·Basic Research·

pigment epithelium-derived factor protects the morphological structure of retinal Müller cells in diabetic rats

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Abstract

• **AIM: To investigate if pigment epithelium -derived factor (PEDF) has any protective effect on the retinal Müller cells of Sprague -Dawley rats suffering from diabetes mellitus.**

• **METHODS: Sixty Sprague-Dawley rats were randomly divided into a negative control group, a group receiving 0.1 μg/μL PEDF, another group receiving 0.2 μg/μL PEDF, and a group receiving balanced salt solution (BSS). Rats in both the PEDF and BSS groups were treated intravitreally based on previously established diabetic models. After 4wk of treatment, morphological alterations of Müller cells and protein expression of glutamine synthase (GS) and glial fibrillary acidic protein (GFAP) were analyzed.**

• **RESULTS: PEDF at either 0.1 μg/μL or 0.2 μg/μL significantly improved the structures of both nuclei and organelles of Müller cells compared to the BSS -treated group. Expression of GS was significantly higher in the 0.2 μg/μL PEDF group than that in the BSS group (P=0.012), but expression of GFAP was significantly lower in the 0.2 μg/μL PEDF group than that in the BSS group (P=0.000); however, there were no significant differences in expression of these proteins between the 0.1 μg/μL PEDF group and the BSS group (P=0.608, P=0.152).**

• **CONCLUSION: PEDF protects the morphological ultrastructure of Müller cells, improves the expression of glutamate synthase and prevents cell gliosis.**

• **KEYWORDS:** diabetes mellitus; pigment epithelium-derived factor; retinal Müller cells; glutamine synthase; glial fibrillary acidic protein

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INTRODUCTION

Diabetic retinopathy is a serious ocular complication associated with diabetes mellitus. The disorder is characterized by generation of reactive oxygen species, reduction of multiple antioxidants, a change in the ratio of NADPH/$NADP^+$, activation of NADPH oxidase, and the generation of an excess of advanced glycation end products[1-5]. The cumulative effect of all these changes disrupts normal signal transduction pathways and results in cellular apoptosis[6-9]. Recent evidence suggests that diabetes-induced structural and physiological changes to retinal Müller cells may precede the onset of diabetic retinopathy[10-12]. Therefore, protection of Müller cells in a patient with diabetes mellitus may delay or reverse further progression of diabetic retinopathy. Pigment epithelium-derived factor (PEDF) is a multifunctional secreted protein [13-15]. While its effects on retinal vascular endothelial cells and pericytes have been extensively studied [16-19], little is known about the effect of PEDF on Müller cells [20-22]. Here, we examine both cellular structure and protein expression of retinal Müller cells in diabetic rats following intravitreal injection of PEDF *via* the pars plana. The data presented here will help improve both prevention and early treatment for diabetic retinopathy.

MATERIALS AND METHODS

Materials

Animals Protocols were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, formulated by the National Research Council[23]. Sixty specific pathogen-free Sprague-Dawley rats (both males and females) weighing between 200-220 g were obtained from the Animal Experiment Center of Xi'an Jiaotong University, China [Animal license number: SCXK (Shaanxi) 2007-001]. The animals were housed at 22±2℃ with a relative humidity of 60%±10% and a 12h light/12h dark cycle.

Methods

Animal classification Sixty Sprague-Dawley rats were randomly divided into a negative control group, a group

receiving 0.1 μg/μL PEDF, another group receiving 0.2 μg/μL PEDF, and a group receiving balanced salt solution (BSS). Each group included 15 rats (30 eyes in total). The rats in the negative control group did not receive any intervention. Both eyes of each rat in every treatment group received intravitreal injection *via* the pars plana with 10 μL(0.1 μg/μL) PEDF (Peprotech company), 10 μL (0.2 μg/μL) PEDF, or 10 μL BSS (Bausch Lomb company).

