Original Article
Effect of vascular endothelial growth factor on retinal ganglion cells of rats with chronic intraocular hypertension

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Abstract: Aim: To investigate the effect of vascular endothelial growth factor (VEGF) on the expression of pigment epithelium-derived factor (PEDF) in retina and the protective effect of VEGF on retinal ganglion cells (RGCs) of rats with chronic elevated intraocular pressure (EIOP) and it's potential mechanism. Methods: 30 females Sprague-Dawley rats were randomly divided into three groups: EIOP + VEGF group (A), EIOP + vehicle group (B) and sham operated + VEGF group (C). The EIOP was introduced by obstructing episcleral veins in Group A and Group B. In the Group C, only the bulber conjunctiva was opened without obstructing episcleral veins. Immediately after surgery, rats in the Group A and Group C were intravitreously injected with 2 μL of VEGF. In the Group B, rats were intravitreously treated with 2 μL of normal saline. At 3 and 14 days after injection, animals were sacrificed and the eyes were collected for preparation of frozen sections. Results: After surgery, the IOP increased significantly in the Group A and Group B. There was no significant difference in the IOP at day 3 and day 14 after operation. The PEDF expression in the Group A and Group B was higher than that in the Group C. TUNEL staining showed the apoptotic RGCs markedly reduced after VEGF treatment when compared with rats without treatment. Conclusion: Intravitreal treatment with VEGF may reduce the apoptosis of RGCs in rats with chronic intraocular hypertension.

Keywords: Vascular endothelial growth factor, pigment epithelium derived factor, ocular hypertension, ganglion cell, apoptosis, β-tubulin

Introduction

Intraocular pressure (IOP), as a risk factor especially to retinal ganglion cells, has made a bad infection to the sight not only for rats but other life. And to our knowledge, elevated IOP is the most significant single risk factor for the development and progression of glaucoma and is the only modifiable risk factor thus far identified conclusively. Many states have show that episcleral veins play an important role in the intraocular pressure which would induce RGCs loss and damage of optic nerve [1].

Vascular endothelial growth factor (VEGF), as a factor that can promote angiogenesis, has been pointed out that it can facilitate the proliferation of endothelial cells. It would increase vascular permeability results in increasing interstitial pressure and reducing perfusion and increasing hypoxia in cancers, which shows a critical role for tumor progression and metas-

tasis in tumor-associated angiogenesis [2]. On the other side, VEGF could change the extracellular matrix and have many other biological effects, which play important roles in angiogenesis [2-4]. And many data in depth also reveal that VEGF can exert trophic ,which would be likely mediated directly by increasing survival and decreasing apoptotic proteins and signals as well as indirectly by modulating release of proteins that affect neuronal viability, and anti-apoptotic effects on neurons and glial cells [5, 6]. These data demonstrate that VEGF is a significant factor in many several physiological processes of vital movement.

Pigment epithelium derived factor (PEDF), as a glycoprotein with a molecular weight of 50 kDa approximately 15 kbp long and includes 8 exons interspersed with 7 introns belonging to the non-inhibitory serpin family, has been stated that own many important effects in physiological processes which include protecting neu-
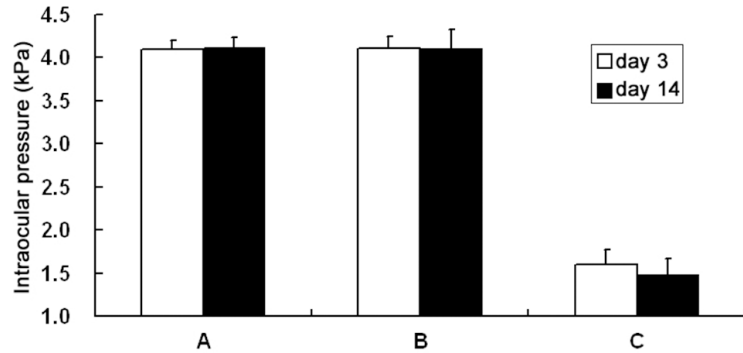
Figure 1. Intraocular pressure at two time points in three groups ($n = 5$). [a]$P < 0.05$ *vs.* C group.

ronal cells against apoptosis, stimulating of retinoblastoma cell differentiation into neuron cells, and facilitating of the growth and viability of photoreceptor cells [7, 8]. Therefore, PEDF is not only a neurotrophic factor, but also a natural angiogenesis inhibitor. Current research shows that PEDF, as a regulatory factor of cell and tissue growth, is expressed in almost all mammalian and avian tissues and has numerous important functions. It as a protein generates great interest in clinical disease pathogenesis and potential treatment like a promising drug for the therapy of a wide range of neurodegenerative, ophthalmological, and oncological diseases. All data suggest that PEDF would maybe act as an important means in the treatment of neurodegenerative disorders, including Parkinson's disease in a while future.

