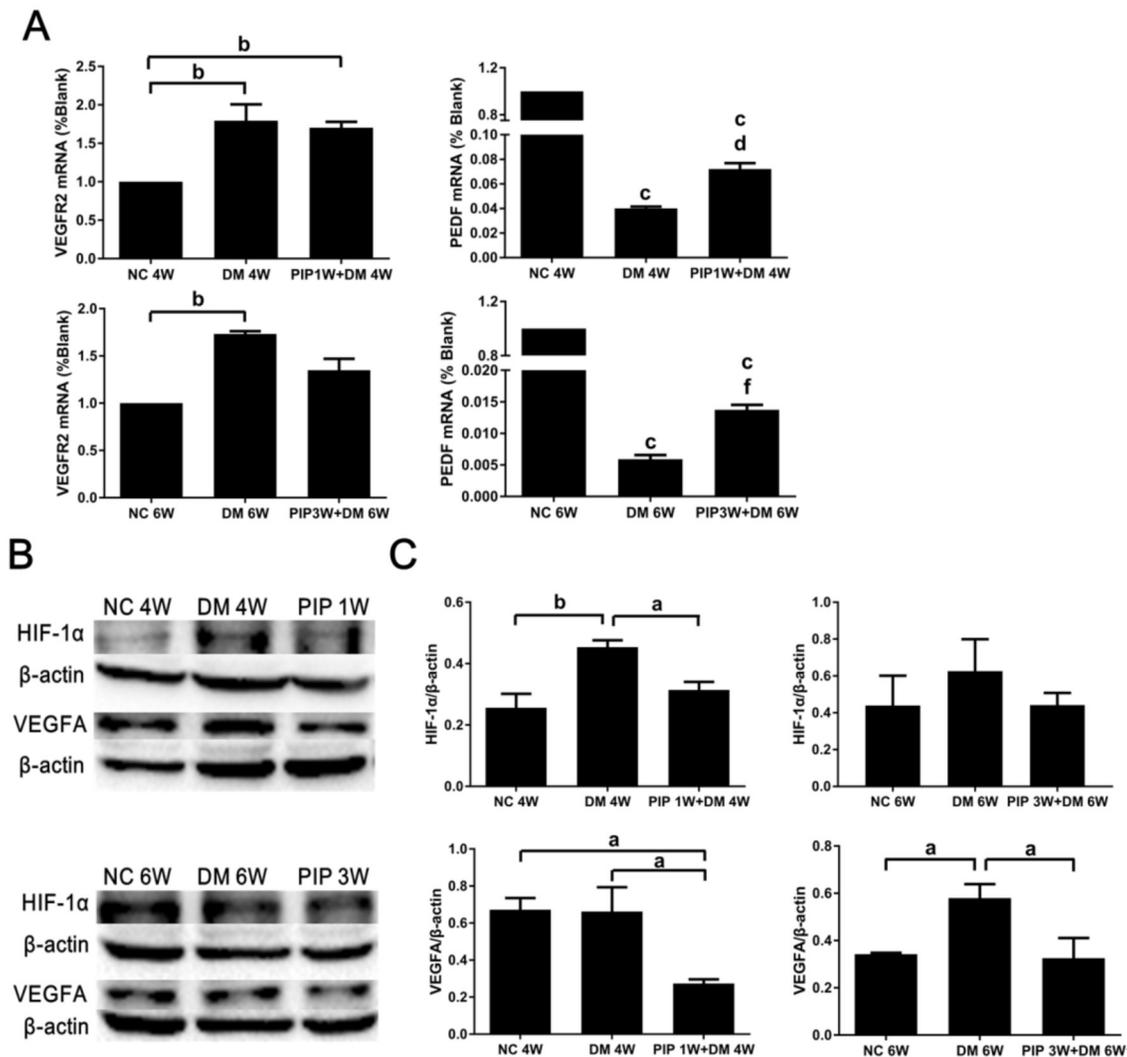


Figure 5 Effect of piperine on relevant mRNA and protein levels in the retina of mice with diabetes. A: The mRNA expression of VEGFR2 and PEDF in different groups of mouse retina in diabetes mellitus (DM) 4wk and 6wk. For mRNA expression of VEGFR2, ^a $P < 0.05$ and ^b $P < 0.01$. For mRNA expression of PEDF, ^c $P < 0.001$ compared with the values of normal control (NC) mice; ^d $P < 0.05$ and ^e $P < 0.001$ compared with the values in control mice with DM, respectively. B: Representative photographs of protein levels in the mouse retina. C: Densitometry analysis of HIF-1 α and VEGFA protein levels in DM 4wk and 6wk mice. ^a $P < 0.05$ and ^b $P < 0.01$. PIP: Piperine ($n = 3$ in each group). Data were expressed as mean \pm SEM.

piperine at any time point ($P < 0.05$ vs DM 4W or DM 6W group; Figure 5B and 5C). As a key receptor of VEGFA, the mRNA expression of VEGFR2 significantly increased after the onset of diabetes, although piperine had no effect on it (Figure 5A).