Diabetes model construction Diabetic rat models are based on the methods of Akbarzadeh *et al*[24] and Xiao *et al*[25]. Rats in the 0.1 μg/μL PEDF group, 0.2 μg/μL PEDF group, and BSS group received a single dose of 60 mg/kg 1% streptozotocin (Sigma) citrate solution *via* intraperitoneal injection. Seventy-two hours after injection, animals with blood glucose levels >16.65 mmol/L were considered to be diabetic. All diabetic models underwent intravitreal administration.

Müller cell morphology After 4wk, rats in each group were transcardially perfused with 4% paraformaldehyde for 25min. Right eyes were removed, fixed with 4% paraformaldehyde, and incubated overnight in glutaraldehyde. After multiple washes in phosphate buffered saline (PBS), a small piece of retinal tissue (approximately 1 mm^3) below the optic disc was removed and dehydrated in gradient alcohol. Tissues were embedded in EPON epoxy 812, cut into 70 nm thick sections, and stained with uranyl acetate and lead citrate for 15min. The sections were photographed and observed by transmission electron microscopy (Hitachi).

Immunohistochemistry Left eyes were removed and fixed for 72h in 4% paraformaldehyde. Eyes were then embedded in paraffin and cut into 5-μm sections. The sections were incubated with rabbit anti-mouse-glutamine synthase antibody (Sigma; 1:200) or rabbit anti-mouse-glial fibrillary acidic protein antibody (Beijing Biosynthesis Biotechnology Co., Ltd; 1:200) for 24h at 4℃. After exposure to horseradish peroxidase marked goat anti-rabbit IgG (Beijing Biosynthesis Biotechnology Co., Ltd; 1:200) for 1h at 37℃, digital images were obtained by with an optical microscope (Nikon).

Digital image analysis Image-Pro Plus 6.0 software (Media Cybernetics) was used to measure the optical density values of glutamine synthase (GS) and glial fibrillary acidic protein (GFAP) in digital images. The procedure was performed as follows: Step 1: Open the analysis software; Step 2: Turn the gray-scale units into optical density units; Step 3: Select a digital image to correct optical density value, set the measurement parameters, and save the data; Step 4: Measure the selected area and cumulative intensive optical density (IOD) and use the formula to calculate the optical density (OD), which equals the IOD divided by the selected area; Step 5: Follow the set parameters to measure the other digital images with the above-mentioned method.

Statistical Analysis SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. All experimental data were expressed as mean±deviation. Statistical differences were made by one-way analysis of variance with Dunnett T3 test. Values with $P<0.05$ were considered statistically significant.

RESULTS

Müller Cell Structure Is Altered in Each Group Normal Müller cells found in the control group displayed smooth nuclear membranes, well-distributed nuclear chromosomes, normal mitochondria with clear cristae, well developed rough endoplasmic reticulums, and plenty of free ribosomes. In contrast, in the 0.1 μg/μL PEDF-treated group, the nuclear membrane was blurred, nuclear chromosomes were basically normal, mitochondria were swollen with disrupted cristae, the rough endoplasmic reticulum was mildly swollen, and the total number of free ribosomes was increased. In the 0.2 μg/μL PEDF-treated group, we observed a clear nuclear membrane, normal nuclear chromosomes, moderately swollen mitochondria with a wide cristae gap, a fairly developed rough endoplasmic reticulum, and a large increase in the number of free ribosomes. Changes of Müller cells in the BSS group were as follows: a distorted nuclear membrane, mild agglutination of nuclear chromosomes, extremely swollen mitochondria, absence of mitochondrial cristae, and a significant decrease in rough endoplasmic reticulum and free ribosomes (Figure 1).