In the present study, chronic elevated intraocular pressure (EIOP) was introduced to rats to establish animal model of chronic intraocular hypertension. Immunohistochemistry and TUNEL staining were employed to detect the protective effect of exogenous VEGF on retinal ganglion cells (RGCs) in rats with EIOP.

At the same time, β-tubulin has been as a marker for neurons. Many studies have stated it and show the necessary for the correct number of neurites a neuron generates in vivo [9]. Immunofluorescence staining was employed to explore the relationship between pigment epithelium derived factor (PEDF) and RGCs.

Materials and methods

Materials

Female adult SD rats were purchased from the Experimental Animal Center in Forth Military

Medical University. TUNEL kit (Teva, USA), recombinant rat VEGF (Boster Biotech), βIII-tubulin (Jingmei Biotech), PEDF antibody (Santa Cruz; USA), VEGF antibody (Jingmei Biotech), and Tonopen XL pen tonometer (Jackson, USA) were used in the present study.

Grouping and treatment

A total of 30 female adult SD rats weighing 220-250 g were randomly assigned into 3 groups: EIOP + VEGF group (A); EIOP + vehicle group (B) and sham operated + VEGF group (C) ($n = 10$ per group). All SD rats were 8 weeks of age, and were age-matched across the groups. One eye was randomly selected for introduction of EIOP. In the Group A and Group B, the EIOP was introduced by obstructing episcleral veins according to previously reported [1, 2]. When the intraocular pressure was higher than 4.0 kPa, rats were used in the following experiments. In the Group C, only the bulber conjunctiva was opened without obstructing episcleral veins. Immediately after surgery, rats in the Group A and Group C were intravitreously injected with 2 μL of 0.05 μg/μL VEGF 164 isoform at 2.0 mm behind the limbus of cornea following aspiration of 2 μL of vitreous humor. In the Group B, rats were intravitreously treated with 2 μL of normal saline. At 3 and 14 days after injection, animals were sacrificed. All the procedures were in accordance with the Guide for the Care and Use of Laboratory Animals. The Guide has been approved by the State Council in 1988 October thirty-one. Detection of intraocular pressure, fixation of samples, preparation of sections, immunohistochemistry for retinal PEDF and PEDF and cell counting were performed according to previously reported [10-12].

Immunofluorescence double staining (PEDF + β-tubulin)