Piperine Protected the Retina of Mice with Diabetes by Promoting the Expression of PEDF The expression of the antiangiogenic factor of PEDF was tested in the mouse retina. The mRNA expression of PEDF decreased sharply at each time point of diabetes, whereas piperine could observably reverse this effect (Figure 5A). In immunofluorescence analysis, PEDF mainly located in the inner nuclear layer (INL), outer nuclear layer (ONL), inner segment (IS), and outer segment (OS). The PEDF level in the diabetic retina decreased gradually with the progression of diabetes (Figure 6A). With the use of piperine, the PEDF level markedly increased in INL, ONL, and IS of

the diabetic retina in the fourth week of diabetes in further analysis. The content of PEDF in each layer significantly augmented in the third week after using piperine (Figure 6B).

DISCUSSION

In the present study, piperine was identified as having protective effects *in vitro* and *in vivo*. In chemical hypoxia conditions, piperine regulated the ARPE-19 cells by inhibiting HIF-1/VEGFA signaling and promoting the expression of PEDF. Accordingly, piperine protected the STZ-induced diabetic retina by inhibiting HIF-1/VEGFA signaling and promoting the expression of PEDF without affecting the weight and blood glucose level in mice with diabetes. The protective effects of piperine in the retina of mice with diabetes probably were exerted *via* regulating pro-angiogenic homeostasis composed by VEGFA/PEDF.

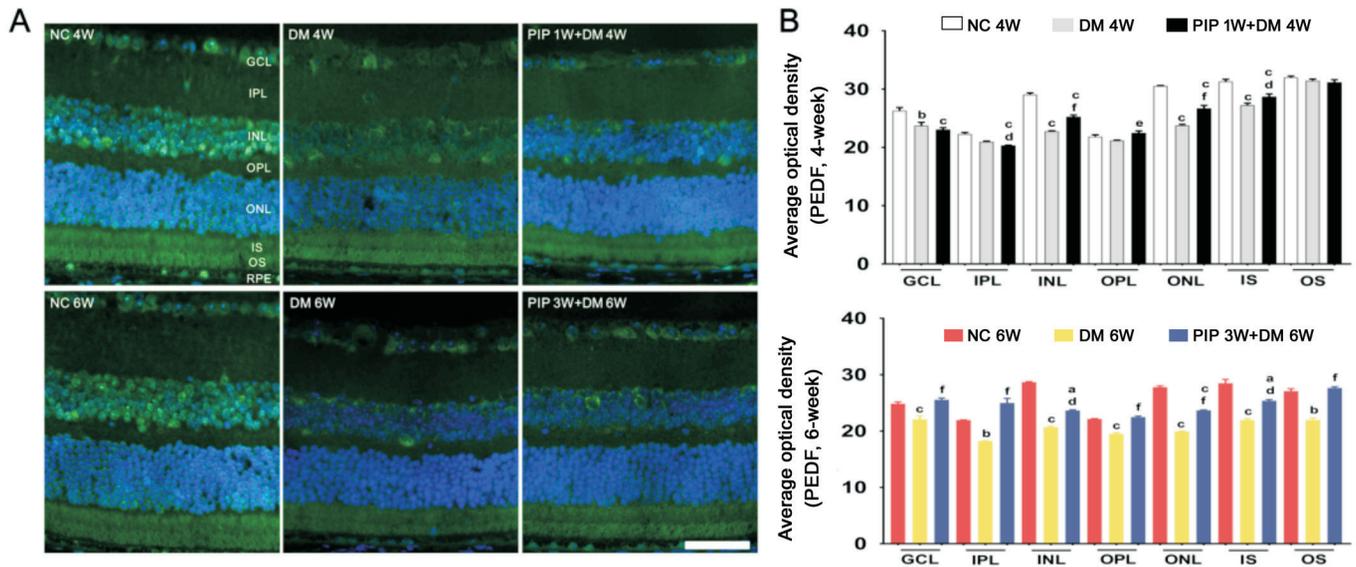


Figure 6 Effect of piperine on protein location and expression in the retina of mice with diabetes. A: Representative photographs of PEDF in immunofluorescence. Normal control (NC) mice according to DM 4wk (NC 4W); DM 4wk of control mouse with diabetes (DM 4W); mouse retina after using piperine for 1wk (PIP 1W+DM 4W); NC mice according to DM 6wk (NC 6W); DM 6wk of control mouse with diabetes (DM 6W); mouse retina after using piperine for 3wk (PIP 3W+DM 6W). B: Retinal average optical density analysis of PEDF in DM 4wk and 6wk. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ compared with the values of NC mice. ^d $P < 0.05$, ^e $P < 0.01$, and ^f $P < 0.001$ compared with the values of control mice with diabetes. PIP: Piperine; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; IS: Inner segment; OS: Outer segment; RPE: Retinal pigment epithelium. Magnification, 400 \times ; scale bar, 50 μ m; $n = 3$ in each group. Data were expressed as mean \pm SEM.