Lutamine Synthase and Glial Fibrillary Acidic Protein Expression Are Changed in Each Treatment Group In the normal control group, GS was expressed in multiple cell layers, including the retinal layer, the ganglion cell layer, the inner plexiform layer, the inner nuclear layer, the outer nuclear layer, and the photoreceptor layer. The optical density value for GS in the control group was 0.300±0.059. In the 0.1 μg/μL PEDF-treated group, GS was primarily expressed in the ganglion cell layer and in the inner plexiform layer; the optical density in this group was 0.215 ±0.059, which was significantly different from the control ($P=0.027$). Expression of GS in the 0.2 μg/μL PEDF-treated group was not significantly different from expression in the normal control group ($P=0.078$); the optical density in this group was 0.240±0.035. Expression of GS in the BSS group was significantly reduced ($P=0.000$); the optical density value was equal to 0.182 ±0.037. Furthermore, there was a statistically significant difference in GS expression between the BSS group and the 0.2 μg/μL PEDF group ($P=0.012$). In contrast, no significant differences were detected between the 0.1 μg/μL PEDF-treated group and either the 0.2 μg/μL PEDF group ($P=0.812$) or the BSS group ($P=0.608$; Figures 2, 3, Table 1).

Little to no GFAP expression was detected in the normal control group (optical density=0.148±0.024). In the 0.1 μg/μL

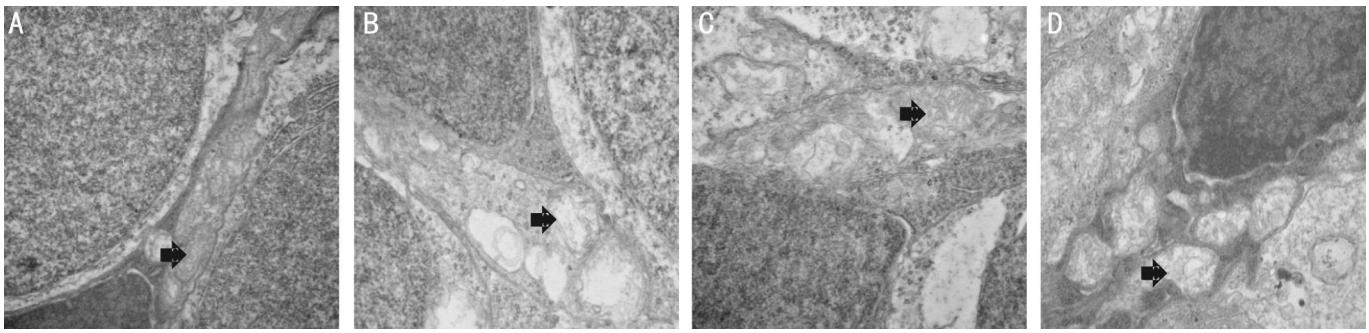

Figure 1 Müller cells observed by transmission electronmicroscopy A: Negative control group: smooth nuclear membrane, normal mitochondria; B: 0.1 μg/μL PEDF group, blurred nuclear membrane, swollen mitochondria with abnormal cristae; C: 0.2 μg/μL PEDF group, clear nuclear membrane, mildly swollen mitochondria with a wide space within the cristae; D: BSS group, distorted nuclear membrane, extremely swollen mitochondria without cristae, newly formed vacuoles. Arrows indicate mitochondria.