At 14 days after injection, 2 sections were collected from each group and dried for 2 hours at room temperature. After washing in 0.01 mol/L PBS thrice (3 minutes for each), sections were treated with Formaldehyde and hydrogen peroxide in formaldehyde at room temperature for 30 minutes. After washing, sections were incu-
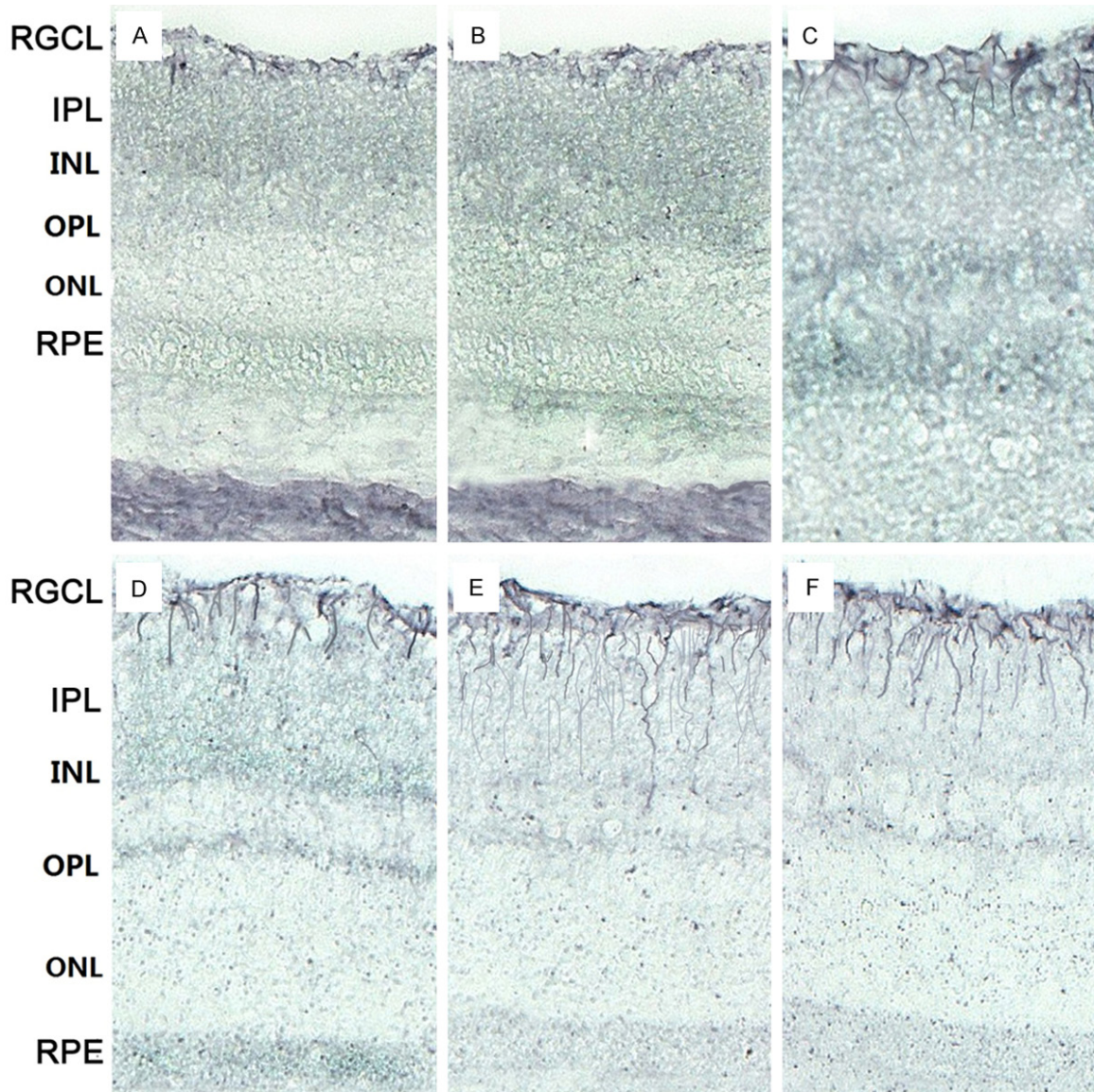
Figure 2. PEDF expression in retina after intravitreous injection of VEGF (×400). A: Group C, day 3; B: Group C, day 14; C: Group A, day 3; D: Group A, day 14; E: Group B, day 3; F: Group B, day 14.

bated with 3% goat serum for 1 hour. Following incubation with primary antibody (goat anti-rat PEDF: 1:50; mouse anti-rat β-tubulin: 1:2500) at room temperature over night, sections were washed with 0.01 mol/L PBS thrice (3 minutes for each). After incubation with secondary antibody (donkey anti-mouse secondary antibody [red fluorophore]: 1:1000; donkey anti-goat secondary antibody [green fluorophore]: 1:400) at room temperature for 4 hours in dark, mounting was performed and sections were observed under a fluorescence microscope. Positive cells presented yellow. Five fields were randomly selected at a magnification of ×400, and the

positive cells were counted. The proportion of positive cells was calculated as follow: proportion of positive cells = ($\sum$positive cells/$\sum$total cells) ×100%.

TUNEL staining

At room temperature, sections were washed with normal saline for 5 minutes. After washing in 0.01 mol PBS at room temperature for 5 minutes, sections were treated with 100 μL of 10 μg/mL proteinase K for 5 minutes. Following treated with 0.01 mol PBS for 5 minutes at room temperature, sections were incubated
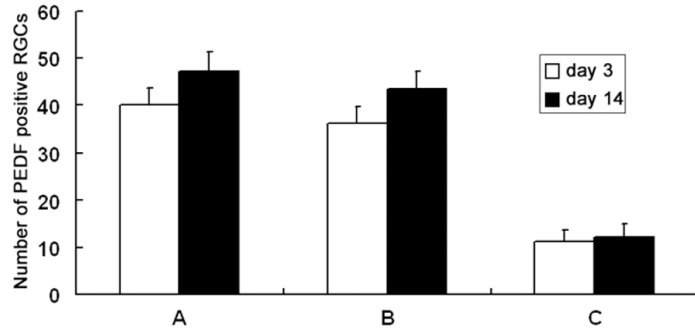
Figure 3. Number of PEDF-positive RGCs after intravitreous treatment in three group ($n = 5$). [a]$P < 0.05$ *vs.* all other groups.