DR is a complication of diabetes involving the eye. As the only visible blood vessels in the body, retinal vessels are important indicators of systemic vascular changes. Meanwhile, pathologic neovascularization is the main reason for the development of DR^[5]. In the very early-stage DM, elevated blood glucose level leads to an increase in plasma osmotic pressure and, subsequently, edema. With disease progression, the destruction of organizational structure and dysfunction emerges in the retina, such as intraretinal hemorrhage, venous string changes, hard and soft exudations, intraretinal microvascular abnormalities, and fibrosis. Similarly, edema in Bruch's membrane in the fourth week of diabetes was significantly alleviated after using piperine. As a structure of outer BRB (oBRB), the destruction of Bruch's membrane in the sixth week of diabetes was also protected by piperine. Likewise, the shielding effects of piperine in the inner BRB (iBRB) quantified using Evan's blue displayed favorable results. HIF-1 is composed of HIF-1 α and HIF-1 β . HIF-1 α is rapidly degraded because of hydroxylation and ubiquitylation by HIF prolyl hydroxylases (PHDs) and von Hippel-Lindau E3 ubiquitin ligase (VHL E3), respectively^[6]. In hypoxia or hyperglycemia conditions, the activity of PHDs is inhibited to ensure the stability of HIF-1 α . Then, HIF-1 α binds to HIF-1 β , and the HIF-1 α/β complex is translocated to the nucleus, where it binds to HIF-responsive elements and induces the transcription of VEGFA^[6]. Although the content of HIF-1 in the early

diabetic retina is controversial^[22-25], its crucial effect on the stage of PDR is certain^[26-28]. In the present study, piperine did not affect the mRNA expression of HIF-1 α , but it decreased the HIF-1 α level in ARPE-19 cells and diabetic retina probably through promoting the degradation of HIF-1 α . VEGFA is mainly expressed in vascular endothelium cells, RPE, Müller cells, and astrocytes in the retina. Then, it mainly binds to VEGFR2 and promotes the progression of DR by augmenting the expression of inflammatory factors and interrupting the tight junctions of BRB^[7]. In non-PDR donors, the expressions of VEGFR2 mRNA in the macular and peripheral retina increased 10 times and 4 times compared with that in normal donors, respectively^[3]. Accordingly, the contents of VEGFA and VEGFR2 protein considerably increased in the peripheral blood of patients with DM^[29]. Therefore, the VEGFA and VEGFR2 are good biomarkers and drug intervention points for DR. Until now, DME and PDR treatments conducted using antiangiogenic reagents, such as ranibizumab and bevacizumab, have achieved encouraging results in clinical applications. Also, piperine drastically inhibited either mRNA or protein expression of VEGFA *in vivo* and *in vitro* in the present study. These results showed that piperine might serve as a drug for DR by suppressing HIF-1 α /VEGFA signaling. As an important neurocyte protection factor, VEGFA binds to VEGFR1 and maintains endothelial homeostasis^[30]. These

mechanisms are the basis of the side effects in intravitreally injected anti-VEGF reagents. Meanwhile, a variety of proangiogenic factors are involved in DR, including insulin-like growth factor 1 (IGF-1), platelet-derived growth factor B (PDGF-B), erythropoietin, angiopoietin 2, interleukin 8, and so forth. Therefore, curing DR only using anti-VEGF reagents is not sufficient. The ideal treatment is to use multitarget drugs in DR pathogenesis, especially in pro-antiangiogenic homeostasis.

PEDF is mainly expressed by RPE in the retina and has effects on the photoreceptor and retinal neuronal cell survival and anti-pathological invasion of neovessels. As an important antiangiogenic factor, the protection mechanisms of PEDF have not been totally elucidated. Some investigators believed that PEDF might bind to PEDF receptor (PEDFR). Then, it plays a protective role by increasing the ratio of B-cell lymphoma-2 (BCL2) / BCL2-associated X (BAX) and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) and decreasing the oxidative stress in the cell and the expression of VEGFA mRNA^[8]. In diabetes, the expression of PEDF is markedly decreased accompanied by an increase in the VEGFA expression, consistent with the results of the present study^[8]. Remarkably, piperine promotes the expression of PEDF at either in mRNA level or protein level, indicating that piperine may be a potential therapeutic drug in DR.

This study had several limitations. First, the chemical hypoxia condition was not optimal, although relevant literature was referred to^[20,31]. However, the expression trends of relevant genes under hypoxia were obvious and piperine could substantially regulate the function of RPE cells. Second, mice with diabetes were not fed long enough, and some of the protective effects of piperine were different at different time points. Last but not the least, whether piperine may protect the diabetic retina *via* immunity, inflammation, oxidative stress, and apoptosis requires further investigation.

In conclusion, as a cheap and safe plant extract, piperine showed powerful protective effects on iBRB and oBRB by regulating the pro-antiangiogenic homeostasis in the retina of mice with diabetes. To the best of our knowledge, this study confirmed the therapeutic effects of piperine in the early diabetic retina for the first time, and it also suggested that piperine might serve as a multitarget drug for the pathogenesis of DR.

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