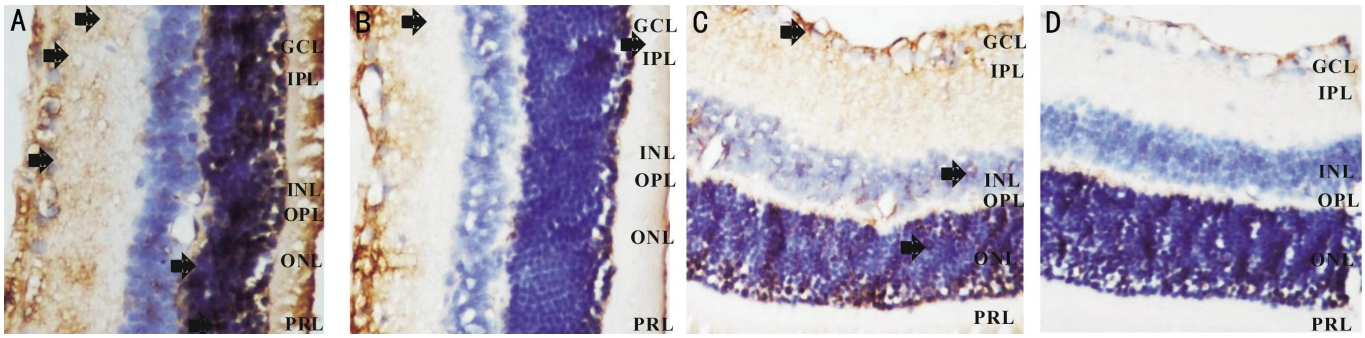

Figure 2 Expression of retinal GS immunohistochemical staining 20×20 A: Negative control group: expression of GS detected in all layers of the retina; B: 0.1 μg/μL PEDF group, GS expression in the ganglion cell layer and the inner plexiform layer; C: 0.2 μg/μL PEDF group, GS expression in the inner plexiform layer, the inner nuclear layer, and the outer nuclear layer; D: BSS group, little to no GS expression detected. Arrows indicate positive areas. GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PRL: Photoreceptor layer.

PEDF-treated group, GFAP was expressed in the ganglion cell layer, in the inner plexiform layer, and in the photoreceptor layer (optical density=0.230±0.059; P=0.010). In the 0.2 μg/μL PEDF group, GFAP was expressed in the inner limiting membrane, the ganglion cell layer, and the inner plexiform layer of the retina (optical density= 0.198 ±0.008; P=0.001). In the BSS group, GFAP was widely expressed in the ganglion cell layer, the inner plexiform layer, the outer plexiform layer, and the photoreceptor layer (optical density=0.283±0.038; P=0.000). There was also a statistically significant difference in GFAP expression between the 0.2 μg/μL PEDF-treated group and the BSS group (P=0.000). In contrast, there were no difference between the 0.1 μg/μL PEDF group and either the 0.2 μg/μL PEDF group (P=0.497) or the BSS group (P=0.152; Figures 4, 5, Table 1).

DISCUSSION

Müller cells are the principal glial cells found in the vertebrate retina, where they assist the metabolic activity of retinal nerve cells and constitute most of the blood-retina barrier. Müller cells help maintain normal retinal structure and function, including providing support for neurons and maintaining the homeostasis of extracellular ions involved in both synaptic signal transmission and the glutamic acid

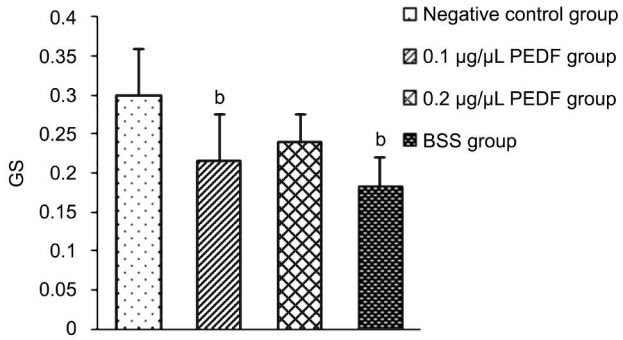

Figure 3 Column chart of GS optical density in each group ($\bar{x} \pm s$) [b]Compared to the negative control group, differences were significant (P<0.01).

Table 1 Optical density of retinal GS and GFAP in each group $\bar{x} \pm s$

Groups	Sample size	GS	GFAP
Negative control group	15	0.300±0.059	0.148±0.024
0.1 μg/μL PEDF group	15	0.215±0.059[a]	0.230±0.059[a]
0.2 μg/μL PEDF group	15	0.240±0.035	0.198±0.008[a]
BSS group	15	0.182±0.037[a,c]	0.283±0.038[a,c]
F		10.345	22.713
P		0.000	0.000

[a]Compared to the negative control group, differences were significant (P<0.05); [c]Compared to the 0.2 μg/μL PEDF group, differences were significant (P<0.05).