Results

Changes in intraocular pressure

Statistical analysis showed the intraocular pressure in the Group A and Group B increased dramatically after surgery ($P < 0.05$) when compared with Group C, and there was no significant difference in the intraocular pressure at day 3 and day 14 (**Figure 1**).

Detection of PEDF expression in retina by immunohistochemistry

PEDF expression was observed in the retina of three groups. PEDF positive cells were mainly found in the retinal pigment epithelium (RPE), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), retinal ganglion cell layer (RGCL) and retinal nerve fiber layer (RNFL). The PEDF expression in the Group A and Group B was higher than that in the Group C and mainly found in the RGCL and RNFL. The positive cells were clear and had protrusions which extended to the inner nuclear layer. Significant changes were also found in the Group B and Group A. In the Group B, the number of PEDF positive cells was larger than that in the Group A, and PEDF positive cells were mainly found in the RGC and RNFL. In addition, marked difference was also noted in the PEDF expression at day 3 and day 14 in the same group. Moreover, the number of PEDF positive cells at day 14 was larger than that at day 3 (**Figure 2**).

PEDF positive RGCs

Significant difference was found in the number of PEDF positive RGCs between Group A and Group B ($P < 0.05$) and between Group B and Group C ($P < 0.05$). The changes in number of PEDF positive RGCs over time were similar in Group A and Group B, and the number of PEDF positive RGCs at day 14 was larger than that at day 3 ($P < 0.05$). There was no marked difference in the number of PEDF positive RGCs in Group C at two time points ($P > 0.05$) (**Figure 3**).

Immunofluorescence double staining (PEDF + β-tubulin)

Under a fluorescence microscope, the PEDF positive cells were green, and β-tubulin positive

with 100 μL of balance buffer at room temperature for 5 minutes. Then, sections were incubated with 100 μL of TdT reaction compound (biotinylated nucleotide, TdT and balance buffer [1:1:98]) in the presence of a plastic cover at 37°C for 2 hours. After recovery to room temperature, sections were treated with 2× SSC. Reaction was stopped by washing with 2× SSC for 15 minutes at room temperature. After washing with 0.01 mol PBS at room temperature twice (5 minutes for each), sections were treated with 0.3% H_2O_2 at room temperature for 3 minutes to inactivate the endogenous peroxidase. Streptavidin peroxidase HRP was diluted with 0.01 mol PBS at 1:500. Sections were incubated with 100 μL of incubation buffer for 30 minutes. After washing in 0.01 mol PBS at room temperature twice (5 minutes for each), visualization was done with DAB. Following washing in deionized water several times, dehydration and mounting were performed. Sections were observed under a light microscope. TUNEL positive cells presented yellow brown granules and the positive cells were counted under a microscope as abovementioned.