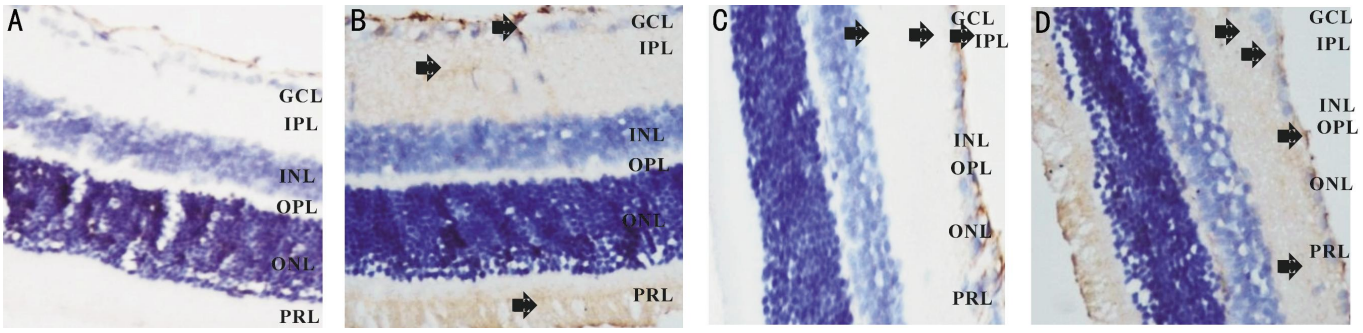

Figure 4 Expression of retinal GFAP immunohistochemical staining 20×20 A: Negative control group, little to no GFAP expression; B: 0.1 μg/μL PEDF group, GFAP expression in the ganglion cell layer, the inner plexiform layer, and the rod and cone layers; C: 0.2 μg/μL PEDF group, GFAP expression in the internal limiting membrane, the ganglion cell layer, and the inner plexiform layer; D: BSS group, GFAP expression in the ganglion cell layer, the inner plexiform layer, the outer plexiform layer, and in the rod and cone layers. Arrows indicate positive areas. GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PRL: Photoreceptor layer.

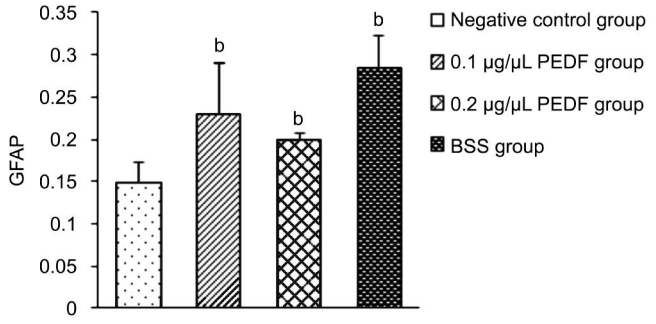

Figure 5 Column chart of GFAP optical density in each group ($\bar{x} \pm s$) [b]Compared to the negative control group, differences were significant (P<0.01).

cycle [26-29]. Previous research has established that morphological and physiological changes to Müller cells occur prior to retinal vascular lesions resulting from diabetes[10,11]. These changes include shrinkage of the nucleus, chromosome margination, expansion of the endoplasmic reticulum, and swelling of mitochondria [30,31]. Additionally, there is abnormal expression of a number of molecules, including glutamate-aspartate transporter and glutamine synthetase [32-34], vascular endothelial growth factor (VEGF), erythropoietin (EPO), erythropoietin receptor (EPOR), angiotensin-Ⅱ (Ang-Ⅱ), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [35-39]. Importantly, there are critical changes in both the expression level and localization of glial fibrillary acidic protein (GFAP), which not only impairs normal nerve cell function (including decreased visual acuity, impaired color vision sensitivity, and abnormal retinal oscillatory potentials of β waves) in patients with diabetes mellitus, but also affects the entire progression of diabetic retinopathy[12,40-43].