Statistical analysis

Statistical analysis was done with SPSS version 12.0. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The intraocular pressure at two time points was compared with analysis of variance, and comparisons between two groups were done with Dunnett t test. Comparisons of TUNEL positive cells were performed with factorial analysis of variance, and comparisons of means between two groups were done with LSD-t test. A value of $P < 0.05$ was considered statistically significant.
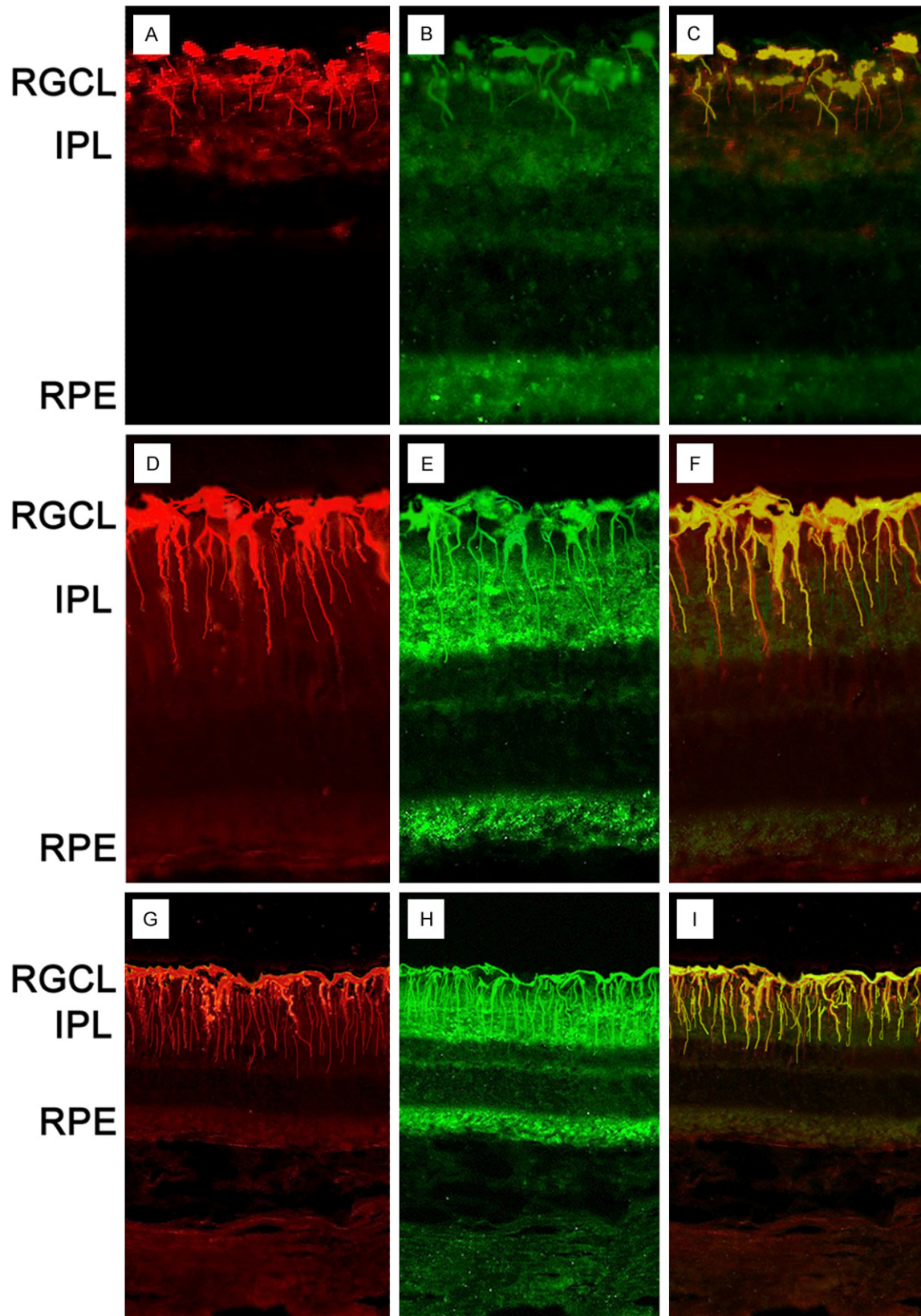
Figure 4. Immunofluorescence double staining of PEDF + β-tubulin at retina at 14 days after surgery (×400). A: β-tubulin staining (Group C, day 14); B: PEDF staining (Group C, day 14); C: β-tubulin and PEDF double staining