PEDF was first isolated from the culture medium of fetal retinal pigment epithelial cells. It is a 50 kD secreted serine protease inhibitor that is widely expressed throughout the body. PEDF is involved in the regulation of neurotrophy and also has anti-angiogenic anti-tumorigenic, and anti-metastatic properties [13-15]. According to previous research *in vitro*, PEDF can inhibit the expression of IL-1β in Müller cells, improve the downregulation of Kir4.1 generated by the hyperglycemia- induced oxidative stress [16]. Meanwhile, PEDF increases the expression of glutamine synthetase in Müller cells under high glucose, improves the function of glutamate-aspartate transporters (GLAST), thereby promotes the glutamic acid cycle [17]. While the effect of PEDF on retinal vascular endothelial cells and pericytes has been well established, its effect on Müller cells is not as well characterized[18-22]. Thus, in this study, we further examine the effect of PEDF on the structure and function of Müller cells *in vivo*.

In this study, we detect altered structure of retinal Müller cells in our diabetic rat model. These changes include, a disrupted nuclear membrane, mildly agglutinated chromatin, vacuolated mitochondria with cristae dissolution, a disorganized rough endoplasmic reticulum, and a significant reduction in the number of free ribosomes. We treated these diabetic rats with PEDF (0.1 μg/μL and 0.2 μg/μL) *via* intravitreal injection and assessed effects on Müller cell structure and function. For the 0.1 μg/μL PEDF-treated group, we detected fuzzy nuclear membranes, normal chromosomes, slightly swollen mitochondria with partially visible cristae, a moderately abnormal rough endoplasmic reticulum, and an increased number of free ribosomes. The conditions in the group treated with PEDF (0.2 μg/μL) were even further improved. Thus, PEDF alleviates the structural damage of retinal Müller cells in diabetic rats so that we suppose this effect was improved in rats treated with PEDF at 0.2 μg/μL. Glutamic acid is an important excitatory neurotransmitter in the mammalian retina involved in signal transduction of ganglion cells. GS is a key enzyme in the glutamate-glutamine cycle, which reduces the extracellular concentration of glutamic acid in the retina [44,45]; it also plays

a protective role to ganglion cells. Previous studies have used GS as a specific marker of Müller cell function and metabolism [46,47]. Furthermore, retinal expression of GS is significantly decreased in diabetic models after three months[48]. In this study, we find decreased GS expression in the retina by 4wk. This may be due to higher average blood glucose levels (21.85 mmol/L) in our model. Previous studies have shown that PEDF protects the function of Müller cells' GLAST and GS; it also improves the glutamate-glutamine cycle and inhibits ganglion cell death under high glucose conditions [49,50]. Importantly, PEDF can also protect the function of GLAST in diabetic rats[20]. While the expression of GS in the 0.2 μg/μL PEDF-treated group was unchanged compared to the negative control group, it was significantly higher compared to the BSS group. The expression of GS in the 0.1 μg/μL PEDF group was lower than in the negative control group but was not significantly different compared to the BSS group. Thus, based on these data, we conclude that PEDF reduces GS-associated damage of retinal Müller cells and improves the glutamate-glutamine cycle in diabetes; importantly, this protective effect was dose-dependent.

Little to no GFAP expression was present in normal Müller cells of the retina [51]. However, GFAP expression is up-regulated in the retina after damage[52]. Such changes may promote mitosis of Müller cells and subsequent reactive glial proliferation [53]. As a result, GFAP serves as a marker of Müller cell damage. Previous work has demonstrated that intravenous injection of PEDF (5 μg/100 g body weight) significantly inhibits GFAP expression in diabetic rats [21]. In this study, we find that GFAP expression in retinal Müller cells is inhibited by 0.2 μg/μL PEDF; in contrast, we did not detect this decreased expression in the 0.1 μg/μL PEDF group. Thus, a particular concentration threshold is likely required to inhibit astrogliosis and prevent Müller cell damage in diabetes mellitus.

In summary, PEDF protects the structure and function of retinal Müller cells in diabetes. While the detailed molecular mechanism governing this effect is not quite clear, some studies have suggested that it may be related to down-regulation of IL-1beta in Müller cells [22,48]. Taken together, PEDF may delay or reverse Müller cell death in diabetes and may serve as a new therapy for the treatment of diabetic retinopathy.

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