(Group C, day 14); D: β-tubulin staining (Group A, day 14); E: PEDF staining (Group A, day 14); F: β-tubulin and PEDF double staining (Group A, day 14); G: β-tubulin staining (Group B, day 14); H: PEDF staining (Group B, day 14); I: β-tubulin and PEDF double staining (Group B, day 14).
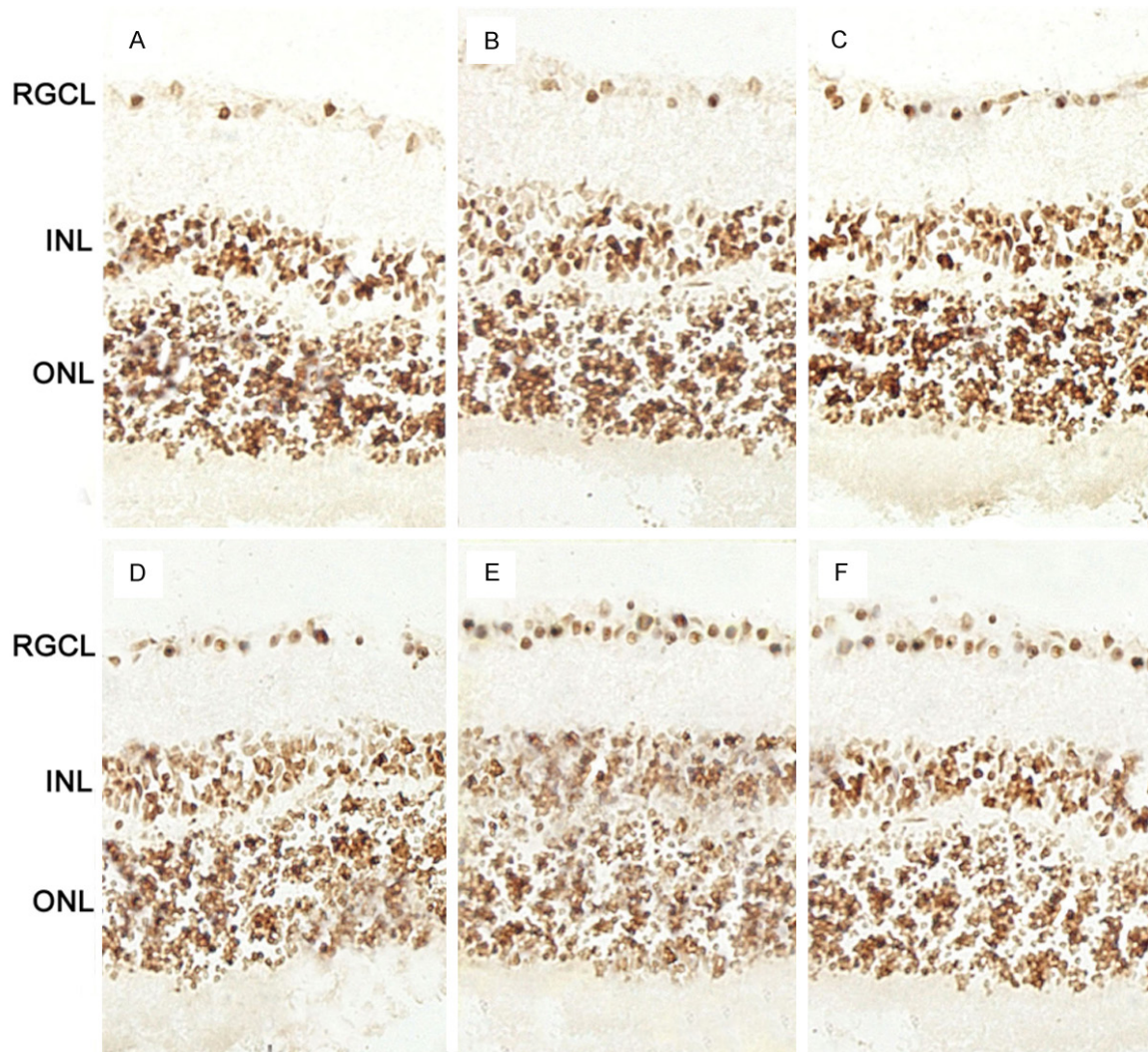

Figure 5. TUNEL staining of retina after intravitreous injection of VEGF (×400). A: Group C, day 3; B: Group C, day 14; C: Group A, day 3; D: Group A, day 14; E: Group B, day 3; F: Group B, day 14.

cells were red. In the Group C, the cells with green fluorescence and red fluorescence were found in the ganglion cell layer. The cell protrusions extended to the inner plexiform layer, and the number of cell body and protrusion was small, but they were evenly distributed. There was overlapping between green fluorescence and red fluorescence. In the Group A, the cells with green fluorescence were mainly found in the ganglion cell layer, inner nuclear layer, inner plexiform layer and pigment epithelium layer. In the ganglion cell layer, PEDF positive cells were observed, had a large number and evenly distributed. Cells with red fluorescence were mainly found in the ganglion cell layer, and cell body was present. The cell protrusion extended to the inner plexiform layer and there was overlapping in the red and green fluorescence. In the Group B, the cell body and protrusion of cells with red and green fluorescence were similar to those in the Group A, but the number of cell body and protrusion was smaller than that in the Group B and higher than that in the Group C (**Figure 4**).

TUNEL staining

In the Group B, a large number of TUNEL positive cells were found in the ganglion cell layer, inner and outer nuclear layer, and there was no difference in these cells at day 3 and day 14. In the Group C, only a small number of TUNEL positive cells were found in the ganglion cell layer, inner and outer nuclear layer, and there was no difference in these cells at day 3 and day 14. In the Group A, a large number of TUNEL positive cells were found in the ganglion cell layer, inner and outer nuclear layer, but the number of TUNEL positive cells in the ganglion cell layer was smaller than that in the Group B. Moreover, the number of TUNEL positive cells at day 14 was smaller than that at day 3 (**Figure 5**).

Discussion

VEGF can exert protective effect on the retina at elevated intraocular pressure, which is characterized by reduction in apoptotic RGCs. In early studies, VEGF was regarded as a cytokine whose expression is induced by ischemia and/ or hypoxia and it can promote angiogenesis especially in tumor tissue [12]. Studies have confirmed that VEGF can facilitate the proliferation of endothelial cells, promote angiogenesis and increase vascular permeability [13, 14]. Recently, a variety of studies demonstrate that VEGF can exert neurotrophic and neuroprotective effects. VEGF could protect endothelial cells against apoptosis [15]. Currently, some studies reveal that VEGF has neurotrophic effect [16] and can prolong the survival time of cells by inducing angiogenesis which then attenuates the brain edema after cerebral ischemia/reperfusion and neuron injury. The neuroprotective effect of VEGF has been a hot topic in studies. Our results showed exogenous could reduce the apoptotic RGCs in retina at elevated intraocular pressure, leading to the attenuation of EIOP induced damage to retina. This protective effect could last for at least 2 weeks. At 14 days after injection, animals were sacrificed and we can find the protective cells and the protective effect at day 14 was more obvious than that at day 3. This might be attributed to that the apoptosis of RGCs in the retina is not obvious, and thus the protective effect of VEGF is not evident at this time point. In addition, VEGF could also inhibit the degeneration of RGCs in the retina at elevated intraocular pressure. In the present animal model, the damage to RGCs

in the retina may cause apoptosis and degeneration/death of RGCs. VEGF exerts dual protective effects to inhibit the apoptosis and degeneration.

VEGF can stimulate the growth of neuron and simultaneously induce angiogenesis. This challenging issue is imperative to resolve. Oosthuyse et al. [17] proposed that the neuroprotective effect of VEGF was unrelated to its angiogenic effect. VEGF can promote the regeneration of neurons after stimulation by regulating the functions of endothelial cells and neurons. The regeneration of neurons together with angiogenesis may improve the repair after injury. VEGF may facilitate the regeneration of neurons after ischemia [18, 19]. Louissaint et al. [20] proposed that VEGF was a factor bridging the neural growth, neural migration and angiogenesis. In the present study, intravitreous injection was done with 2 μL of 0.05 μg/μL VEGF, but angiogenesis was not observed, which might be related to low dose of VEGF or the purity or quality of VEGF or the short-term observation. In addition, in rats with elevated intraocular pressure, the retinal ischemia due to elevated intraocular pressure is a major cause of damage to RGCs in the retina. On the basis of angiogenic effect of VEGF, VEGF can improve the nutrient supply, which is consistent with its neurotrophic effect and neuroprotective effect.

Intravitreous injection of VEGF can induce the PEDF expression in the retina. PEDF is another neurotrophic factor which is extensively investigated in studies [21, 22]. PEDF can promote the survival and differentiation of neurons, and specifically protect neurons against apoptosis and H_2O_2 or glutamate induced cytotoxicity. Our results showed the neuroprotective effect might be partially attributed to the up-regulation of PEDF expression. Of note, we cannot exclude that the PEDF expression might act as an acute response and thus is not associated with neuroprotective effect of VEGF.

Disclosure of conflict of interest

None.

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References

[1] Wang J, Ge J, Sadun AA and Lam TT. Characteristics of optic nerve damage induced by chronic intraocular hypertension in rat. Yan Ke Xue Bao 2004; 20: 25-29.

[2] Kim SH, Cho YR, Kim HJ, Oh JS, Ahn EK, Ko HJ, Hwang BJ, Lee SJ, Cho Y, Kim YK, Stetler-Stevenson WG and Seo DW. Antagonism of VEGF-A-induced increase in vascular permeability by an integrin alpha3beta1-Shp-1-cAMP/PKA pathway. Blood 2012; 120: 4892-4902.

[3] Zhou JL, Zou MH, Chen YC, Lu CF, Shi JW and Dong NG. [PEG-mediated covalent binding of VEGF to decellularized aortic valves promotes adhesion and proliferation of endothelial progenitor cells]. Nan Fang Yi Ke Da Xue Xue Bao 2011; 31: 1474-1479.

[4] Rivron NC, Vrij EJ, Rouwkema J, Le Gac S, van den Berg A, Truckenmuller RK and van Blitterswijk CA. Tissue deformation spatially modulates VEGF signaling and angiogenesis. Proc Natl Acad Sci U S A 2012; 109: 6886-6891.

[5] Foehring D, Brand-Saberi B and Theiss C. VEGF-induced growth cone enhancement is diminished by inhibiting tyrosine-residue 1214 of VEGFR-2. Cells Tissues Organs 2012; 196: 195-205.

[6] Sanchez A, Wadhwani S and Grammas P. Multiple neurotrophic effects of VEGF on cultured neurons. Neuropeptides 2010; 44: 323-331.

[7] Minkevich NI, Lipkin VM and Kostanyan IA. PEDF - A Noninhibitory Serpin with Neurotrophic Activity. Acta Naturae 2010; 2: 62-71.

[8] Falk T, Gonzalez RT and Sherman SJ. The yin and yang of VEGF and PEDF: multifaceted neurotrophic factors and their potential in the treatment of Parkinson's Disease. Int J Mol Sci 2010; 11: 2875-2900.

[9] Laggner U, Pipp I, Budka H, Hainfellner JA and Preusser M. Immunohistochemical detection of class III beta-tubulin in primary brain tumours: variable expression in most tumour types limits utility as a differential diagnostic marker. Histopathology 2007; 50: 949-952.

[10] Ling ZH and Sun XH. [Expression of glutamate receptor 2 in retinal ganglion cells in the eyes with chronic elevated intraocular pressure: experiment with rats]. Zhonghua Yi Xue Za Zhi 2007; 87: 2727-2729.

[11] Brar VS, Sharma RK, Murthy RK and Chalam KV. Bevacizumab neutralizes the protective effect of vascular endothelial growth factor on retinal ganglion cells. Mol Vis 2010; 16: 1848-1853.

[12] Nakano M, Satoh K, Fukumoto Y, Ito Y, Kagaya Y, Ishii N, Sugamura K and Shimokawa H. Important role of erythropoietin receptor to promote VEGF expression and angiogenesis in peripheral ischemia in mice. Circ Res 2007; 100: 662-669.

[13] Cao X, Geradts J, Dewhirst MW and Lo HW. Up-regulation of VEGF-A and CD24 gene expression by the tGLI1 transcription factor contributes to the aggressive behavior of breast cancer cells. Oncogene 2012; 31: 104-115.

[14] Gyurkovics M, Lohinai Z, Gyorfi A, Szekely DA, Dinya E, Fazekas A and Rosivall L. [Investigation of the venodilatory effect of vascular endothelial growth factor (VEGF) in rat gingiva]. Fogorv Sz 2013; 106: 53-59.

[15] Morrison JC, Johnson E and Cepurna WO. Rat models for glaucoma research. Prog Brain Res 2008; 173: 285-301.

[16] Zheng XR, Zhang SS, Yin F, Tang JL, Yang YJ, Wang X and Zhong L. Neuroprotection of VEGF-expression neural stem cells in neonatal cerebral palsy rats. Behav Brain Res 2012; 230: 108-115.

[17] Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, Acker T, Vleminckx V, Van Den Bosch L, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D and Carmeliet P. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet 2001; 28: 131-138.

[18] Ma Y, Qu Y and Fei Z. Vascular endothelial growth factor in cerebral ischemia. J Neurosci Res 2011; 89: 969-978.

[19] Zan LK, Song YJ, Teng GX, Li H, Liu W, Jia Y, Zhou M, Sun YL and Qi JP. [Expression and function of vascular endothelial growth factor and angiopoietins in rat brain after cerebral ischemia]. Zhonghua Bing Li Xue Za Zhi 2011; 40: 834-839.

[20] Louissaint A Jr, Rao S, Leventhal C and Goldman SA. Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 2002; 34: 945-960.

[21] Volpert KN, Tombran-Tink J, Barnstable C and Layer PG. PEDF and GDNF are key regulators of photoreceptor development and retinal neurogenesis in reaggregates from chick embryonic retina. J Ocul Biol Dis Infor 2009; 2: 1-11.

[22] Kim SY, Mocanu C, McLeod DS, Bhutto IA, Merges C, Eid M, Tong P and Lutty GA. Expression of pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in sickle cell retina and choroid. Exp Eye Res 2003; 77: 433-445.